Department of Clinical and Experimental Medicine
Division of Cell Biology

Final Thesis

The interaction between the Wnt –and Notch-pathways in colorectal cancer development

John Grünberg

LiTH-IFM-Ex

Supervisors: Jonas Ungerbäck, Linköpings Universitet
Peter Söderkvist, Linköpings Universitet
Examinator: Jordi Altimiras, Linköpings Universitet

Department of Clinical and Experimental Medicine
Faculty of Health Sciences, Linköpings Universitet
SE-581 85 Linköping, Sweden

Presentationsdatum	Institution och avdelning	
090529 **Publiceringsdatum (elektronisk version)**	**IKE, Avd för Cellbiologi**	Linköpings universitet

Språk	Typ av publikation	
___ Svenska _x_ Annat (ange nedan) Engelska **Antal sidor** 19	____ Licentiatavhandling _x_ Examensarbete ____ C-uppsats _x_ D-uppsats ____ Rapport ____ Annat (ange nedan)	**ISBN (licentiatavhandling)** **ISRN** **Serietitel (licentiatavhandling)** **Serienummer/ISSN (licentiatavhandling)**

Publikationens titel
The interaction between the Wnt –and Notch-pathways in colorectal cancer development

Författare
John Grünberg

Sammanfattning
Both Notch and the Wnt pathways are key regulators in maintaining the homeostasis in the intestine. Defects on the key tumor suppressor adenomatous polyposis coli, APC a gene in the Wnt pathway is most frequently mutated in colorectal cancer. Previous studies have indicated that there is a crosstalk between these two pathways. We investigate if there is correlation by first using bioinformatics to find Lef1/Tcf sites in several of the Notch pathway gene promoters. Bioinformatically we found that a lot of the genes contained theses sites controlled by the APC's destruction target β-catenin. By using semi quantitative PCR and western blot we found that Hes 1, Hes 7, JAG 2, MAML 1, Notch 2, NUMB, NUMBL, RFNG and LFNG was downregulated in HT29 colon cancer cells carrying a vector containing wild type APC. All but JAG 2 contains at least one Lef1/Tcf site in their promoter region. The results were verified in HT29 cells transfected with siRNA against β-catenin.
We also investigated what would happen to the Tcf/Lef target gene program of the Wnt pathway, if the Notch pathway was inhibited through the gamma-secretase inhibitor DAPT. Results showed no downregulution of β-catenin or its target gene Cyclin D1.
Taken together, these results demonstrate that the Wnt pathway can be placed upstream of the Notch pathway and regulates the latter through β-catenin and the Tcf/Lef target gene program. However, preliminary results indicate that there is no regulation of APC/β-catenin by the Notch pathway.

Nyckelord
Notch, Wnt, APC, β-catenin, Colorectal cancer, HT29, LEF1/Tcf

Content

1 Abstract

Both Notch and the Wnt pathways are key regulators in maintaining the homeostasis in the intestine. Defects on the key tumor suppressor adenomatous polyposis coli, APC a gene in the Wnt pathway is most frequently mutated in colorectal cancer. Previous studies have indicated that there is a crosstalk between these two pathways. We investigate if there is correlation by first using bioinformatics to find Lef1/Tcf sites in several of the Notch pathway gene promoters. Bioinformatically we found that a lot of the genes contained theses sites controlled by the APC's destruction target β-catenin. By using semi quantitative PCR and western blot we found that Hes 1, Hes 7, JAG 2, MAML 1, Notch 2, NUMB, NUMBL, RFNG and LFNG was downregulated in HT29 colon cancer cells carrying a vector containing wild type APC. All but JAG 2 contains at least one Lef1/Tcf site in their promoter region. The results were verified in HT29 cells transfected with siRNA against β-catenin.
We also investigated what would happen to the Tcf/Lef target gene program of the Wnt pathway, if the Notch pathway was inhibited through the gamma-secretase inhibitor DAPT. Results showed no downregulution of β-catenin or its target gene Cyclin D1.
Taken together, these results demonstrate that the Wnt pathway can be placed upstream of the Notch pathway and regulates the latter through β-catenin and the Tcf/Lef target gene program. However, preliminary results indicate that there is no regulation of APC/β-catenin by the Notch pathway.

2 List of abbreviations

APC - Adenomatous polyposis coli	Hes – Hairy enhancer of split
JAG – Jagged	MAML- Mastermind like
NUMBL – Numb like	RFNG – Radical fringe
LFNG – Lunatic fringe	MFNG – Manic fringe

3 Introduction

One of the most investigated organ systems in the human body is the intestine, for both normal intestinal development and cancer formation (Braletz et al. 2009). The most common form of cancer in the intestine is colorectal cancer (CRC) and it is the second most common type of cancer in both men and women in Sweden (Martling et al. 2008).
Dividing cells of the mammalian intestine are restricted to finger-like invaginations of the epithelium called the crypts of Lieberkühn. All gut epithelium cells originates from stem cells in these crypts. Each stem cell can give rise to four different types of cells: absorptive, goblet, enteroendocrine and Paneth cells. The progeny of the stem cells migrates upward, from the cryps towards finger-like outgrows into the lumen called *villi*. In the villi tips the division stagnates and the differentiation is completed (Crosnier et al 2006).
The organization to form the cryps and villi is maintained by a two-way communication system, where the hedgehog, PDGF and BMP signalling pathways are key mediators. Cell to

cell signalling in the epithelium is mainly carried out by Wnt, Notch and ephyrin pathways (Crosnier et al 2006).

The Wnt's are cytosine rich glycoproteins that in their active form bind locally to cellular receptors. In the mammalian Wnt family, there are nineteen members who are expressed in both embryos and adults (Kelleher et al 2006). The name Wnt comes from a discovery in *Drosophila*, where it was found that the polarity gene *Wingless* and the proto-oncogene *Int-1* had a common origin, the Wnt signaling pathway.
The Wnt cascade is a canonical pathway and a critical regulator of stem cells (Reya & Clevers 2005). It maintains cells in the crypts of Lieberkühn proliferative (Logan & Nusse 2004), but upon dysregulation of the activation, cancer ensues in tissues where Wnt controls stem cells (Reya & Clevers 2005).
Signaling cells releases or presents Wnt proteins on their surfaces to act on target cells by binding to a receptor complex called Frizzled/LDL/LRP. This complex mediates the signaling to several intracellular proteins including adenomatous polyposis coli, APC which is a part of a complex that is controlling the cytoplasmic β-catenin levels. Normally the β-catenin levels are low (Logan & Nusse 2004), but Wnt induces destabilization of this complex called β-catenin destruction complex leading to stabilization of β-catenin (Kelleher et al 2006). This raises the intranuclear levels of β-catenin which activates transient transcription of a Tcf/Lef target gene program. A complex of β-catenin and Tcf4 drives a genetic program in colorectal cancer cells, that are physiologically expressed in crypt stem- and progenitor-cells in the small intestine (Reya & Clevers 2005). The most studied disorder with Wnt pathway mutation is familial adenomatous polyposis, FAP that's causes hundreds or thousands polyps in the colon and rectum. The mutations are most frequently on adenomatous polyposis coli, APC a protein that stabilizes the β-catenin destruction complex. This causes a constitutive activation of the Wnt pathway which in turns leads to an increased cell proliferation leading to adenomatous lesions (Logan & Nusse 2004).

Notch is a cell surface receptor and is named Notch because a haploinsufficiency of the gene gives notched wings in *Drosophila*. There are four Notch genes in mammals, 1-4, and all are heterodimers consisting of an intracellular and extracellular part (Kelleher et al 2006). The receptor is single transmembrane and its extracellular region consist of conserved arrays of epidermal growth factors (EGF) like repeats (Fiúza & Arias 2007). These repeats bind ligands on the extra cellular surface of neighboring cells, the ligands are called delta-like and jagged (Kelleher et al 2006). Both Notch and its ligands are glycoproteins, and glycosylations on the EGF-like reapeats affects the Notch ligand-binding properties. OFUT1 and Fringe are two glycosyltransferases that can act on Notch and vertebrates have three homologues; Radical, Manic and Lunatic Fringe (Fiúza & Arias 2007).
Both delta-like and jagged are transmembrane proteins like Notch, which make cell to cell contact important to set off the Notch pathway. Interaction with a ligand results in release of the intracellular domain, NICD by a gamma-secretase complex. NICD translocates to the nucleus where it binds to CSL, also called RBP-J kappa.

CSL is a DNA-binding transcriptions factor, which upon formation of a ternary complex with NICD and Mastermind converts from being a transcriptional repressor to an activator (Fiúza & Arias 2007).

Formation of this complex results in transcription of the Notch signaling pathways target genes (Kelleher et al 2006). Many proteins of the Notch pathway expressed in the intestinal crypt and several genes of the Notch pathway are involved in the control intestinal homeostasis. The signaling is required for establishing the fate of the differentiating cells migrating from the crypts and also for maintain the crypts progenitors in an undifferentiated proliferative state which is essential for the crypt (Van Es & Clevers 2005).
The Notch pathway is very important in the developmental processes, and therefore there are a lot of diseases linked to defect genes involved in the Notch signaling, like Alagille syndrome, CADASIL syndrome and T-cell acute lymphoblastic leukaemia, where mutation in the Notch receptor is a common event (Fiúza & Arias 2007).

The crosstalk between these two pathways is relatively unknown but it is known that there are Tcf/Led binding elements in the Notch ligand Jagged 1 (S. Estrach et al. 2006). It has also been suggested that the Notch-pathway should be placed upstream of Wnt, where it has been seen that over expression of Notch-1 gave rise to higher level of cytoplasmic β-catenin and Wnt-dependent gene expression (Deregowski et al 2005). In one study, van Es and co-workers demonstrated that inhibition of the Notch pathway, while the Wnt is active drives differentiating cells exiting the crypt towards a secretory fate (goblet, enteroendocrine and Paneth cells), while inhibition of the Wnt pathway leads to an enterocyte fate (absorptive cells). They also demonstrated that one of Notch target genes, *Hes1*, an indicator of Notch signaling was expressed in high levels uniformly in adenomas of all sizes in the intestines of mice with trunked APC (APC^{min}). This suggests that the Wnt and Notch pathway is simultaneously active in proliferative adenoma cells, as in the intestinal cryps (Van Es JH et al. 2005). van Es and coworkers also suggest that the Wnt and Notch pathway works in synergy as a gatekeeper of self-renewal in the intestine epithelium, but that the Wnt signaling is the major driving force (Van Es JH et al 2005).

The aim of the study is to investigate the interaction between the Wnt –and Notch-pathways with focus of Wnt pathway regulation of the Notch-pathway, and if this process is important for colorectal cancer development and/or progression.
We will use human colorectal cell line HT29 were both endogenous APC alleles contain truncated mutations, leading to continuously Wnt pathway activation. By inserting a vector containing a zinc-inducible APC gene (HT29-APC) makes us control the deactivation of the Wnt pathway. We will also use a control cell line containing an analogous inducible lacZ gene (HT29-βgal) (T.-C. He et al 1998).
By treating the HT29 cells with DAPT, a gamma-secretase inhibitor which prevents release of Notch intracellular domain (H. Zhang et al 2008), we will examine what happens with the Wnt/β-catenin signaling when the Notch pathway is inhibited.

4 Materials and Methods

4.1 Identifying LEF1-TCF and RBPJ –kappa sites

Identification of potential promoter regions, LEF1-TCF sites and RBPJ-kappa sites was performed by using Genomatix software (http://www.genomatix.de, Genomatix Software GmbH, Munich, Germany) (Quandt et al., 1995). The Gene2Promoter software (Models: library Vertebrate_Modules Version 4.5) was used to retrieve and identify promoters 2500 bp upstream the first exon of each gene (2500 bp upstream after the first exon). The highest quality sequence obtained was then used in the MatInspector software (MatInspector Release professional 7.7.3) to search and identify putative transcription factor binding sites (Cartharius et al 2005).

The Matrix Family Library Version 7.0 (October 2007) was used and the selected groups was ALL vertebrates.lib were the standard (0.75) core similarity and the optimized matrix similarity was used. The number of LEF1-TCF sites and RBPJ-kappa sites in each gene was counted.

4.2 Cell cultivation

4.2.1 Cell cultivation of HT29 cells harbouring a vector

HT29 cells harbouring a vector containing the wild type APC gene(HT29-APC) or the control gene β-galactosidase(HT29- βgal), regulated by a zinc inducible promoter (generous gift from B. Vogelstein) was cultivated in McCoy´s 5A media (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum at 37ºC in 5% CO2. The vector-harbouring HT29 cells were selectively grown by the addition of 0.6 mg hygromycine B (Invitrogen) per mL media. Twenty-four hours before harvest, the cells were split with 0,05% Trypsin-EDTA (Gibco/Invitrogen) and counted. HT29-APC and HT29- βgal was seeded with the concentration of 1,0 x 10^6 cells/well in MULTIWELL™ 6 well (Falcon, BD Bioscience, San Jose, CA) and 100 μM $ZnCl_2$ was added to the medium in the interwall of 24, 18, 12, 6, 3 and 0 hours before cell harvest and RNA and protein isolation.

4.2.2 Cell cultivation of HT29 cells

HT29 colon cancer cells (ATCC, Manassas, VA, USA) was cultivated in McCoy´s 5A media (Gibco/Invitrogen) supplemented with 10% foetal bovine serum at 37ºC in 5% CO2. Twenty-four hours before transfection, the cells were split with 0,05% Trypsin-EDTA (Gibco/Invitrogen) and counted. Cells for siRNA transfection was seeded with a concentration of 2,5 x 10^5 cells/well in MULTIWELL™ 12 well (Falcon, BD Bioscience, San Jose, CA) and cells for DAPT treatment was seeded with a concentration 1 x 10^6 cells/well in MULTIWELL™ 6 well (BD Bioscience).

4.3 siRNA treatment of HT29 cells

A pool of four different siRNA oligonucleotides targeting β-catenin mRNA (siGENOME SMARTpool™, Dharmacon, Chicago, IL, USA) with the sequence: GAUCCUAGCUAUCGUUCUU, UAAUGAGGACCUAUACUUA, GCGUUUGGCUGAACCAUCA, GGUACGAGCUGCUAUGUUC, was transfected into HT29 with DharmaFECT4™ transfection reagent (Dharmacon) according to the

manufacturer's recommendations. The final siRNA concentration was 100 ηM. As control experiment, cells were in parallel mock transfected with siGLO® transfection indicator (Dharmacon) only or a non-specific siRNA pool (siCONTROL™ Non-Targeting siRNA pool, Dharmacon).

4.4 DAPT treatment

4.4.1 Determination of DAPT concentration for HT 29 cells with MTS assay

The gamma-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester) (Sigma, St. Louis, MO, USA) was dissolved in DMSO at a concentration 40 mM (stock solution), stored in at -70^{o}C. Cells were seeded in triplicates in an ELIZA plate, Tissue Culter Plate 96-well Flat bottom with lid (Sarstedt Inc. Newton, NC, USA) with concentrations of 10000, 20000 and 40000 cells/well in McCoy´s 5A media (Gibco/Invitrogen) supplemented with 10% foetal bovine serum at 37ºC in 5% CO2. After 24h different concentrations of DAPT were added to the wells or mock treatment with grows media. 48h later the MTS assay was preformed,10 uL CellTiter 96®AQ^{ueous} One Solution Cell Proliferation Assay (Promega Corpation, Madison, WI, USA) was added and absorbance at 490 nm was measure after 1 hour by THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) with SOFTMAX software (Molecular Device).

4.4.2 DAPT treatment of HT 29 cells

Cells were seeded in duplicates with the concentration of 1,0 x 10^6 cells/well in MULTIWELL™ 6 well (Falcon, BD Bioscience, San Jose, CA) in McCoy´s 5A media (Gibco/Invitrogen) supplemented with 10% foetal bovine serum at 37ºC in 5% CO2 for 24 h. 1,6 µL DAPT was added to the medium to a final concentration of 12,5 µM and as controls, cells were in parallel treated with the corresponding DMSO concentration or mock treated with growth mediums for 48h.

4.5 RNA and protein isolation

RNA and protein was isolated from HT29 cells with the PARISTM Kit (Ambion Inc., Austin, TX, USA) according to the supplier´s recommendations. The RNA concentration was measured with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), 2 µL sample was loaded and the concentration was calculated in the ND 1000 V3.1.0 software (NanoDrop Technologies). The protein concentration was spectrophotometrically measured using Bradford Reagent (Sigma). 2 µL sample was mixed with 98 uL Milli-Q H_2O and 1000 2 µL Bradford Reagent. As standard curve 0/1/2/4/8 µL of 1 mg/mL BSA (Sigma) was mixed with Milli-Q H_2O to a final volume of 100 µL and then 1000 µL Bradford reagent was added. The samples were immediately vortexed and 200 µL in double samples was added to an ELIZA plate and measured by THERMOmax microplate reader (Molecular Devices) with SOFTMAX software (Molecular Device).

4.6 Semi-quantitative PCR

200 ηg RNA from each sample were reversely transcribed into cDNA with SuperScript^{TM}III (Invitrogen) according to the supplier's recommendation. To determine the expression of APC or silencing of β-catenin, the well-established target gene of the WNT pathway Cyclin

D1 was used as a positive control (Logan & Nusse 2004). For DAPT treated cells, Hes 1 was used as a positive control for silencing of the Notch pathway. As negative control the endogenous gene GAPDH was used. 1 µL of cDNA solution was added to 19 µL of a standard master mix containing 0.5 units of ThermoWhite DNA Polymerase (Saveen Werner AB, Limhamn, Sweden) and 1µM of the appropriate primers. The PCR was followed by separation of the PCR products in a 1.5% agarose gel and detection with ethidium bromide and UV-light. The program Quantity One (Bio-Rad Laboratories, Hercules, CA, USA) was used for semi-quantitative analysis.

4.7 Western blot

Cell lysates containing equal amounts of total protein were denatured in NuPAGE® LDS Sample Buffer (Invitrogen) at 70^{o}C for 10 min with NuPAGE® Sample Reducing Agent (Invitrogen). The proteins were separated at 200 V for 35 min in NuPAGE® 4-12% Bis-tris gels (Invitrogen) held in NuPAGE® MES DS Running Buffer (20x) diluted 1:20 with H_2O, 0,25% NuPAGE® Antioxidant (Invitrogen) was added to the inner chamber. After the separation, the proteins were blotted to PVDF membranes (Pierce, Rockford, IL, USA) at 30 V for 60 min in NuPAGE® Transfer Buffer (20x) (Invitrogen) diluted 1:20 in H_2O with 20% methanol and 0,1 % NuPAGE® Antioxidant. The PVDF membranes were blocked with 5% Blotto (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) desolved in TBST, tris-buffered saline with 0,1 % Tween®- 20 (Sigma) for 60 min in room temperature. The primary antibodies added to the PVDF membranes were diluted in TBST as follows: anti-APC 1:500 (Abcam, Cambridge, UK), anti-β-catenin 1:1000 (Millipore Corp., Mosheim, France), anti-GAPDH 1:2000 (Millipore), anti-Hes 1 1:200 (Santa Cruz). The antibodies were incubated for 60 min in room temperature or over night in 4^{o}C, washed in TBST, followed by incubation for 60 min in room temperature with appropriate secondary antibodies, goat-anti-mouse 1:10000 (Jackson ImmunoResearch Europe Ltd.,Suffolk, UK) and goat-anti-rabbit 1:10000 (Cayman Chemical, Ann Arbor, MI, USA). The PVDF membranes were exposed to ECL Western Blotting Substrate (Pierce) and photographed digitally. For reuse of the membranes, the antibodies were stripped with Restore™ Western Blot Stripping Buffer (Thermo SCIENTIFIC, Rockford, IL, USA) for 15 min in room temperature and washed in TBST.

4.8 Statistic and graphs

All statistics and graphs are computed in GraphPad Prisma 5 (GraphPad Software Inc., San Diego, CA, USA). Error bars describe the standard error of the mean (SEM).

5 Results

5.1 Identifying LEF1-TCF and RBPJ –kappa sites

Genes of the Notch pathway that contains LEF1/TCF sites and genes of the Wnt pathway that contains RBPJ-kappa sites are shown in Table 1.

Genes highlighted in bold font was the genes investigated in the semi quantitative PCR and also the receptors Notch 1 and Jagged 2. Noteworthy is that these sites are theoretical and haven't been confirmed *in vivo*.

Table 1. Genes of the Notch pathway with LEF1/TCF sites and genes of the Wnt pathway with RBJK-Kappa sites. Genes highlighted in bold was further investigated with semi quantitative PCR.

Notch pathway	LEF1/TCF	Wnt pathway	RBPJ-Kappa
Jagged 1	3	Frizzled 3	9
Delta 1	3	Frizzled 4	2
HES 7	2	Frizzled 6	2
Delta 3	3	SFRP 1	1
Delta 4	1	SFRP 2	1
NUMB	1	SFRP 3	2
NUMBL	4	SFRP 4	2
HEY 2	6	Math1	1
HEYL	2	APC	12
Fringe lunatic	4	DVL 2	3
Fringe radical	1	Axin-1	6
Fringe manic	5	Axin-2	7
MAML1	5	GSK3-beta	2
Notch 2	4	CK1	3
Notch 3	1	LRP5	2
Notch 4	1	LRP6	1
HES 1	3	Bcl9-2	5
HES 3	2	HDAC 2	1
HES 5	1	Beta-TrCP	1
DLK 1	1	Wnt 1	5
RBK-JK	5	Wnt 2	1
SHARP	2	Wnt 3a	2
Gamma secretase ABP-1A	3	Wnt 4	3
Gamma secretase ABP-1B	2	Wnt 5a	7
Gamma secretase PEN2	2	Wnt 5b	11
		Wnt 6	2
		Wnt 9a	2
		Asef	13
		Dkk1	3

5.2 Analysis of Notch genes in HT29 cells with APC and β-galatosidase vector

To confirm that APC protein is upregulated in HT29-APC cells a western blot analysis was performed against APC, antibodies against GAPDH was used as a negative control as well as a control for equal loading. The results are shown in Figure 1 and there was a clear upregulation of full length functional APC in HT29-APC 24-6h.

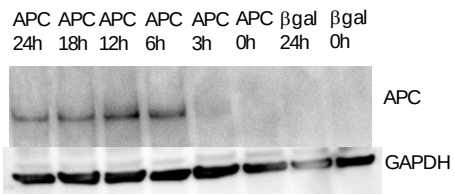

Figure 1. Western blot of Ht29-APC 24-0h and HT29-β 24h,0h against APC and GAPDH.

The RNA from each time point in the HT29-APC and HT29-βgal was transcribed into cDNA and the relative mRNA expression of each gene was determined by semi quantitative PCR. Each primer pair was optimized to appropriate annealing temperature and number of cycles to give a product that gives a reliable signal on the gels. The results from the Notch pathway genes with a downregulation of mRNA levels in HT29-APC are shown in Figure 2.
From Figure 2 we can see that the negative endogenous control gene GAPDH is homogenously expressed at all time points. The positive controls cyclin D1 for the Wnt pathway and Hes 1 for the Notch pathway were both downregulatedated in the time points of 24h-6h, confirming that both pathways are inhibited.

5.3 Analysis of Notch genes in HT29 cells treated with siRNA against β-catenin
To confirm that these downregulations are accomplished through the Tcf/Lef target gene program, siRNA transfection against β-catenin were done. The transfections were carried out at the time points 0, 24, 48, 72 and 96h. To confirm that the protein levels of β-catenin were downregulatedated a western blot analysis were done; results are shown in Figure 3. GAPDH was used as a control for equal loading.

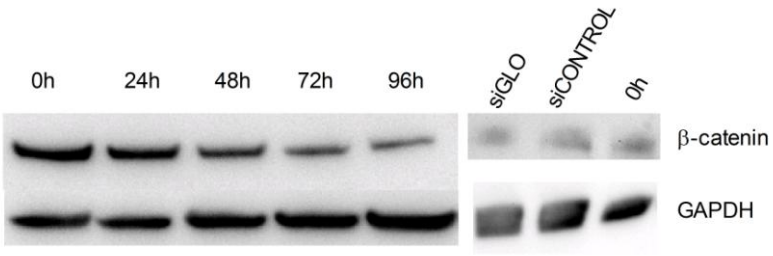

Figure 3. Western blot of HT29 cells transfected with siRNA against β-catenin in the interval of 0-96h The controls siGLO® transfection indicator and siCONTROL was transfected 96h before cell harvest.

The levels of β-catenin are clearly downregulatedated at the time points of 24-96h. At time point 0h after transfection the β-catenin levels were as high as in the controls.
The RNA from each time point of siRNA transfected HT29 cells were transcribed to cDNA and the relative mRNA expression was determinate by semi quantitative PCR. The primers for the genes that was downregulatedated in HT29-APC was used and the results are shown in Figure 4. The negative control GAPDH was homogenously expressed at all time points. The positive control cyclin D1 for Wnt pathway activity and the positive control Hes 1 for Notch pathway activity was downregulatedated in time points of 24-96h after siRNA transfection.

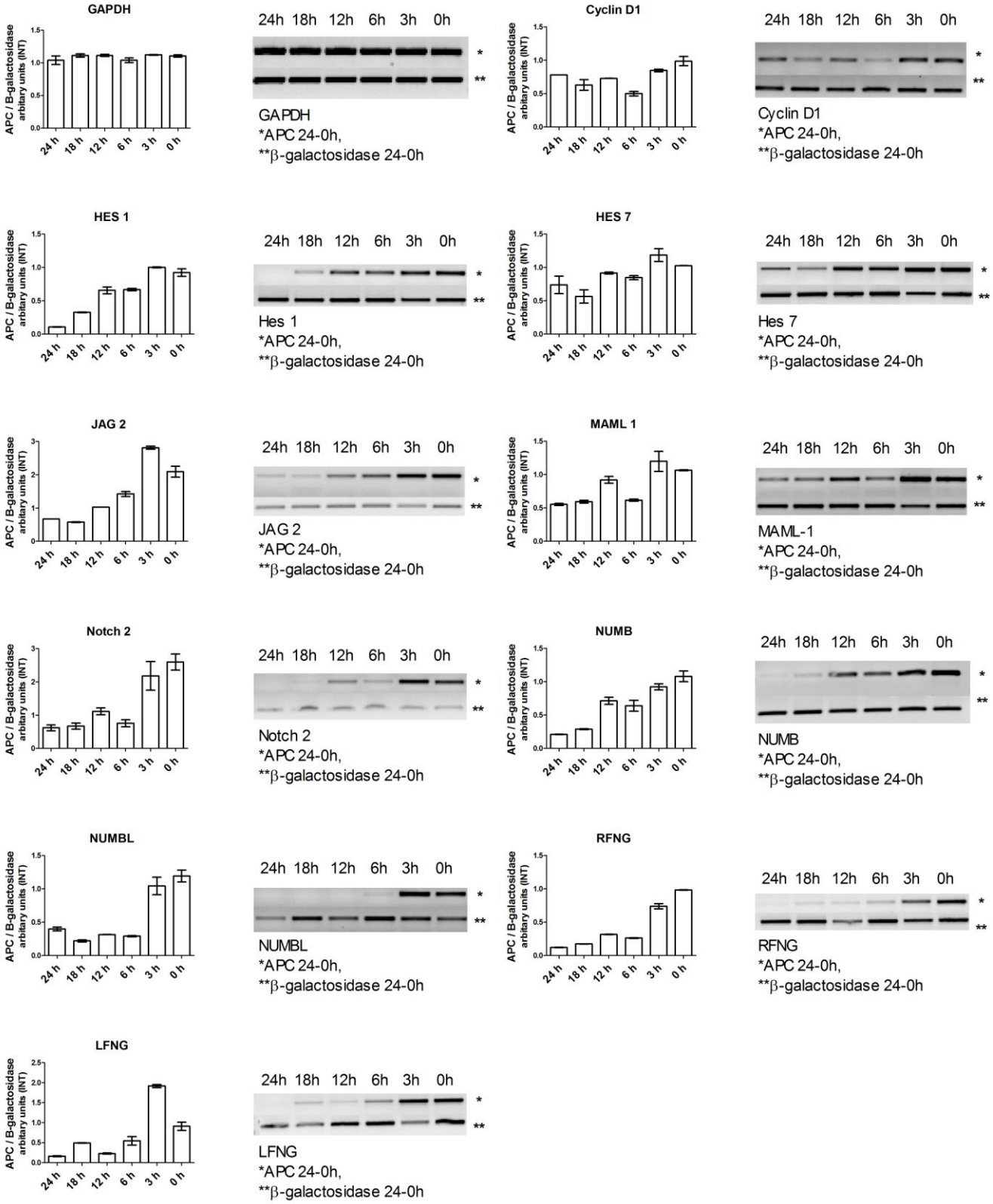

Figure 2. Results from semi quantitative PCR with genes from the Notch pathway in HT29-APC and HT29-βgal. Diagrams are showing the intensity signal from HT29APC/HT29-βgal at different time points. There is a clear trend of downregulation, lowering of signal in 24h-6h samples for all genes except GAPDH. Experiments were repeated twice.

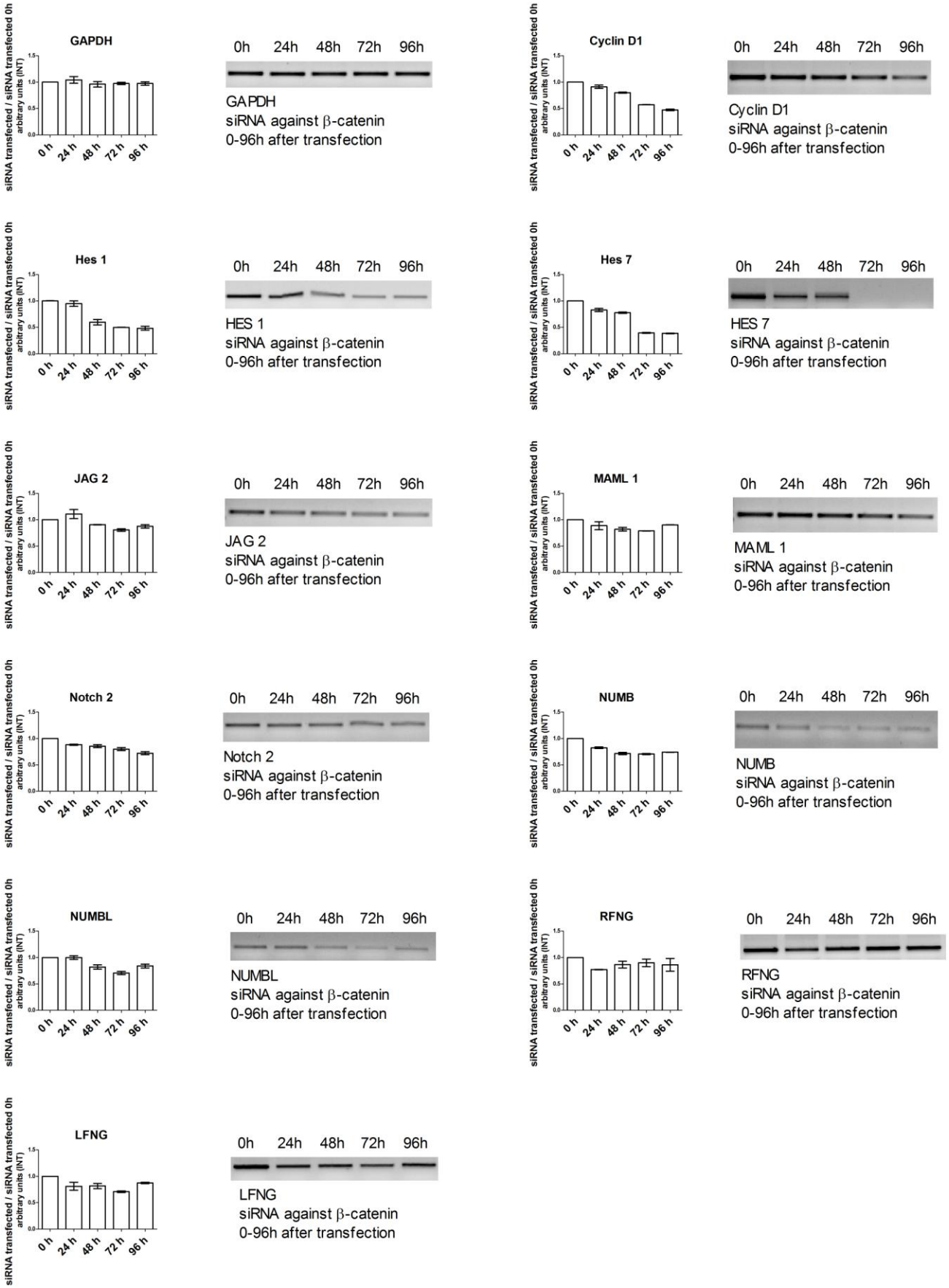

Figure 4. Results from semi quantitative PCR with genes from the Notch pathway in HT29 cells transfected with siRNA against β-catenin. Diagrams are showing the intensity signal from siRNA transfected/siRNA transfected 0h in different time points. There is a clear trend of downregulation, lowering of signal in 24h-96h samples. Experiments were repeated twice.

5.4 Determination of DAPT concentration for HT29 cells with MTS assay

40000 cells per well gave a higher signal than 20000 and 10000 cells per well, and also a better proliferation curve shown in Figure 5. The proliferations decreases until the DAPT concentrations reaches approximately 12,5 µM. From this result we choose to use the concentration 12,5 µM for the trails in the in 6-well plates. 40000 cells in a ELIZA plate well (~0,32 cm^2) gives about the same cell density as 1 x 10^6 cell/well in a 6-well plate (area~9,6 cm^2)

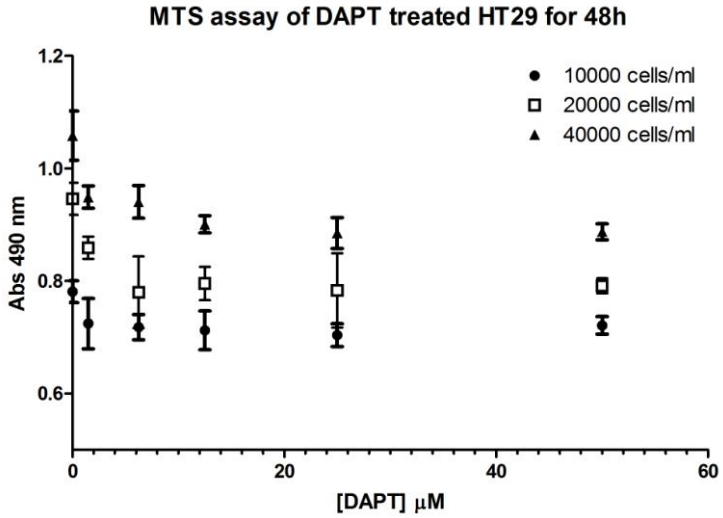

Figure 5. MTS assay measuring proliferation in 48h DAPT treated HT 29 cells. Filled circles are 10000 cells/well, squares are 20000 cells/well and filled triangles are 40000 cells/well.

5.5 Analysis of DAPT treated HT29 cells

To confirm that the gamma-secretase inhibitor DAPT inhibited the Notch pathway, the downstream target Hes 1 was analyzed with western blot. GAPDH was used as a negative control as well as a control for equal loading. The results are shown in Figure 6A.

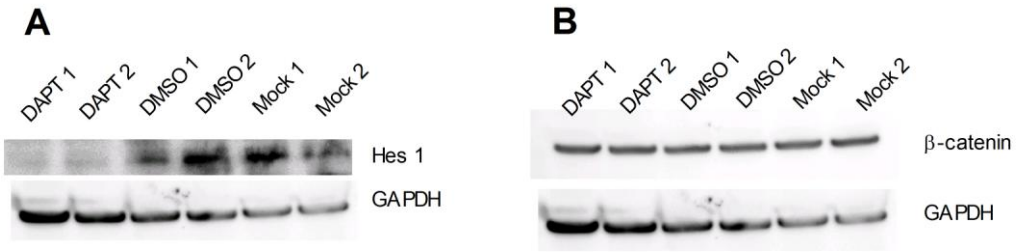

Figure 6.Western blot of 48h DAPT, DMSO and mock treated HT29 cells. A) Antibodies against Hes 1 and GAPDH. B) Antibodies against β-catenin and GAPDH.

DAPT treated cells clearly show downregulation of Hes 1 protein levels. To see if downregulation of the Notch pathway affected the β-catenin levels and, a western blot against β-catenin was carried out. Results are shown in Figure 6B.
There was no downregulation of β-catenin in the DAPT treated cells and the protein levels were equal to the two controls; DMSO and mock.

The RNA for the different treatment of HT29 was transcribed to cDNA to determine relative mRNA levels with semi quantitative PCR. Primers against Hes 1, Cylin D1 and GAPDH was used, results are shown in Figure 7.

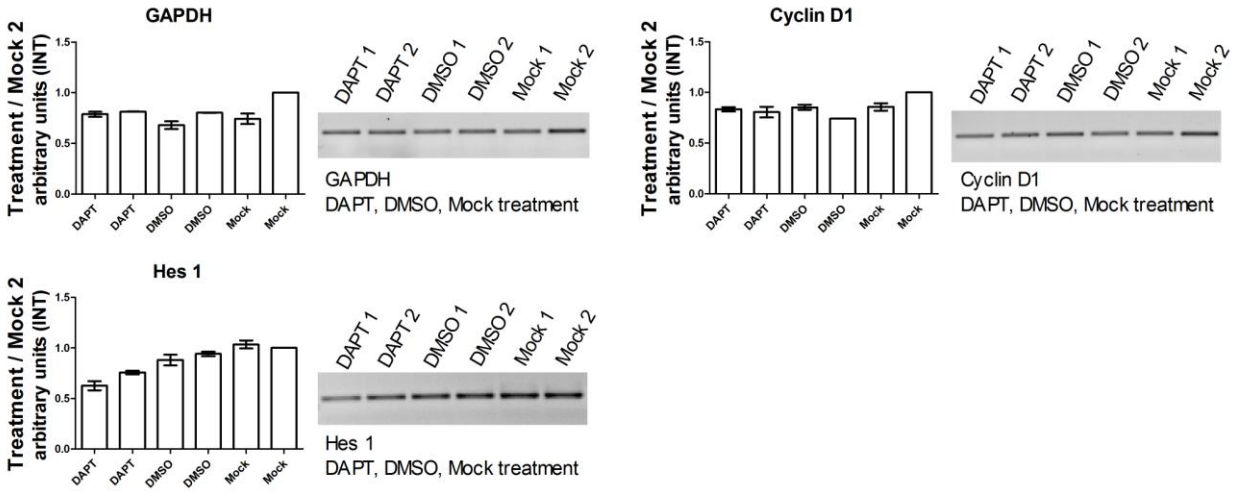

Figure 7. Results from DAPT,DMSO and Mock treatment of HT29 cells for 48h. Diagrams are showing intensity signal from the different treatments/Mock 2. Experiments were repeated twice.

The levels of Hes 1 mRNA was downregulatedated in the DAPT treated cells, but the levels of Cyclin D1 mRNA had an equal expression for all treatments.

6 Discussion

6.1 Numbers of LEF1/TCF sites

There was no correlation between the number of LEF1/TCF sites found by the bioinformatic search in the genes of the Notch pathway and downregulation of these upon Wnt inactivation through induction of wildtype-APC. This may be because they are just theoretical and have not been proven *in vivo*. It would be interesting to *in vivo* promoter characterize these sites with EMSA (Electrophoretic mobility shift assay) or ChIP display (Chromatin immunoprecipitation assay).

6.2 Expression of Notch genes in Wnt pathway inhibited HT29 cells

Through activation of full length ACP in HT29-APC the Wnt pathway is deactivated. In the western blot analysis against APC in Figure 1 the APC was upregulated in 24-6h before cell harvest. This coincides with the results from the semi quantitative analysis in of HT29-APC and HT29-βgal in Figure 2 where the trend of downregulation was most significant in HT29-APC 24h-6h, the HT29 APC expression is lower than the HT29-βgal expression. The downregulation of Cyclin D1 was expected (Logan & Nusse 2004) and so Hes 1 downregulation if Notch pathway was affected (Van Es JH et al. 2005). Hes 7, JAG 2, MAML 1, Notch 2, NUMB, NUMBL, RFNG and LFNG was also downregulated. All but

JAG 2 contains one or more theoretical LEF1/TCF sites. JAG 1 does have LEF1/TCF sites and have been proven to be upregulated upon β-catenin activation in human hair follicles (Estrach et al. 2006). But both JAG 1 and JAG 2 gene expression have been seen to be upregulated in human colorectal cancer and so has also Notch 1, LFNG, while Notch 2 also seems upregulated, however not as greatly as the other genes (M. Reedijk et al 2008). It is interesting to see that JAG 1 and Notch1 are not downregulated in our experiments. Notch 1 does not contain any theoretical LEF1/TCF sites and may be regulated in an alternative way. JAG 1 does contain 3 theoretical sites, and has also bee shown to be upregulated in hairy follicles by β-catenin activation (Estrach et al 2006). The molecular mechanism may be different in the colon and JAG 1 may not be regulated by β-catenin in colon.
Both NUMB and NUMBL are known to inhibit Notch signaling (Fiúza & Arias 2007) and are here shown to be downregulated. Since JAG 2, MAML 1, Notch 2, RFNG and LFNG are all downregulated and so are the target genes Hes 1 and Hes 7, the effects of the downregulation of the inhibitors NUMB and NUMBL would be insignificant for the outcome of expression of Notch pathway downstream targets.
Same genes as in HT29-APC are also downregulated in the trails with siRNA against β-catenin (Figure 4), but the trends are not as clear. This may be because there is still detectable levels of β-catenin at all time points (Figure 3), and these levels can be sufficient to affect β-catenin target genes. We can conclude that these effects are due to decreased intracellular levels of β-catenin and hence an inhibition of the Tcf/Lef target gene program.

6.3 Correlation analysis of Notch and Wnt pathways in DAPT treated HT 29 cells

The determination of proliferation by using a MTS assay showed us the best concentration of cells/well (Figure 5) and the best concentration of the gamma-secretase inhibitor DAPT that was going to be used. The concentration of cells/well did not differ much from each other, but the more cells the more proliferation. So we decided to use 1×10^6 cells/well in a 6-well plate. The concentration of DAPT was decided to be 12,5 µM , because after 12,5 µM of DAPT the proliferation halts.
To confirm that Notch is inhibited trough DAPT treatment we did a western blot analysis against Hes 1 protein (Figure 6A) (H. Zhang et al 2008). From that result we can conclude that the Notch inhibition that Hes 1 protein was downregulated. However, a western blot against β-catenin(Figure 6B) showed that the intracellular levels of the protein was not affected. This was also confirmed by a semi quantitative PCR (Figure 7) were β-catenin target gene Cyclin D1 had a homogenous expression in all treatment, but Hes 1 was downregulated. These preliminary results indicate that there is no regulation of APC/β-catenin by the Notch pathway. Deregowski et al (2005) concluded from their experiments on osteoblastogenesis, that overexpression of the Notch intracellular domain decreases the levels of intracellular β-catenin and inhibits the activity of Wnt3a. We do not see these effects in colon cancer cell lines, which could be due to different tissue types. However, further studies are warranted to elucidate the mechanism fully.

6.4 Conclusion

By inhibiting the Wnt pathway, our results have shown downregulation of one ligand (JAG 2), one receptors (Notch 2), one transcriptional activator (MAML 1), two target genes (Hes 1,

Hes 7), two inhibitors (NUMB, NUMBL) and two of Notch glycosyltransferases (LFNG, RFNG) of the Notch pathway. We have also shown that this is accomplished by the Tcf/Lef target gene program by transfecting the cells with siRNA against β-catenin.
This total down regulation of the Notch pathway upon Wnt deactivation shows a correlation between the two signaling pathways in colorectal cancer. But we did not find any correlation the other way around, between Notch inhibition and Wnt pathway through β-catenin signalling.
There have been suggestions that gamma-secretase inhibitors may provide a targeted-drug strategy for treating human colorectal cancer, because of the close correlation between the Notch and Wnt pathway (M. Reedijk et al 2008) (Van Es & Clevers 2005). Inhibition of Notch signalling gives a decrease in Hes 1 gene expression, leading to halt of cell proliferation and generation of apoptosis (H. Zhang et al 2008), which our MTS assays with DAPT treated HT29 cells supports. Gamma-secretase inhibitors has also an inhibitory effect in Alzheimer's disease, were it are reduces the accumulation of β-amyloid aggregates hat causes the onset and progression of the disease. Preclinical studies for Alzheimer's disease in rodents have shown that a side effect of gamma-secretase inhibitors is macroscopic abnormalities in the gastrointestinal tract, where the small and large intestine is distended and with an excess of mucus (D.M Barten et al 2006). This may be one way of inhibiting the upregulation of the Notch pathway in colorectal cancer.

7 Acknowledgments

First I want to thank my supervisor Jonas Ungerbäck for his help and support in this project. Peter Söderkvist and Nils Elander for the consult and help concerning methodology, I also want to thank the rest of Peter Söderkvist lab group for all the help and Jan-Invar Jönsson for the consult concerning MTS assays.

8 References

Brabletz S., Schmalhofer O., Brabletz T. 2009
Gastrointestinal stem cells in development and cancer Journal of Pathology, 217 (2), pp. 307-317

Martling, A., Granath, F., Cedermark, B., Johansson, R., Holm, T. 2008
Gender differences in the treatment of rectal cancer: A population based study
European Journal of Surgical Oncology, In press

Crosnier C., Stamataki D., Lewis J. 2006
Organizing cell renewal in the intestine: Stem cells, signals and combinatorial control
Nature Reviews Genetics, 7 (5), pp. 349-359.

Kelleher, F., Fennelly, D., Rafferty, M. 2006
Common critical pathways in embryogenesis and cancer
Acta Oncologica 45 (4), pp. 375-388

Reya, T., Clevers, H. 2005
Wnt signalling in stem cells and cancer
Nature 434 (7035), pp. 843-850

Logan C.Y., Nusse R. 2004
The Wnt signaling pathway in development and disease
Annual Review of Cell and Developmental Biology, 20, pp. 781-810.

Fiúza, U.-M., Arias, A.M. 2007
Cell and molecular biology of Notch
Journal of Endocrinology 194 (3), pp. 459-474

Van Es J.H., Clevers H. 2005
Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease
Trends in Molecular Medicine, 11 (11), pp. 496-502.

Estrach S., Ambler C.A., Lo Celso C., Hozumi K., Watt F.M. 2006
Jagged 1 is a β-catenin target gene required for ectopic hair follicle formation in adult epidermis
Development, 133 (22), pp. 4427-4438

Deregowski V., Gazzerro E., Priest L., Rydziel S., Canalis E. 2005
Notch-1 Overexpression Inhibits Osteoblastogenesis by Suppressing Wnt/β-catenin but None Morphogenetic Protein Signaling
The Journal of Biological Chemistry, 281, p. 6203-6210.

Van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. 2005
Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells
Nature 2005 Jun 16;435(7044):959-63.

He T.-C., Sparks A.B., Rago C., Hermeking H., Zawel L., Da Costa L.T., Morin P.J., Morin P.J., Vogelstein B., Kinzler K.W. 1998
Identification of c-MYC as a target of the APC pathway
Science, 281 (5382), pp. 1509-1512.

Zhang, H., Ye, Y., Bai, Z.and Wang, S. 2008
The COX-2 Selective Inhibitor-Independent COX-2 Effect on Colon Carcinoma Cells is Associated with the Delta1/Notch1 Pathway
Digestive Diseases and Sciences 53 (8) pp. 2195-2203

Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Research (1995) 23:4878–4884.

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics (2005) 21:2933–2942.

Reedijk, M., Odorcic, S., Zhang, H., Chetty, R., Tennert, C., Dickson, B.C., Lockwood, G., Gallinger, S., Egan, S.E 2008
Activation of Notch signaling in human colon adenocarcinoma
International journal of oncology 33 (6), pp. 1223-1229

Barten D.M., Meredith Jr. J.E., Zaczek R., Houston J.G., Albright C.F.
γ-secretase inhibitors for Alzheimer's disease: Balancing efficacy and toxicity
(2006) Drugs in R and D, 7 (2), pp. 87-97

9 Appendix A

Fringe lunatic (LFNG)

Forward Primer: 5’-ACGTCTACGTCGGCAAGC
Reverse Primer: 5’-AGGTTCTCCAGGTGGGAGTG

Fringe radical (RFNG)

Forward Primer: 5’-ACCCAGCCAGGACGTCTAC
Reverse Primer: 5’-CCTCCACGATGTAGCCAACT

HES 1

Forward Primer: 5’-GCATCTGAGCACAGAAAGTC
Reverse Primer: 5’-GAGGTGCTTCACTGTCATTT

HES 7

Forward Primer: 5’-CTGGAAGAGCTGAGGCTGCT
Reverse Primer: 5’-GGTTTGGGGCGCAGATAG

Jagged 2

Forward Primer: 5’-TCTGCCTTGCTACAATGGTG
Reverse Primer: 5’-GCGATACCCGTTGATCTCAT

Mastermind-1

Forward Primer: 5’-ACAGGGTCCTCTGCTGCCGT
Reverse Primer: 5’-AGCCACACCCCGGTCCTGTT

Notch 2

Forward Primer: 5’-TCAGGGGTTAATTGTGAAAT
Reverse Primer: 5’-ATATACAGCGGAAACCATTC

NUMB

Forward Primer: 5’-AAAATGCAGTGCCAGAAGTA
Reverse Primer: 5’-GAAGGTAGGAGATTGTGGTG

NUMBL

Forward Primer: 5’-GCGACAGTGACAGCATCAAC
Reverse Primer: 5’-CTCAGCCTCTGAAGGTGTCC

GAPDH

Forward Primer: 5’-GAGTCAACGGATTTGGTCGT
Reverse Primer: 5’-GACAAGCTTCCCGTTCTCAG

Cyclin D1

Forward Primer: 5’-AACTACCTGGACCGCTTCCT
Reverse Primer: 5’-CCACTTGAGCTTGTTCACCA

Delta 1

Forward Primer: 5’- GGGTGGAGAAGCATCTGAAA
Reverse Primer: 5’- TGGGGCATATATCCTTGGAA

Delta 3

Forward Primer: 5’-GAGCTGCGCTTCTCGTACC
Reverse Primer: 5’-TCAAAGGACCTGGGTGTCTC

Delta 4

Forward Primer: 5’-TATTGGGCACCAACTCCTTC
Reverse Primer: 5’-AACCAGTTCTGACCCACAGC

Hes 3

Forward Primer: 5’- ACAGGGACTATTTTCAGCAC
Reverse Primer: 5’- TGTAGGTGGAGCTAGAGTGG

Hes 5

Forward Primer: 5’- GACTGCGGAAGCCGGTGGTG
Reverse Primer: 5’- CTGGTGCAGGCTCTTGGGGC

HEY2

Forward Primer: 5’- CAACATCTCAGATTATGGCAAGA
Reverse Primer: 5’- TTGCCTGAAGCATCTTCAAAT

HEYL

Forward Primer: 5’- AGATGCAAGCCAGGAAGAAA
Reverse Primer: 5’- GGCATGGAGCATTTTCAAGT

Fringe manic (MFNG)

Forward Primer: 5’- CCATGTGGACGATGACAACT
Reverse Primer: 5’- TATAGCCCATGGTGCAGTCA

Notch 1

Forward Primer: 5’- CAGGATGTCAACGAGTGTGG
Reverse Primer: 5’- GTTCTGGCAGGCATTTGG

Notch 3

Forward Primer: 5’- TGTGGACGAGTGCTCTATCG
Reverse Primer: 5’- AATGTCCACCTCGCAATAGG

Notch 4

Forward Primer: 5’- CAGAGGGCTGCTGTGTGGGA
Reverse Primer: 5’- GAGAGGGAGAGCTGGGGAGC