RESEARCH

Inhibition of Notch signaling attenuates pituitary adenoma growth in Nude mice

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Abstract

Preclinical and clinical studies support that Notch signaling may play an important oncogenic role in cancer, but there is scarce information for pituitary tumors. We therefore undertook a functional study to evaluate Notch participation in pituitary adenoma growth. Tumors generated in Nude mice by subcutaneous GH3 somatolactotrope cell injection were treated *in vivo* with DAPT, a γ-secretase inhibitor, thus inactivating Notch signaling. This treatment led to pituitary tumor reduction, lower prolactin and GH tumor content and a decrease in angiogenesis. Furthermore, *in silico* transcriptomic and epigenomic analyses uncovered several tumor suppressor genes related to Notch signaling in pituitary tissue, namely *Btg2*, *Nr4a1*, *Men1*, *Zfp36* and *Cnot1*. Gene evaluation suggested that *Btg2*, *Nr4a1* and *Cnot1* may be possible players in GH3 xenograft growth. *Btg2* mRNA expression was lower in GH3 tumors compared to the parental line, and DAPT increased its expression levels in the tumor in parallel with the inhibition of its volume. *Cnot1* mRNA levels were also increased in the pituitary xenografts by DAPT treatment. And the *Nr4a1* gene was lower in tumors compared to the parental line, though not modified by DAPT. Finally, because DAPT *in vivo* may also be acting on tumor microenvironment, we determined the direct effect of DAPT on GH3 cells *in vitro*. We found that DAPT decreases the proliferative, secretory and migration potential of GH3 cells. These results position selective interruption of Notch signaling as a potential therapeutic tool in adjuvant treatments for aggressive or resistant pituitary tumors.

Key Words
- DAPT
- pituitary
- angiogenesis
- prolactin
- GH

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Introduction

Pituitary adenomas are mostly benign intracranial tumors, which do not metastasize but may recur after surgical removal, compress nearby structures or produce considerable morbidity related to hormonal dysfunction. A subset of these tumors may be aggressive, atypical or recurrent, and presently, there is a paucity of molecular

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markers that could improve diagnosis, treatment and prognosis. Stem-like cell activation of different components of the Notch pathway have been consistently detected in pituitary tumors (Mertens *et al.* 2015) suggesting potential therapeutic benefit for targeting Notch in tumoral pituitaries. Nevertheless, functional studies linking the Notch pathway with pituitary tumorigenesis are lacking.

Notch signaling regulates numerous cellular processes, including stem cell maintenance, proliferation, cellular differentiation and apoptosis (Artavanis-Tsakonas & Muskavitch 2010). It maintains precursor cells by balancing cellular proliferation, cell fate decisions and differentiation in several tissues such as brain, muscle, intestine and the hematopoietic system. It is therefore not surprising that Notch pathway dysfunction is implicated in the pathogenesis of adult human disease, including cancer (Ranganathan *et al.* 2011).

The mammalian Notch receptor family consists of four type 1 transmembrane receptors (termed NOTCH 1–4), which are synthesized as precursor forms and cleaved by a furin-like convertase to generate the mature receptor, composed of two subunits: an extracelluar and an intracelluar domain (NICD) held together by non-covalent interactions. Notch signaling is initiated by cell-to-cell contact of the receptor with the neighboring-cell Notch ligands Jagged1 and 2 (JAG1 and JAG2) and Delta-like 1,3 and 4 (DLL1,3,4). Ligand binding initiates a series of cleavages and a final cleavage mediated by the γ-secretase complex, which releases NICD from the plasma membrane so that it can translocate into the nucleus where it recruits a transcriptional activation complex activating and repressing genes. Classical target genes are the transcriptional factors of the Hairy Enhancer of Split (HES) family (HES 1,5,6, and 7), the Hairy-Related Transcription factor family (HRT1,2 and 5; also known as HEY), Notch receptors, Notch ligands, cyclin D1 and MYC (Bray 2006, Gordon *et al.* 2008), among others.

Substantial evidence derived from preclinical and clinical studies support that Notch signaling may play an important oncogenic role in several types of cancer. In particular, most patients with T cell acute lymphoblastic leukaemia (T-ALL) harbor activating mutations in the *NOTCH1* gene, which result in ligand-independent proteolytic cleavage of the receptor and increased stability of the NICD (Ellisen *et al.* 1991). This leads to constitutive activation of the Notch pathway and neoplastic transformation of T cells. Nevertheless, in solid tumors, there is little evidence for genetic alterations in Notch genes, even though Notch signaling seems to be crucial in the generation and progression of breast, colon, pancreas and prostate cancer (Radtke & Raj 2003). Intriguingly, Notch signaling may also have a tumor suppressor role as it was described in mouse keratinocytes, pancreatic and hepatocellular carcinoma (Koch & Radtke 2010, Ranganathan *et al.* 2011).

The versatility and pleiotropic effects, which result from aberrant Notch activity may be interpreted based on contextual and developmental cues. Moreover, each tissue and even every cellular component within a tissue express different proportions of Notch paralogs and target genes, which may ultimately determine cell fate during Notch dysfunction. Activity and outcome of increased Notch signaling may therefore depend on the specific paralog involved as found in medulloblastoma tumors (Castro *et al.* 2003) and in breast (Harrison *et al.* 2010) and pancreatic carcinomas (Avila & Kissil 2013). Complexity is increased when target genes are considered, leading to the concept that Notch activity outcome depends on cellular context.

In the search for targets for pituitary adenoma combinatorial treatment, elucidation of relevant Notch signaling components within each adenoma type would be highly valuable. Knowledge on the participation of the Notch system in pituitary tumor generation and progression is scarce. In general, links between pituitary adenomas and Notch have been revealed by the description of expression levels of Notch pathway elements, but to our knowledge, no functional study has been performed so far. Notch 3 was increased in prolactinomas and non-functioning adenomas (Moreno *et al.* 2005, Evans *et al.* 2008, Miao *et al.* 2012, Lu *et al.* 2013) and decreased in somatotropinomas (Lu *et al.* 2013). Furthermore, HES1 expression was decreased in prolactinomas and non-functioning adenomas (Evans *et al.* 2008), and levels of Jagged1 were increased (Lu *et al.* 2013). Importantly, in pituitary adenomas, the side population with stem cell characteristics showed increased levels of *HES1*, *JAGGED1* as well and *NOTCH 1,2* and *4* (Mertens *et al.* 2015). Furthermore, pituitary adenoma-derived stem-like cells express higher levels of *NOTCH4*, *JAG2* and *DLL1* and are more resistant to chemotherapeutics than their differentiated daughter cells (Xu *et al.* 2009).

We recently found that all four Notch receptors are expressed in the pituitary gland and also demonstrated enhanced gene expression of the Notch ligands *Jag1* and *Dll1*, and the target gene *Hey1*, as well as activated Notch2 intracellular domain N2ICD in the somatolactotrope cell line GH3 compared to normal rat pituitaries (Perrone *et al.* 2017). Furthermore, in prolactinomas harbored by

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lacDrd2KO female mice an activated Notch signaling pathway was found (Perrone *et al.* 2017). Therefore, in the present study, we undertook a functional approach to evaluate Notch participation in pituitary adenoma growth. GH3 somatolactotrope tumors generated in Nude mice were treated with a γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)- L-alanyl]-S-phenylglycine t-butyl ester (DAPT), thus inactivating Notch signaling. Tumor development, Notch signaling component expression and angiogenic and proliferative markers were assessed. Furthermore, because regulation of Notch signaling pathways is specific for each tumor type, we undertook a bioinformatic approach based on a combined epigenomic and transcriptomic analysis to identify Notch target genes with a potential role in tumor suppression, which may be relevant to pituitary tumors. We next determined their expression in treated and untreated GH3 xenografts. Finally, because DAPT *in vivo* may be acting not only on pituitary tumor cells, but also on endothelial cells or modifying the extracellular matrix of the tumor, we determined the direct effect of DAPT on GH3 cells *in vitro*. Our results demonstrate that inhibiting Notch signaling *in vivo* leads to pituitary tumor reduction and a decrease in tumor angiogenesis. In addition, DAPT acts directly on GH3 cells decreasing their proliferation, secretory and migration potential. These results position selective interruption of Notch signaling as a potential therapeutic tool in the search for adjuvant treatments in aggressive or resistant pituitary tumors.

Materials and methods

Cell line and culture conditions

GH3 rat somato-prolactinoma cell line (ATCC, CCL-82.1) was cultured in adhesion as reported (Vela *et al.* 2007) in DMEM/F12K medium, supplemented by 2.5% (v/v) fetal bovine serum, 15% (v/v) horse serum, 1% glutamine and 1% (w/v) penicillin/streptomycin and fungizone, pH 7.3 and maintained at 37°C and 5% CO_2. After incubation in serum-free medium for 18–24 h cells were treated with DAPT 1, 5 and 10 μM (Calbiochem Cat No: 565770) or vehicle. Medium was refreshed every 24 h with the appropriate stimuli. Aliquots of supernatant were collected for GH and prolactin measurements at 24 and 48 h. To analyze gene and protein expression, cells were detached and dissociated using trypsin (0.05%) with EDTA (0.02%; Life Technologies).

For GH3 sc injections in Nude mice, GH3 cells were cultured and detached as indicated, and trypsin was inactivated with excess (20 mL) F12K medium (supplemented with 15% (v/v) horse serum, 2.5% (v/v) bovine fetal serum). Cells were centrifuged for 10 min at 950 rpm, 23°C, the pellet resuspended in 1 mL PBS or F12K medium and cells were counted.

Experiments with athymic Nude mice

Nude mice BALB/c NU/NU were housed at the Animal House Facility of the Instituto de Biología y Medicina Experimental. Experimental tumors were induced by sc injection of 700,000 GH3 cells suspended in 100 μL PBS in one flank of adult female Nude mice. DAPT treatment was started when the tumor volume had reached about 70 mm^3 in size (approximately 21 days after GH3 injection). DAPT was dissolved in 0.5 μM DMSO–PBS, and 8 mg/kg BW per mouse was administered i.p., thrice a week. Vehicle-treated animals served as controls. The tumor volume was regularly determined with a caliper until the animals were killed after 3 weeks of treatment. Tumors were excised, weighed and frozen at −70°C for mRNA and protein studies and a portion was embedded in paraffin for immunohistochemical studies.

All experimental procedures were carried according to guidelines of the Institutional Animal Care and Use Committee of the Instituto de Biología y Medicina Experimental, Buenos Aires (in accordance with the Animal Welfare Assurance for the Instituto de Biología y Medicina Experimental, Office of Laboratory Animal Welfare, NIH, A#5072-01). Study #07/2016 was approved by IBYME IACUC.

RNA extraction and cDNA synthesis

Xenotransplant tissue or GH3 cells cultured *in vitro* were processed for recovery of total RNA using TRIzol reagent (Invitrogen). Reverse transcription was performed as previously described (Perrone *et al.* 2017).

Real-time PCR

Measurements were performed as previously described (Garcia-Tornadu *et al.* 2009, Perrone *et al.* 2017). Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA or by the use of PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Oligonucleotides were obtained from Invitrogen. The sequences are described in Supplementary Table 1 (see section on supplementary data given at the end of this article).

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Western blot

Xenotransplant and cell lysates were homogenized in a motor microtissue mixer in 80–300 µL of lysis buffer (50 mM HEPES (pH 7.4), 140 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1% Triton X-100), and 1 mM phenymethylsulfonyflouride and protease cocktail inhibitor (Roche Diagnostic) were added to the buffer just before use. The homogenate was then centrifuged at 12,000 rpm for 30 min at 4°C. An aliquot of the supernatant was taken to quantify proteins by the Qubit Quant-it protein assay kit (Invitrogen).

Thirty to forty micrograms of proteins in 20 µL of homogenization buffer were mixed with 5 µL of 5× sample buffer (312 mM Tris-HCl, 10% SDS, 25% glycerol, 0.002% bromophenol blue and 1% Beta-mercaptoethanol, pH 6.8). Samples were heated 5 min at 95°C and separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (G&E, Little Chalfont, UK). After blocking with 3% nonfat dry milk solution in phosphate saline buffer – Tween (PBST) (10 mM sodium phosphate, 2 mM potassium phosphate pH 7.4, 140 mM NaCl, 3 mM KCl, and 0.1% Tween 20) blots were incubated overnight at 4°C with primary antibodies. Antibodies used were rabbit polyclonal anti-Notch 1 (1/1000, EMD-Millipore, Cat #07-1232), anti-Notch2 (1:1000, Merck Millipore): anti-Hes1 (1:1000, EMD-Millipore, Cat. #AB5702).

Membranes were washed with PBST and incubated with the corresponding horse radish peroxidase (HRP)-conjugated secondary antibody, and protein bands were detected in a G:box chemi HR16 (Syngene, Frederick, MD, USA). The monoclonal beta-Tubulin (1:7000, Sigma-Aldrich, Cat #T0198) was used to validate equal amount of protein loaded and transferred. For repeated immunoblotting, membranes were incubated in stripping buffer (62.5 mM Tris, 2% sodium dodecyl sulfate and 100 mm mercaptoethanol, pH 6.7) for 40 min at 55°C and reprobed. Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

NOTCH1-2 expression levels were evaluated by the semi-quantification of two bands, the active intracellular domain (NICD) of 80 kDa and the membrane domain plus the NICD of the receptor of 110 kDa.

Prolactin and GH RIAs

Serum

Aliquots (10 µL) of serum obtained from Nude mice were used to assay serum prolactin and GH by RIA.

Tissue

Xenotransplanted GH3 tumoral samples (1–5 mg) were homogenized in ice-cold PBS and centrifuged at 3000 rpm for 5 min. Supernatant protein contents were measured with the QUBIT Fluorometer and the QUANT-IT protein Assay Kit (Invitrogen). Aliquots of equal quantity of protein were used to assay GH and prolactin content.

In vitro supernatants from cultured GH3 cells, 10 µL diluted 1/20–1/40, were kept at −20°C for GH and prolactin RIA assays.

RIA assays were performed using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Dr. A.F. Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA). Results are expressed as ng/mL for *in vitro* studies and serum, and ng/µg protein for xenograft content, in terms of rat prolactin standard RP3 and GH standard AFP-10783B. Intra- and inter-assay coefficients of variation were 7.2 and 12.8% and 8.4 and 13.2%, for prolactin and GH, respectively. Sensitivity threshold was 0.02 and 0.04 ng for prolactin and GH, respectively.

Quantification of cell proliferation

MTS proliferation assay

Proliferation of GH3 cells was colorimetrically determined at 490 nm using a commercial proliferation assay kit CellTiter 96 (AQueous Non-Radioactive Cell Proliferation Assay, Promega Corp.) following the manufacturer's instructions. Cell cultures were repeated four times and each had duplicate samples.

Cell motility assay

Cell motility was evaluated using the 'scratch assay'. After reaching 90% confluence, GH3 cells were serum-starved for 24 h and then treated with mitomycin C (10 µg/mL; Calbiochem 475820) to inhibit cell proliferation. A straight scratch was created, and cells were further kept in DMEM/F12K (2% horse serum, 1% fetal bovine serum), together with DAPT (5 and 10 µM) or vehicle. Medium was changed every 24 h. The migration of cells into the scratch was evaluated by light microscopy, and live pictures were taken with an Olympus CKX 41 microscope at different time points. The open area was calculated using the image processing and analysis software ImageJ http://rsbweb.nih.gov/ij/.

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Tumor microvessel density, vascular area and vessel size assessment

Immunohistochemistry

Xenotransplants were deparaffinized and dehydrated in graded ethanols. A microwave pre-treatment for antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary antibody (goat polyclonal antibody PECAM for CD31 endothelial cell detection (1:200, sc-1506 Santa Cruz Biotechnologies Inc.) or rabbit polyclonal SMA antibody (1:200; α-SMA ab15734; Abcam) for vessel mural cell detection) was incubated overnight at 4°C. After incubation with biotin-conjugated secondary antibody for 1 h, the reaction was developed using an avidin-biotin kit coupled to peroxidase (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine as a chromogen substrate. Samples were counterstained with hematoxylin and mounted with permanent mounting medium. Each immunohistochemical run included negative controls replacing the primary antibody with PBS. As a measure of angiogenesis, we determined the microvascular density (MVD) by counting the number of $CD31^+$ or αSMA+ vessels per square millimetre, the vascular area determined by the cumulative area of the tumor occupied by $CD31^+$ or αSMA+ vessels and expressed as % vessel area/total area and the average vessel size. Images of randomly selected fields were recorded using 40× or 100× objective, using a Zeiss Axiostar Plus microscope and a Canon PowerShot G6 digital camera. Three slides per tumor (4 tumors per group) were analyzed and at least five images per slide at 400× of total magnification were counted by the image processing and analysis software: Image J, http://rsbweb.nih.gov/ij/.

Bioinformatic analysis

Publicly available raw RNA-seq datasets were obtained from the Sequence Read Archive (SRA) database as listed in Supplementary Table 2. Human normal pituitary datasets used for the analyses described in this manuscript were obtained from dbGaP at http://www.ncbi.nlm.nih.gov/gap through dbGaP accession number phs000424.v6.p1. Raw read sequence alignment and gene expression quantification was performed with the Tuxedo suite (Langmead *et al.* 2009, Trapnell *et al.* 2009, 2012). In brief, raw reads were first aligned to the human genome (hg19 version) using TopHat v2.0.12 (Trapnell *et al.* 2009) with default parameters, and Cufflinks (Trapnell *et al.* 2012) was used with default settings to quantify the expression levels as fragments per kilobase of exon per million fragments mapped (FPKM). Further analysis included normalization of transcript levels and differential expression analysis using Cuffnorm and Cuffdiff tools (Trapnell *et al.* 2012).

Publicly available raw ChIP-seq datasets were obtained from the Sequence Read Archive (SRA) database as listed in Supplementary Table 3, and aligned and processed as follows to infer putative Notch1-bound regulatory regions in pituitary GC cells. Sequence reads were aligned to the rat genome (version rn4) using Bowtie 1.1.1 (Langmead *et al.* 2009). Only sequences uniquely aligned with ≤1 mismatch were retained. Post-alignment processing of sequence reads included *in silico* extension and signal normalization based on the number of million mapped reads. Reads were extended to a final length equal to MACS fragment size estimation (Zhang *et al.* 2008), and only unique reads were retained. For signal normalization, the number of reads mapping to each base in the genome was counted using the genomeCoverageBed command from BedTools(Quinlan & Hall 2010). Processed files were visualized in the UCSC genome browser (Kent *et al.* 2002). ChIP-seq enrichment sites were detected with MACS v1.4.0beta (Zhang *et al.* 2008) using default parameters and a *P* value of 1e-5. A control dataset derived by sequencing input DNA samples was used to define a background model.

Next, active regulatory regions in GC cells were defined as H3K27ac-enriched genomic sites that overlapped with H3K4me1 signal in rat GC cells. To infer putative Notch1 binding, we lifted over the Notch1 peaks as published by the group of Dr Pear (Zhang *et al.* 2008) to the rat genome (rn4) and searched for overlap among these sites and the active regulatory regions profiled in GC cells. For gene ontology analysis, the putatively Notch1-bound active regulatory regions in GC cells were lifted over to the mouse (mm9) genome, regions were associated to genes and gene ontology analysis was performed using GREAT with default settings. To gain further insights into the tissue specificity of the regulatory regions of interest for this work, we also downloaded, re-aligned and analyzed a Pit1 ChIP-seq dataset profiled in GC cells.

Combination of *in silico* transcriptomic and epigenomic analyses allowed us to choose several tumor suppressors associated with enhancers potentially bound by Notch1 (and thus putative downstream targets of Notch signaling) to be evaluated in our experiment, namely *Btg2*, *Nr4a1*, *Men1*, *Zfp36* and *Cnot1*.

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Statistical analysis

Results are expressed as means ± S.E.M. The differences between means were analyzed by the unpaired Student's *t*-test in the case of only two groups. Two-way ANOVA with repeated-measures design was used to analyze tumor volume *in vivo*, prolactin and GH secretion *in vitro*, protein and gene expression *in vitro*, motility and proliferation assays, for the effects of drug and time. *Post hoc* Tukey's test was employed when necessary. Parametric or nonparametric comparisons were used as dictated by data distribution. $P<0.05$ was considered significant.

Results

Notch signaling inhibition decreased xenograft tumor growth and prolactin and GH content in GH3-inoculated Nude mice

Mice s.c. inoculated with 700,000 GH3 cells developed visible tumors 21 days after inoculation (Volume 61.0 ± 7.1 mm^3); at this point, i.p. DAPT treatment was started (day 0). Xenograft volumes in DAPT-treated mice were consistently smaller at all time points, and statistical significance was achieved beginning on day 16 after the initiation of treatment (Fig. 1A). Body weight remained unaltered during treatment (Fig. 1B). Average tumor volume was 42% lower in DAPT-treated mice at killing (Fig. 1A), and prolactin and GH tumor content and serum prolactin levels were significantly decreased in the DAPT group at the end of the treatment period (Fig. 1C).

DAPT treatment decreased Notch 2 intracellular domain, the target protein HES1 and target gene *Hey2*

By Western blot, we identified NOTCH 1 and 2 active intracellular domain (NICD 80 kDa) and the membrane domain (110 kDa). N2ICD significantly decreased after inhibition of γ-secretase by DAPT (Fig. 2A), while membrane domain levels were not modified. Furthermore, DAPT treatment decreased HES1, a Notch target protein (Fig. 2B). These results indicate that i.p. DAPT effectively reduced Notch activation and signaling in xenografts.

Messenger RNA levels of *Notch2* receptor, Notch ligands (*Jagged 1* and *Dll1*) and several Notch target genes (*Hey1* and *2*, *Hes1* and *5*, *Cyclin D1* and *D3* and *Tgfb1*) were measured in *ex vivo* xenotransplants by RTqPCR at the end of the treatment. No differences in *Notch2* mRNA levels (which measures both active and membrane domains) or Notch ligands were found in response to DAPT treatment *in vivo* (Fig. 3A). The target gene *Hey2* was markedly decreased ($P=0.020$), while no differences were encountered for *Hey1*, *Hes1*, *Cyclin D1*, *Cyclin D3* or *Tgfb1* mRNA expression (Fig. 3B and C). On the other hand, *Hes5* and *Dlk1* could not be detected in the xenotransplants (not shown).

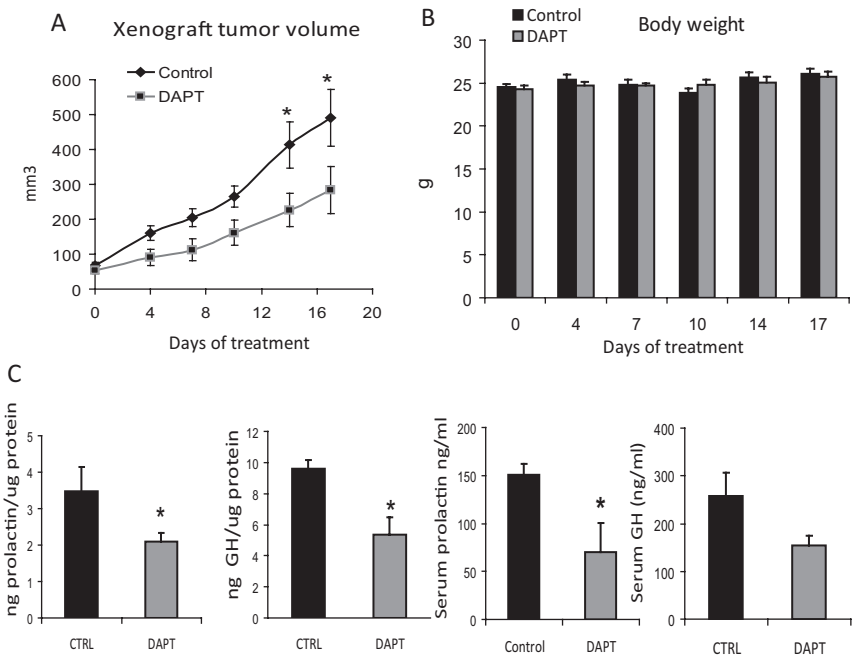

Figure 1
Notch signaling inhibition decreased xenograft volume and prolactin and GH tumor content in GH3 inoculated Nude mice. (A) Tumor volume (in mm^3) in DAPT and vehicle-treated GH3 inoculated Nude mice. DAPT treatment (8 mg/kg, three times a week) was begun on day indicated as 0. *$P \leq 0.05$. $N = 11$ control, and 12 DAPT. (B) Body weight was not modified by the treatment. (C) Prolactin and GH content in the excised tumor (ng prolactin/μg protein), and serum prolactin and GH on day 17 (ng/mL). $N = 11$ and 12 for hormone content, and 10 and 10 for serum hormones, control and DAPT, respectively. *$P \leq 0.05$, DAPT vs control.

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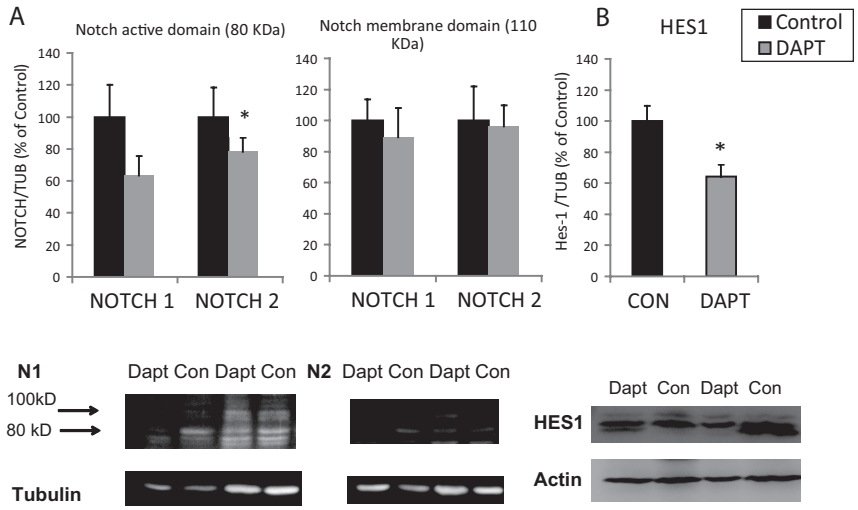

Figure 2
DAPT treatment decreased Notch 2 intracellular domain and the target gene *Hes1*. (A) Effect of DAPT treatment on NOTCH 1 and 2 intracellular domains (80 kDa, Western blot), and NOTCH 1 and 2 membrane domain (110 kDa, Western blot) in tumors excised after 17 days of DAPT treatment. *$P \leq 0.05$, DAPT vs control, $N = 11$ and 12 for NOTCH 1, and 7 and 8 for NOTCH 2. (B) Effect of DAPT treatment on the target HES1 (Western blot), $N = 11$ and 12 , $P = 0.033$. Below representative blots for Notch1 (N1, left) Notch 2 (N2, middle) and HES1 (right); Con, Control.

Bioinformatic analyses identified specific Notch targets in pituitary

The results prompted us to search for additional Notch target genes in pituitary tissue. To that end we undertook a bioinformatic approach which consisted in analyzing the information of existing ChIP-seq data to infer putative Notch1-bound regulatory regions in pituitary cells. For this purpose, we first defined 53,695 active regulatory regions in pituitary GC cells as those genomic regions co-enriched for H3K4me1 and H3K27ac signals, as previously reported (Heintzman *et al.* 2007, Pasquali *et al.* 2014). In these regions, 2699 putative Notch1-bound sites were identified, that resulted from the intersection of regulatory regions in GC cells with Notch1-binding sites (lifted over from T-ALL, see Materials and methods). The rationale for this choice was that, given that Notch ChIP-seq in pituitary samples was not available, some of the Notch-binding sites in other tissue samples could overlap with those of pituitary cells, as long as both cell types have accessible chromatin (as in the case for the active regulatory regions profiled in GC cells). We are aware that this approach may not detect all tissue-relevant Notch1-binding sites in pituitary cells. Rather, it was useful in the context in which it was applied, to infer a subset of regions that might have a shared relevant role in tumorigenesis not only in T-ALL and pituitary, but potentially in other tissues. And, furthermore, in order to assess the relevance of the genes analyzed for the function of pituitary cells, we checked for the presence of Pit1-binding sites, a pituitary-specific transcription factor, at their nearby regulatory regions.

Then, the genes associated with the putative Notch1-bound regulatory regions in GC cells (see Materials and methods) were functionally annotated. Our results revealed, among others, significant enrichment for categories related to 'Genes involved in positive regulation of mRNA catabolic process' ($P = 1.1E-7$) and 'histone lysine methylation' ($P = 5.8E-6$). Noteworthy, these analyses revealed putative Notch1-target genes *Btg2*, *Cnot1*, *Men1*, *Nr4a1* and *Zfp36* with previously reported or suspected tumor suppressor functions (Rouault *et al.* 1996, Farioli-Vecchioli *et al.* 2007, Hafner *et al.* 2011, Wenzl *et al.* 2015, Montorsi *et al.* 2016).

We next compared the list of genes associated with putative Notch1-bound regulatory regions in GC cells with transcriptome information obtained by comparing human control pituitaries (six samples) and three pituitary adenomas (a PRL/GH adenoma and two GH adenomas) (Table 1 and Supplementary Table 4). We quantified gene expression from RNA-seq datasets, and by performing comparisons between Control and PRL/GH+GH adenomas, we found 1778 differentially expressed genes (Supplementary Table 4). These included *BTG2*, *ZFP36* and *NR4A1*, which were significantly downregulated in human pituitary somato/somatolactotrope adenomas when contrasted to the control human pituitaries (Table 1). Noteworthy, *NR4A2* and *NR4A3* expression was also highly downregulated in the adenomas (Supplementary Table 4), further supporting a relevant role for the *NR4A* genes in suppressing adenoma development.

Figure 4 shows the epigenomic profiles for the loci containing the five putative Notch target genes in pituitary cells, which emerged from our strategy: *Men1*, *Zpf36*, *Btg2*, *Cnot1* and *Nr4a1*. These five genes had active regulatory regions nearby (i.e. co-enrichment of H3K4me1, brown signal and H3K27ac, yellow signal, in the plots), and putatively bound by Notch1 (grey boxes). The presence of binding sites

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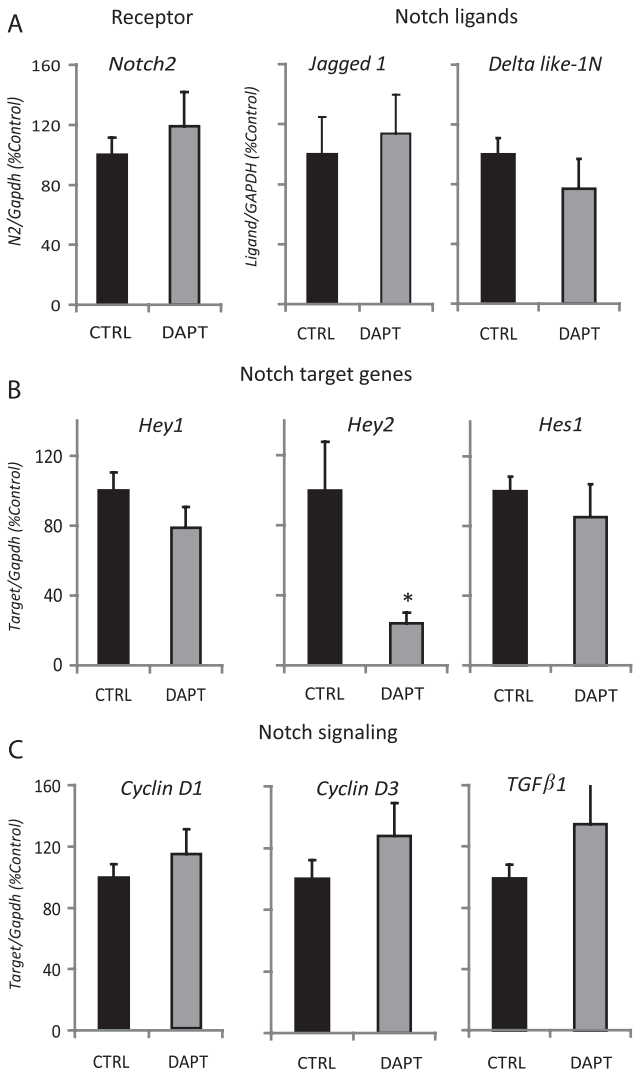

Figure 3
Effect of DAPT treatment on Notch receptors, ligands and target genes. (A) mRNA levels of *Notch 2* receptor, and the ligands Jagged1 and Delta like 1N in xenografts from control and DAPT treated mice; (B) mRNA of Notch target genes and (C) mRNA levels of Notch target genes involved in proliferation or epithelial-to-mesenchymal transition. *$P = 0.020$, N between 8 and 12.

for Pit-1 (blue signal) validates pituitary specificity of the analysis). Noteworthy, active regulatory regions that could bind Notch1 were found especially in *Btg2* and *Nr4a1*, which had 3 and 4 regions, respectively; *Cnot1* and *Men1* had 2 each and *Zfp36* had only one (Table 1).

Taken together, these *in silico* transcriptomic and epigenetic analyses prompted us to evaluate the tumor suppressors *Btg2*, *Nr4a1*, *Men1*, *Zfp36* and *Cnot1* as targets of Notch signaling in our model.

DAPT treatment increased mRNA levels of the tumor suppressors *Btg2* and *Cnot1* in xenografts

A direct interrogation of gene expression in GH3 cells compared to pituitary tumors originated by xenograft transplants of GH3 cells showed that all suppressor genes presented a downregulation trend in the xenografts, which indeed achieved significance for *Btg2* and *Nr4a1* mRNA expression levels (Fig. 5A). Conversely, *in vivo* inhibition of Notch (DAPT treatment) significantly increased the expression of the tumor repressor genes *Btg2* and *Cnot1* in the xenografts (Fig. 5B), advancing them as potential mediators of the DAPT-induced pituitary tumor growth inhibition and suggesting new putative therapeutic targets for pituitary adenoma treatment. mRNA but not protein levels were evaluated in the absence of adequate commercial antibodies for all transcription factors, therefore, results should be interpreted with caution.

DAPT treatment decreased *in vivo* angiogenesis in xenotransplants

Immunohistochemical analysis of xenotransplants at the end of treatment showed that microvascular CD31+relative area was reduced by DAPT (Fig. 6A), with no significant differences in vessel size or density (Fig. 6B and C). Moreover, αSMA+ vascular area and vessel size but

Table 1 Transcriptomic analysis of selected genes based on a combined differential gene expression (control pituitaries and a somatolactotrope/somatotrope adenomas) and ChIP-seq analysis.

Gene	Signaling	Control	Ad-PRL-GH/GH	*q*_value	#of Active Regulatory Regions bound by Notch 1
MEN1	Suppressor	22.5	17.5	N.S.	2
ZFP36	Suppressor	258.8	22.0	**0.011**	1
BTG2	Suppressor	276.5	18.4	**0.002**	3
CNOT1	Suppressor	30.1	29.8	N.S.	2
NR4A1	Suppressor	1046.1	12.0	**0.002**	4

q values indicate significant differences between transcriptomes as evaluated by Trapnell *et al*. (2012). The number of Active Regulatory Regions putatively bound by Notch 1 (determined by ChIP-seq analyses as described in Materials and methods) is also shown.
Ad-PRL-GH/GH, somatolactotrope and somatotrope adenomas.

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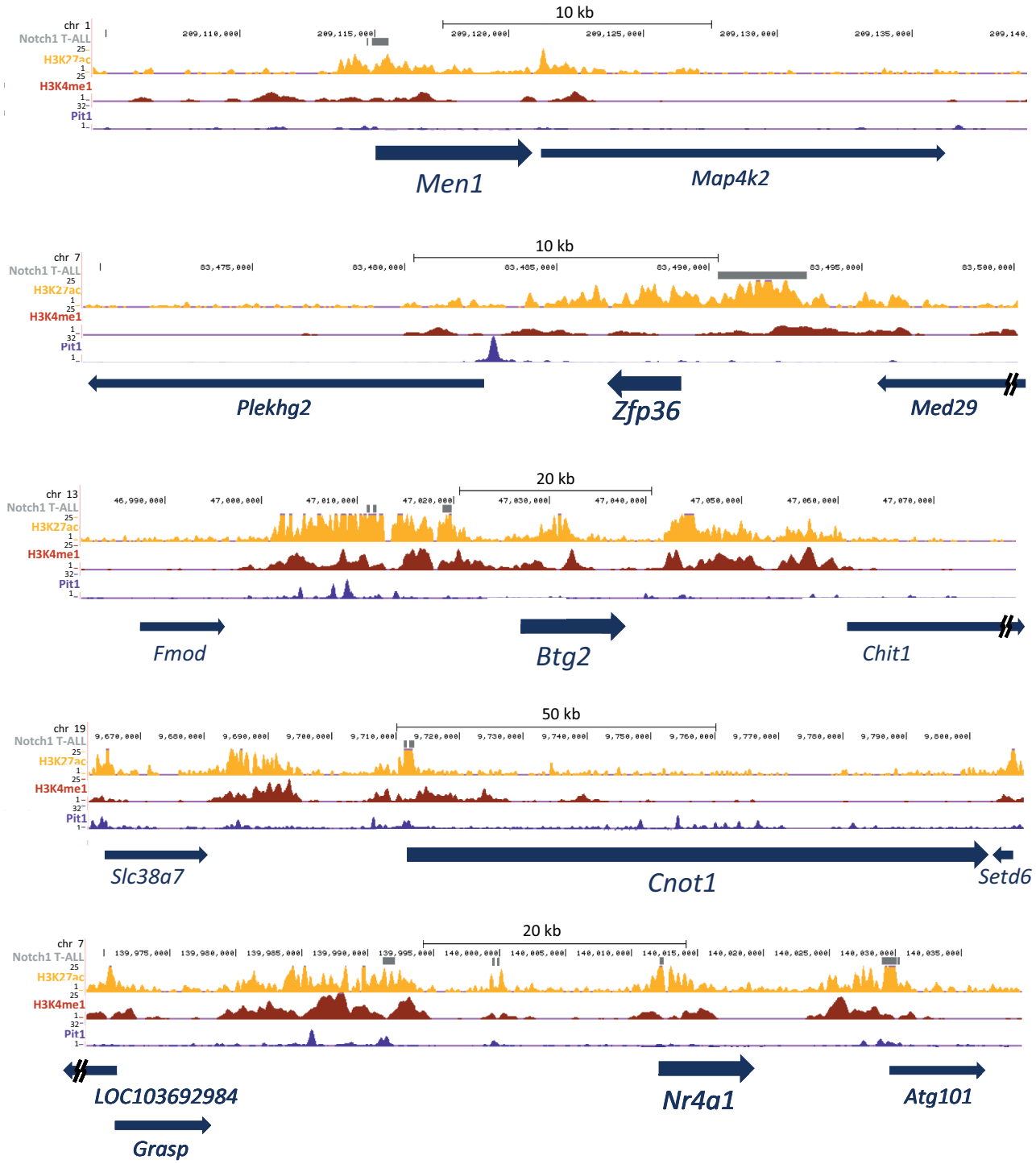

Figure 4

Genome Browser screenshots showing the epigenomic profile in the vicinity of selected genes. The enrichment profiles for H3K4me1, H3K27ac and Pit1 in GC cells, as well as the putative binding sites for Notch1 (gray boxes on top) are presented. A full colour version of this figure is available at https://doi.org/10.1530/ERC-18-0337.

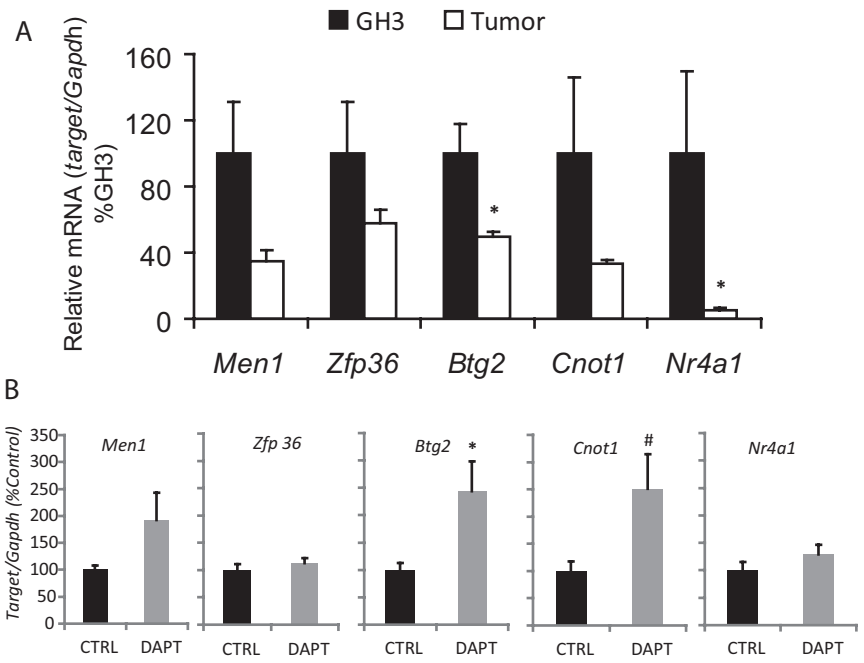

Figure 5
DAPT treatment increased the tumor suppressors *Btg2* and *Cnot1*. (A) Comparative mRNA levels of rat *Men1*, *Zfp36*, *Btg2*, *Cnot1* and *Nr4a1* in GH3 cells and xenografts resulting from GH3 inoculation (tumor).*$P \leq 0.020$, *N* 3 and 3. (B) Effect of DAPT treatment on mRNA levels of Notch targets in excised tumors at the end of the treatment. *$P \leq 0.01$, and #$P = 0.06$; *N* 7 and 8, control and DAPT, respectively.

not microvessel density were decreased in xenotransplants of DAPT-treated mice (Fig. 6D and E). These findings suggest an anti-angiogenic effect of Notch inhibition in pituitary xenotransplants.

Effect of *in vitro* DAPT treatment on Notch system components

We next tested a direct effect of DAPT on Notch signaling in GH3 cells. After a 48-h incubation period, DAPT (10 µM) decreased N2ICD but not HES1 protein or NOTCH2 membrane domain (Fig. 7A). mRNA levels of the target gene *Hes1* was also decreased *in vitro* by DAPT treatment (at 1 and 5 µM: Fig. 7B) while no significant differences were found for *Jag1*, and the target genes *Hey1* and *2*, *Cyclin 3* and *Tgf b1* (Fig. 7B and not shown).

DAPT decreased hormone secretion in cultured GH3 cells

We evaluated prolactin and GH secretion after *in vitro* DAPT treatment for 24 and 48 h of GH3 cell cultures.

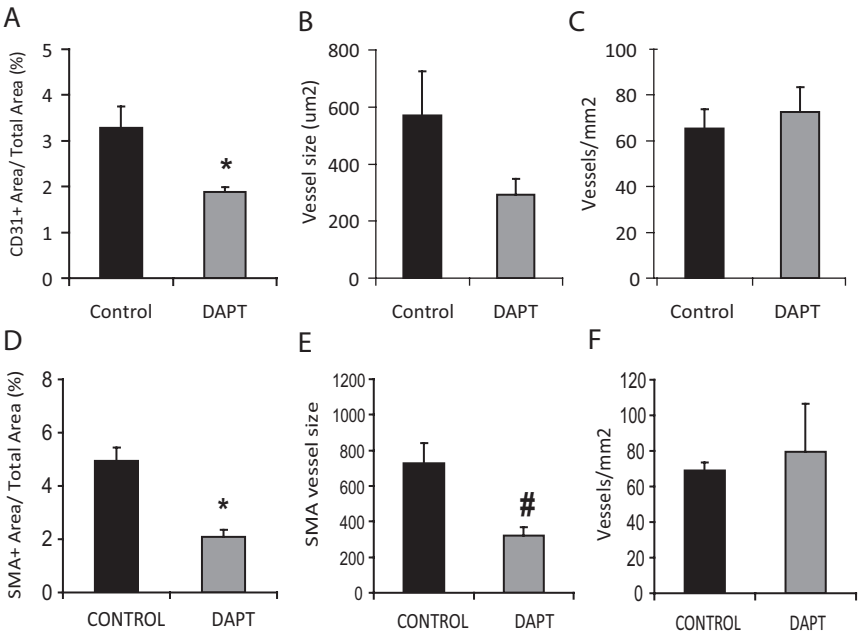

Figure 6
DAPT treatment decreased angiogenesis in GH3 xenografts. Effect of DAPT treatment on (A) CD31+ vessel area/total area %, (B) average vessel size (µm²) and (C) microvessel density (number of vessels per mm² in immunohistochemical evaluation of excised tumors at the end of the treatment. *$P = 0.046$; *N* 4 and 4, control and DAPT, respectively. (D, E and F) αSMA vessel area/ total area %, average vessel size (µm²), and microvessel density, respectively. *$P = 0.015$; #$P = 0.052$; *N* 3 and 3, control and DAPT, respectively.

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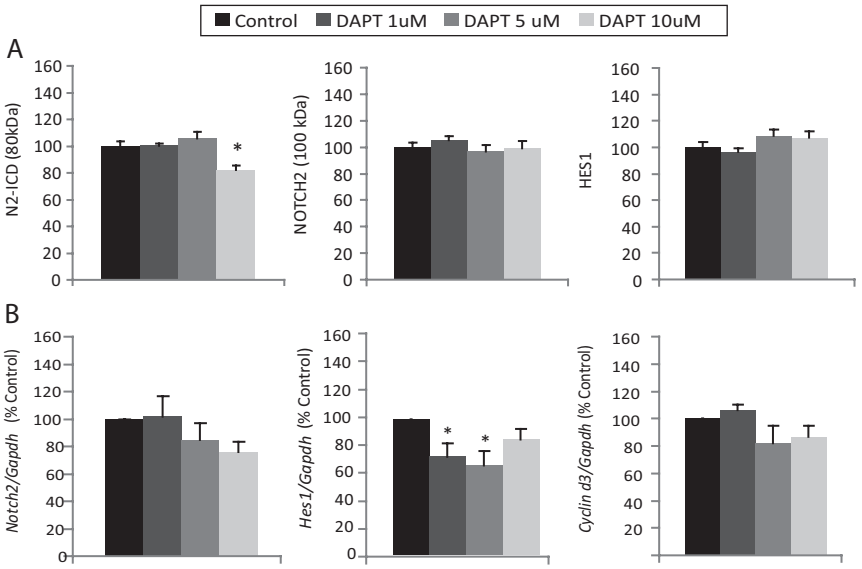

Figure 7
In vitro DAPT treatment decreased *NOTCH2* intracellular domain and Hes1 mRNA levels in GH3 cells. (A) Effect of 48-h treatment with DAPT (1, 5 and 10 μM) on active and membrane Notch 2 receptor (80 and 100 kDa respectively), and Hes-1 measured by Western blot analysis. $N = 4$ independent cultures, of duplicate samples. (B) Effect of DAPT (1, 5 and 10 μM) on mRNA levels of Notch signaling components. $N = 4$ independent cultures of duplicate samples. $^*P \leq 0.05$ vs control group.

Prolactin secretion was decreased at 24 and 48 h by DAPT incubation in a concentration-related manner (Fig. 8A). On the other hand, no significant differences were observed for GH secretion (Fig. 8B).

DAPT decreased proliferation and prevented cell motility in GH3 cells *in vitro*

DAPT (10 μM) decreased cellular proliferation, as measured by MTS assay, at 24 but not at 48 h of incubation (Fig. 9A).

Finally, DAPT (10 μM) prevented cell motility or wound healing in GH3 cells at 24, 48 and 72 h of incubation as evaluated by a scratch assay (Fig. 9B and C). Both results indicate an active participation of Notch signaling in GH3 cell proliferation and migration.

Discussion

Various components of the Notch pathway are expressed during pituitary development, including Notch2 and 3 receptors, the ligand Jagged1 and the downstream effector Hes1 (Raetzman *et al.* 2006). *NOTCH2* is expressed in the periluminal cells of Rathke's pouch that are undergoing rapid proliferation but not in the differentiated cells that are able to secrete glycoprotein hormones (Raetzman *et al.* 2006), and its expression, as well as that of several Notch family members, decrease as pituitary development proceeds, indicating an inverse correlation with cell differentiation (Raetzman *et al.* 2004). Nevertheless, in the adult pituitary gland, components of the Notch signaling pathway persist, particularly in locations containing progenitor/stem cells, both in hormone-producing and hormone-null cells (Chen *et al.* 2005, 2006, Kelberman *et al.* 2009, Tando *et al.* 2013, Mertens *et al.* 2015, Perrone *et al.* 2017). This aspect is in line with the role of Notch in maintaining progenitor cells in an undifferentiated state (as documented in the brain and intestine) and may be important in pituitary plasticity.

Insights into pituitary tumorigenesis may be gained from studies on pituitary development and cell differentiation. Genes that are important during development or differentiation often contribute to tumor promotion, survival or resistance when they become uncontrolled. Indeed, cancer may be considered a developmental disease, and pathways such as Notch that can affect cell fate, and the balance between differentiation, apoptosis and proliferation, are known to be involved in tumorigenesis. Therefore, the Notch pathway is evolving into an actively pursued drug target in cancer.

In numerous types of experimental models of cancer blocking Notch activation by γ-secretase inhibitors, like DAPT and others, was effective in reducing proliferation or resistance to chemotherapeutics (Espinoza & Miele 2013). For example, in colon cancer cells, (Akiyoshi *et al.* 2008), in ER-negative breast cancer (Lee *et al.* 2008), glioma stem cells (Wang *et al.* 2010), prostate cancer (Wang *et al.* 2011), renal cell carcinoma (Sjolund *et al.* 2008) or experimental brain tumors (Gilbert *et al.* 2010). Nevertheless, it has become apparent that altered Notch status may be associated with both pro- and anti-tumor-suppressive roles. For example, it had a suppressive role in the formation of vascular tumors in the liver, in mouse keratinocytes, pancreatic and hepatocellular carcinoma (Koch & Radtke 2010, Liu *et al.* 2011, Ranganathan *et al.* 2011),

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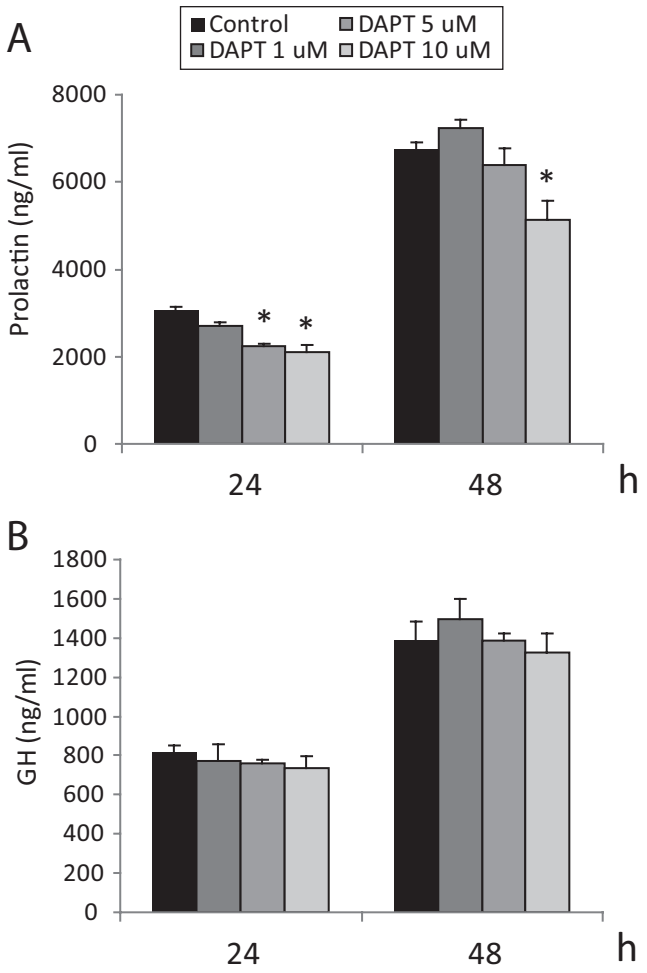

Figure 8
In vitro DAPT treatment decreased prolactin secretion by GH3 cells. Effect of DAPT (1, 5 and 10 µM) on (A) prolactin and (B) GH secretion by GH3 cells cultured *in vitro* for 24 or 48 h. $N = 3$ independent cultures, of duplicate samples. *$P \leq 0.05$ vs control group.

among others. Therefore, it is important to determine the functional direction of Notch activation in each tumor type as its effects are dependent on the cellular context and the interaction with other signal transduction pathways. In this context, the role of Notch signaling in pituitary adenoma development and growth has not yet been addressed. Recent evidence revealed a differential sensitivity to Notch activation within and between pituitary endocrine cell lineages during development (Cheung *et al.* 2018), which further stresses the necessity to establish its role within each pituitary adenoma histotype.

Descriptive but not functional data of Notch pathway in pituitary tumors suggest that Notch may be implicated in the pathogenesis of human pituitary adenomas. By microarray analysis, Evans *et al.* identified increased *NOTCH3*, *DLK1* and decreased HES1 in prolactinomas compared to normal pituitaries (Evans *et al.* 2008). Data from Runchun *et al.* indicated non-significant increases in NOTCH3 and JAGGED1 expression in prolactinomas compared to normal pituitaries; however, only 4 prolactinomas were used in the study (Lu *et al.* 2013). Functional studies of pituitary tumor generation or maintenance using Notch inhibition are lacking in prolactinomas, somatotropinomas, somatolactotropinomas or corticotropinomas. One *in vitro* study was performed by Tando *et al.* (2013) who described that DAPT treatment of anterior pituitary cells in culture decreased *Hes1* mRNA levels, and proliferation but only in the non-hormone-producing S100 cells of the S100b-GFP rat (Tando *et al.* 2013). Nevertheless, the fact that many pituitary cell types co-exist in the normal pituitary is an important caveat that should be kept in mind when interpreting results presented in this work, as in the other mentioned studies that compare tumoral and normal pituitaries. To address this limitation, we performed a combined transcriptomic and epigenomic approach, which allowed focusing our functional analysis and ultimately validate part of our findings.

In a previous study, we showed that in prolactinomas which develop in lacDrd2KO female mice, *Notch1* and *Notch3* mRNA levels and also NOTCH 2–3 membrane and N1ICD were highly expressed compared to pituitaries of control animals (Perrone *et al.* 2017). We also determined that all four Notch receptors were expressed in somatolactotrope GH3 cells, and that N2ICD, and *Jagged1*, *Dll1* and *Hey1* were upregulated in the cell line compared to rat pituitary cells. We therefore sought to determine if inhibition of Notch signaling would modify GH3 xenotransplant growth and angiogenesis in Nude mice. We used DAPT a γ-secretase inhibitor, which prevents cleavage of intracellular Notch domains, and therefore, modifies target-specific transcription factors in the nucleus.

Our results show that inhibition of γ-secretase lowered tumor burden by 42% and decreased tumor angiogenesis by 26% in somatolactotrope xenotransplants. It effectively decreased active N2ICD formation, expression of the target protein HES1 and the *Hey2* gene indicating a blockade of Notch signaling, and suggesting a novel strategy in the treatment of aggressive or resistant prolactinomas. Nevertheless, the lack of specificity of targeting γ-secretase may constitute a significant limitation (Lamy *et al.* 2017), and therefore, specific tumor-related targets activated by Notch signaling in different tumor types are under the spotlight. The classical Notch targets, such as HES and

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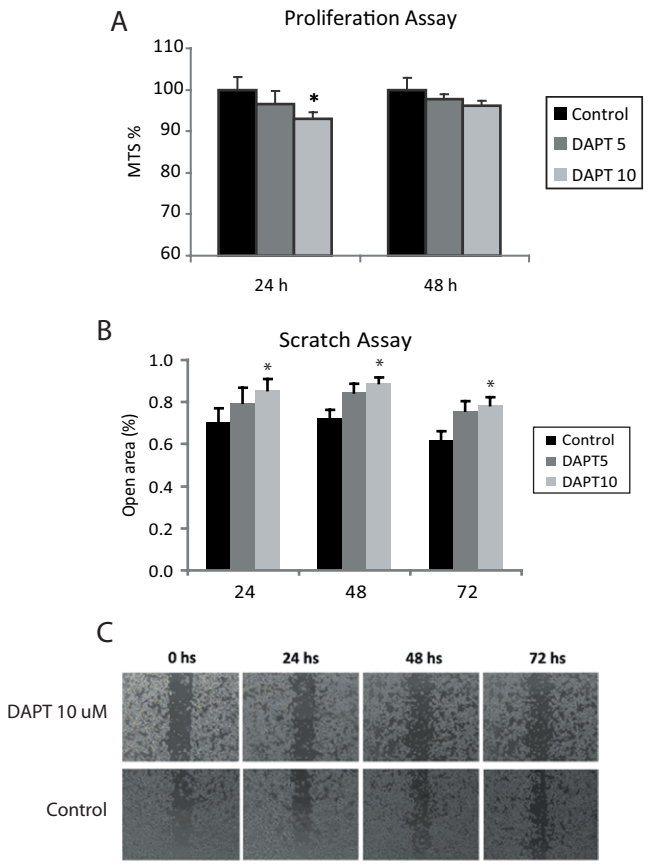

Figure 9

In vitro DAPT treatment decreased proliferation and migration in GH3 cells. (A) Effect of 24 and 48-h DAPT treatment (1, 5 and 10 μM) on cell proliferation assessed by MTS assay. *$P \leq 0.05$ vs control group, $N = 4$ independent cultures of duplicate samples; (B) Effect of 24, 48 and 72-h treatment with DAPT (1, 5 and 10 μM) on remaining open area in culture in a scratch assay (expressed as % of initial open area). *$P \leq 0.05$ vs control group, $N = 4$ independent cultures of duplicate samples. (C) Representative images.

HEY families, are recurrently found in many tissues, while others seem to be tissue specific. In this context, the inventory of Notch targets has begun to expand (Hurlbut *et al.* 2009). Recent gene expression studies combined with chromatin immunoprecipitation arrays revealed the existence of a large number of genes that can directly be regulated by Notch in different solid tumors (Koch & Radtke 2010). The challenge will be to identify and distinguish driver target genes from passenger ones in each cancer type. In this context, our bioinformatic approach combining epigenomic and transcriptomic information obtained from public databases uncovers potential genes activated by Notch, which may be specific to pituitary adenoma development and growth.

By epigenomic profiling of active regulatory regions (enhancers and promoters, which might also present mild enrichments in H3K4me1 Heintzman *et al.* 2007) in a pituitary somatotrope cell line we were able to infer putative Notch-bound regions and novel target genes. Combining this information with the differential gene expression profiles obtained from human normal and somatotrope+somatolactotrope pituitary samples allowed us to focus on some interesting putative Notch targets genes, whose regulation was next validated experimentally. Epigenetic analysis revealed that the genes *Btg2*, *Nr4a1*, *Men1*, *Zfp36* and *Cnot1*, presented active regulatory regions associated to Notch-binding sites. Particularly, the Notch-related tumor suppressor genes selected by epigenetic analysis, *Btg2*, *Zpf36* and *Nr4a1* were downregulated in all somatotrope and somatolactotrope adenomas when compared to normal pituitaries. Therefore, these *in silico* transcriptomic and epigenomic analyses allowed us to select several tumor suppressors of Notch signaling in pituitary tissue to be evaluated in our study, namely *Btg2*, *Nr4a1*, *Men1*, *Zfp36* and *Cnot1*.

Our results point to *Btg2*, *Nr4a1* and *Cnot1* as possible players in GH3 xenograft development and growth. In particular, *Btg2* mRNA expression was lower in xenografted GH3 cells compared to the parental line, and DAPT increased its expression in the xenograft in parallel with the inhibition of tumor volume. *Nr4a1* was also decreased in xenotransplants compared to the parental line, similar to results uncovered in RNA-seq analysis comparing human somatolactotrope adenomas and normal pituitaries, but its expression was not modified by DAPT treatment. Finally, *Cnot1*, also a suppressor gene was increased by DAPT treatment in the pituitary xenografts.

B-cell translocation gene 2, *BTG2*, is a tumor suppressor gene whose overexpression leads to decreased proliferation and arrest of cells at the G1 phase of the cell cycle (Rouault *et al.* 1996). It is downregulated in preneoplastic and neoplastic lesions in various cancers (Farioli-Vecchioli *et al.* 2007, Mao *et al.* 2015), and it intersects with the Notch pathway (Farioli-Vecchioli *et al.* 2014). It is found in the embryonic and adult anterior pituitary (Terra *et al.* 2008), and, using the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank/) to analyze the differentially expressed genes in plurihormonal and GH pituitary adenomas compared with healthy pituitaries, *BTG2* was found downregulated (Jiang *et al.* 2010, 2012). In accordance, our experimental and bioinformatic results support its role as a tumor suppressor in the pituitary and suggest its regulation by Notch.

Nuclear receptor (NR) subfamily 4 group A (NR4A) is a family of three highly homologous orphan nuclear

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receptors that have multiple physiological and pathological roles. These NRs are reportedly dysregulated in multiple cancer types, with many studies demonstrating pro-oncogenic roles for NR4A1 (Nur77) and NR4A2 (Nurr1), while tumor suppression roles have been suggested for NR4A1 and NR4A3 (Nor-1) in leukemia (Wenzl *et al.* 2015). In the pituitary, NR participates in CRH-induced proopiomelanocortin expression in corticotrophs (Kovalovsky *et al.* 2002), and *Nur77* gene expression levels may be critical in the different autonomy of ACTH production between Cushing's syndrome and subclinical Cushing's syndrome (Tabuchi *et al.* 2016). Our results demonstrate that *Nr4a1* is decreased in GH3 xenografts, and our re-analysis of public RNA-seq datasets showed that expression of all NR4A genes (*NR4A1*, *NR4A2* and *NR4A3*) is severely downregulated in somatoprolactinomas and somatotropinomas when compared to normal human pituitaries, indicating a possible suppressive role for this gene in the pituitary.

CNOT1 is a scaffold protein of the CCR4–NOT complex. This complex participates in various physiological functions, including cell proliferation, apoptosis, mitotic progression, fertility, bone formation, heart function, energy metabolism (Zukeran *et al.* 2016) and miRNA-mediated mRNA repression (Hafner *et al.* 2011). Furthermore, CCR4-NOT deadenylase activity contributes to induction of pluripotent stem cells (Zukeran *et al.* 2016). No relation has been yet established with pituitary regulation, and our results show that it can be modulated by Notch inhibition to potentially activate a putative tumor-suppressive role.

Menin is a putative tumor suppressor associated with multiple endocrine neoplasia type 1 (MEN-1 syndrome), and the development of tumors in target neuroendocrine tissues. Even though epigenetic analysis suggested that it may be regulated by Notch signaling, no difference for this gene was found in DAPT-treated tumors. Finally, the mRNA-destabilizing protein ZFP36, which had been previously described as a tumor suppressor and impairs the epithelial-to-mesenchymal transition (Montorsi *et al.* 2016) was not modified in the present experimental model.

Among the signaling pathways involved in tumor angiogenesis, Notch signaling stands as a crucial player. This pathway does not just participate in physiological angiogenesis during development, wound healing or pregnancy, but is also involved in pathological vascularization, such as in tumor angiogenesis. Importantly, experimental evidence revealed that Notch may be involved in anticancer drug resistance, indicating that targeting this pathway could be a novel therapeutic approach to the treatment for cancer by overcoming drug resistance. Notch receptors and ligands are widely expressed in the vasculature, but as described for tumor proliferation, it has been reported that Notch has angiogenic properties, but may also act in anti-angiogenesis in vascular tumors (Liu *et al.* 2011). It is therefore paramount to validate its angiogenic action in each tumor type.

We show that DAPT treatment decreased microvascular area determined by $CD31^+$ and αSMA+cells, indicating that in pituitary tumors Notch increases angiogenesis, as described for neck squamous cell or breast carcinoma models (Zeng *et al.* 2005, Funahashi *et al.* 2008), among others. This is a novel finding for pituitary tumors and should be highlighted in the context of anti-angiogenic therapies, which have been successful in experimental prolactinomas (Luque *et al.* 2011), as well as in a particularly aggressive Cushing tumors (Ortiz *et al.* 2012, Touma *et al.* 2017). Furthermore, the fact that DAPT reduced the expression of the smooth muscle cell marker αSMA is an indication that the Notch system may participate in vasculature remodeling and vessel maturation through interaction of mural and endothelial cells, as described for NOTCH3 (Liu *et al.* 2009).

We have previously shown that expression of different components of the Notch system vary when comparing GH3 *in vivo* tumors generated by GH3 inoculation, and GH3 cells. GH3 tumors showed higher activation of NOTCH1 and lower of NOTCH2 receptor than isolated GH3 somatolactotropic cells (Perrone *et al.* 2017). Differences in *Dll1* ligand expression were also observed, suggesting that tumor vasculature and/or extracellular matrix components, which are absent in cell lines may be important modulators of Notch signaling in xenografted somatoprolactinomas. The extracellular matrix plays a critical role in tumor development in various cancers, and its importance in xenograft growth cannot be disregarded. Therefore, in order to ascertain whether the Notch system cell-autonomously participated in GH3 tumor development, we performed *in vitro* studies inhibiting γ-secretase directly in cultured GH3 cells. Our results clearly indicate that Notch signaling in GH3 cells is positively involved in cellular proliferation and migration. Similarly, results using DAPT treatment of pituitary explants *in vitro* or postnatal mice *in vivo* suggested that Notch signaling allows pituitary cell proliferation during postnatal development, even though a direct effect on dispersed cells was not verified in this study (Nantie *et al.* 2014). Furthermore, we show that inhibition of Notch activation led to decreased prolactin

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but not GH secretion, suggesting a differential activity in the production of both hormones. In human GeneChip microarrays and proteomics analyses, increased expression of *NOTCH3* was found in prolactin and non-functioning secreting adenomas while in somatotropinomas, a significantly reduced expression of *NOTCH3* was found (Moreno *et al.* 2005, Evans *et al.* 2008). Furthermore, in GH3 cells, it was described that the non-canonical Notch ligand Dlk1 is expressed in some clones, in which it represses GH expression and secretion but does not affect prolactin production (Ansell *et al.* 2007). Therefore, it may be hypothesized that Notch manipulation may have a differential outcome for prolactin and GH-secreting tumors.

Personalized molecular treatments based on specific genetic markers may improve diagnosis, treatment and outcome in resistant and aggressive somatotropinomas and somatoprolactinomas. In this context, salient features identify Notch as a candidate diagnostic and prognostic biomarker and a promising target for cancer therapy (Espinoza & Miele 2013). Currently, most Notch-directed therapies involve the use of γ-secretase inhibitors, but the lack of substrate specificity and associated toxicity found in clinical studies constitute limitations to their therapeutic use (Lamy *et al.* 2017). Antibodies have emerged as powerful biological therapeutics due to their specificity and efficacy; and soluble decoys which compete with natural ligands of Notch signaling but lack the transmembrane domain are being tested (Espinoza & Miele 2013). Our results suggest that interruption of Notch-selective pituitary targets might be a novel strategy when designing combinatorial treatment regimens in aggressive or atypical prolactin and GH-secreting adenomas.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0337.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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