doi:10.4149/neo_2011_04_343

Gamma-secretase inhibitor, a potential target therapy for MUC2-positive colorectal carcinoma

X. BU, N. LI, X. TIAN, P. HUANG

Department of Pathology, School of Medicine, Southeast University, Dingjiaqiao Road, Nanjing 210009, Jiangsu Province, China, e-mail: hplwpp@yahoo.cn

Received January 2, 2011

Notch signaling may be mechanistically involved the colorectal carcinogenesis. Blocking of Notch signaling by gamma-secretase inhibitor may constitute a novel molecular therapy for cancer. In the present study, we blocked the Notch signaling by DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester, a gamma-secretase inhibitor) and investigated the effects on the proliferative and invasive potential of human colorectal cancer LS174T cells, a goblet cell-like colorectal cancer cell line which produces high-levels of MUC2 continuously. DAPT inhibited the proliferation and invasion of LS174T cells. Blocking of Notch signaling by DAPT could down-regulate its downstream target gene Hes1, while enhancing the expression of Math1and MUC2 in LS174T cells. In conclusion, we demonstrated that blocking of Notch signaling by DAPT could inhibit the proliferation and invasion of human colorectal cancer LS174T cells and suggested that gamma-secretase inhibitors may provide a targeted therapy for MUC2-positive colorectal tumors.

Keywords cancer, colorectum, invasion, proliferation, Notch signaling

The vast majority of published references indicate that Notch signaling controls stem or progenitor cell proliferation, as well as cell fate specification and differentiation in the colorectum [1,2]. Notch signaling genes are not only highly expressed in colorectal cancer tissues but are also functionally active. Inhibition of Notch signaling may be of therapeutic benefit against colorectal cancer. Consequently, multiple groups have studied the effects of inhibiting Notch signaling by targeting the gamma-secretase protein complex, which cleaves the Notch receptor to activate the pathway. Gamma-secretase inhibitors are able to sensitize colon cancer cells to chemotherapeutic agent induced cell killing [3].

The Notch pathway is important for the development of the secretory cell lineages of the intestine.Loss of function of Notch in the intestinal epithelium results in a massive conversion of epithelial cells into goblet cells [4]. Absorptive cells arise from cells in which a high level of Notch activation is maintained. These cells express high levels of the Notch target gene Hes1. Conversely, secretory-type cells, including goblet cells, arise from cells expressing Notch ligands in which Notch signaling is inactivated. These cells instead express high levels of Atoh1, and inhibit neighboring cells from achieving the same cell fate [5]. Hes1 is a basic helix-loop-helix (bHLH) transcriptional repressor induced by the Notch signal, while mouse atonal homolog 1 (Math1) is another bHLH transcription factor that is suppressed by Hes1 [6]. It suggested that Notch-Hes1 signaling is involved in regulating the differentiation of intestinal cells into absorptive and goblet cells through a mechanism called lateral inhibition. Leow et al. demonstrated the expression of Hath1, a bHLH transcription factor homologous to the *Drosophila* atonal and mouse Math1 and significantly down-regulated in all of the colorectal cancer samples except for mucinous carcinoma. Only minimal Hath1 expression was detected in various colorectal cancer cell lines except for elevated levels in the LS174T cell line [7]. Interestingly, it has been found that mucinous carcinomas of colorectal cancer are positive for MUC2,a goblet cell marker, in contrast to MUC2 down-regulation in non-mucinous adenocarcinomas [8,9]. Rarely literature reported whether the gamma-secretase inhibitor will affect the biological behavior of colorectal mucinous carcinoma. In the present study, we blocked the Notch signaling by DAPT and investigated the effects on the proliferative and invasive potential of human colorectal cancer cell line, LS174T, a goblet cell-like colorectal cancer cell line and produces high-levels of MUC2 continuously.

Materials and methods

Cell line and treatment with gamma-secretase inhibitor. LS174T cells, a human colorectal adenocarcinoma cell line, were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen,USA). *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT, Sigma,USA), a potent gamma-secretase inhibitor, was used to block Notch signal. LS174T cells were treated with 20μM DAPT reconstituted in dimethylsulfoxide (DMSO) as final concentration or DMSO alone as control for various times points.

Cell proliferation assay. Cells were seeded in 96-well plates at 1×10^5 cells/well. The 3-(4,-dimethy -lthiazol-2-yl)-2,-diphenyl-tetrazoliumbromide (MTT) assay was used to detect viable proliferating cells at various time points (12,24,36,48h), using five wells per time point. Cell viability was expressed as optical density (OD), which was detected using an enzyme-linked immunoabsorbent assay reader (Thermo, MK3, USA) at 492nm wavelength.

***In vitro* invasion assay.** The invasive activity of these cancer cells was measured *in vitro* by a transwell assay using a transwell chamber (Corning, Corning, NY) fitted with a polyethylene terephthalate filter membrane with 8μm pores, in 24-well plates. The filter membranes were coated with Matrigel. Cancer cells were added to the upper chamber at a density of 5×10^4 cells/200μl, and the lower chambers were filled with 500μl of RPMI-1640 medium containing 10% FBS as a chemoattractant. After incubation for 24 h, the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed and stained with Giemsa. The invaded cells were then visualized and counted from five randomly selected fields (× 200 magnification) under an inverted microscope. Three invasion chambers were used for each experimental condition.

Quantitative real-time polymerase chain reaction. After treated with 20μM DAPT for 48h, total RNA was isolated from LS174T cells using Trizol reagent (Invitrogen). cDNA synthesis was performed using a reverse transcription system kit (Invitrogen), according to the manufacturer's instructions. PCR detection of gene expression was performed using a fluorescence quantitative PCR instrument (BioRad,USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primer sequences for Hes1,Math1,MUC2 and GAPDH are described in Table 1. The cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 amplification cycles of 95°C for 10 s, 60°C for 30 s, and 70°C for 45 s. Each real-time PCR assay contained 1.2 μl cDNA template, SYBR green 0.5 μl, and 0.5 μl of each forward and reverse primer in a 25 μl reaction mixture. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method [10].

Table 1 Primers used for real-time RT-PCR

Gene	Orientation	Sequence (5'–3')
Hes1	Forward	TGGAGAGGCGGCTAAGGTGTT
	Reverse	TGGAAGGTGACACTGCGTTGG
Math1	Forward	GCTGGACGCTCTGCACTTCT
	Reverse	CTTGCCTCATCCGAGTCACTGTAA
MUC2	Forward	CTTCCACCAACCACCACTTCCA
	Reverse	GAATCCAGCCAGCCAGTCCAAT
GAPDH	Forward	GAAGGTCGGAGTCAACGGATT
	Reverse	CGCTCCTGGAAGATGGTGAT

Western blot analyses. To determine the protein expression of Hes1 and Math1, equal aliquots of total protein (30 μg per lane) were electrophoresed using sodium dodecyl sulfate polyacrylamide (12%) gels(SDS–PAGE). In the analyses of MUC2,we used NUPAGE NOVEX MIDI 3-8% Tris-acetate gradient gel (Invitrogen) for electrophoresis because the molecular weight of the MUC2 was more than 200 kD. The gels were transferred onto nitrocellulose membranes (Invitrogen), and blotted using primary antibody directed against human Hes1,Math1, MUC2 (1:1,000 Santa Cruz, CA). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000 Santa Cruz, CA), immunoreactive bands were visualized by chemiluminescence solution and exposure to X-ray film. The expression of GAPDH (Santa Cruz, CA) was used as a normalization control for protein loading. Quantification of the western blots was performed densitometrically using SensiAnsys software (Shanghai PeiQing Science & Technology, China). The signal for the protein of interest was standardized to its respective GAPDH loading control.

Statistical analysis. Statistical comparisons were performed using SAS, version 9.1, software (SAS Institute, Cary, NC). Values are presented as means ± standard error of the mean. The statistical significance of differences was determined by analysis of variance and Student-Newman-Keuls test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Effect of DAPT on the growth of LS174T cells. The capacity of the LS174T cells for proliferation after DAPT treatment was assessed using a MTT assay. As shown in Figure 1, The OD values measured at 2 days showed that the proliferative capacity of DAPT treatment group cells was significantly lower than that of control group cells ($P < 0.05$).

Effect of DAPT on invasion capabilities of LS174T cells. The abilities of LS174T cells to invade a reconstituted extracellular matrix were assessed using transwell assays. DAPT treatment group cells grown on Matrigel for 24h produced significantly less invasive cells than control group cells, indicating that LS174T cells after DAPT treatment had a lower invasive ability than the control cells ($P<0.05$) (Figure 2). The average number of DAPT treatment group cells that invaded through the Matrigel was 33.00±1.40 cells/site, compared with 76.27±1.92 cells/site for control-blank cells or 83.07±2.61 cells/site for control-DMSO cells.

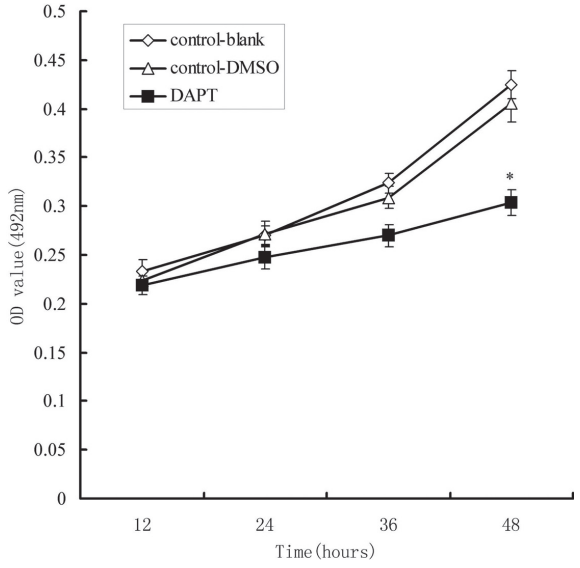

Figure 1. DAPT inhibited the proliferative capacities of LS174T cells. * *P* < 0.05 compared with control-DMSO group.

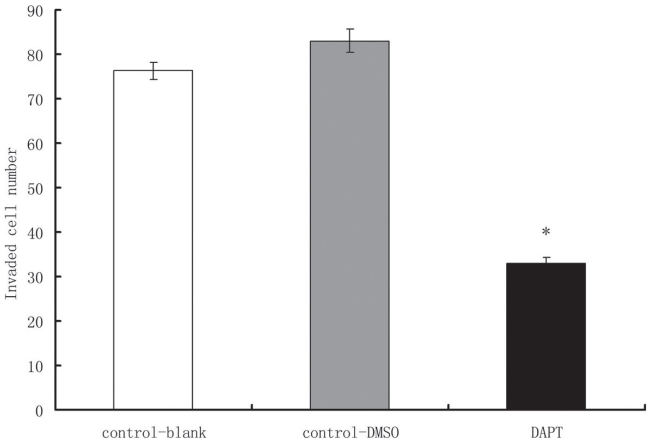

Figure 2. DAPT inhibited the invasive capacities of LS174T cells. * *P* < 0.05 compared with control-DMSO group.

DAPT treatment inhibited the expression of Hes1,while enhancing that of Math1 and MUC2 in LS174T cells. We used real-time RT-PCR and western blot to detect the Hes1,Math1 and MUC2 levels in LS174T cells treated with DAPT. Our study revealed that 20μM DAPT resulted in significant down-regulation of Hes1 mRNA and western blot analysis also showed that the protein level of Hes1 was down-regulated in LS174T cells,confirming that the intracellular Notch-Hes1 pathway had been further inactivated while, expression of Math1 and MUC2 was significantly upregulated in treated cells (Figure 3).

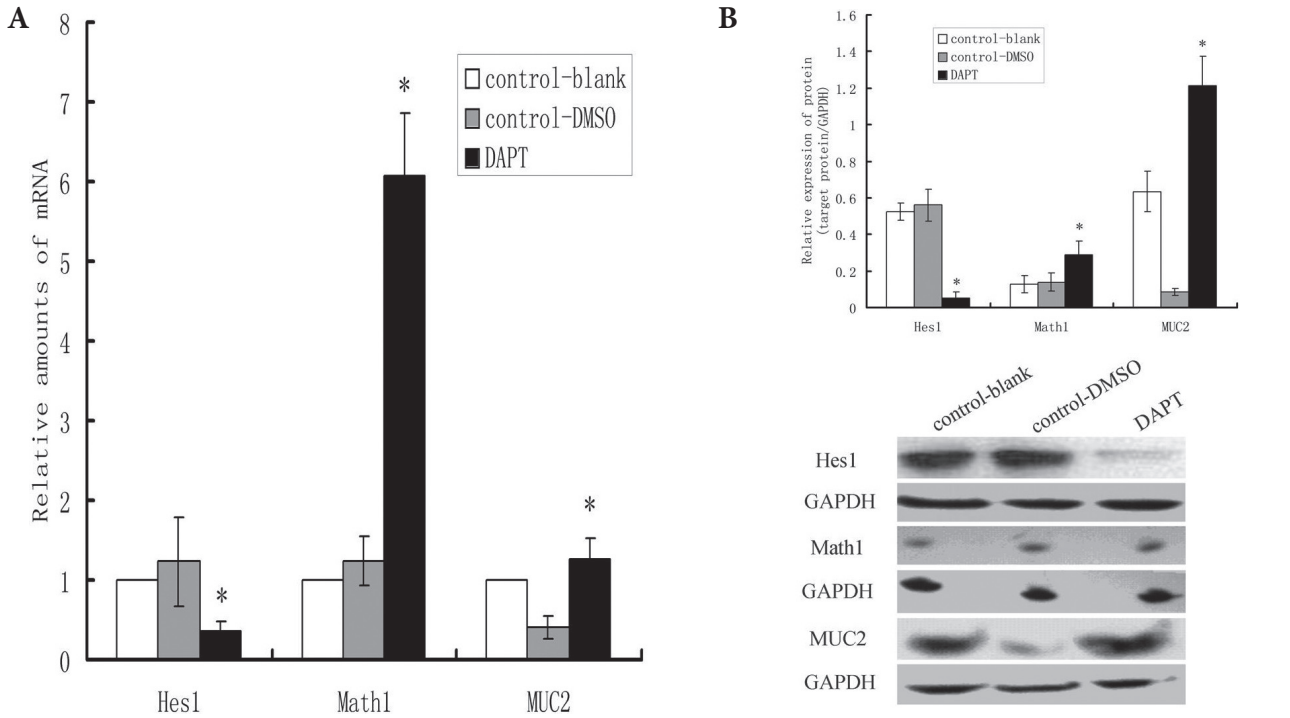

Figure 3. DAPT inhibited the expression of Hes1,while enhancing that of Math1 and MUC2 in LS174T cells. (A) Quantification of mRNA expression levels after treated with 20μM DAPT for 48h. (B) Western blotting results of Hes1,Math1 and MUC2 proteins after treated with 20μM DAPT for 48h. The upper panel in B showed quantification of the western blot. * *P* < 0.05 compared with control-DMSO group.

Discussion

The Notch family consists of four receptors (Notch1–4) and five ligands (Jagged-1, Jagged-2, Delta-like-1, Deltalike-3, and Delta-like-4). Receptor-ligand interaction between two neighboring cells leads to gamma-secretase-mediated proteolytic release of the Notch intracellular domain (NICD), the active form of Notch. Translocation of NICD into nucleus and subsequent binding to transcriptional factors such as Hes1 leads to the activation of Notch signaling,which in turn affect numerous pathways involving cell fate determination [11]. Canonical Notch signaling in colon is implicated in the maintenance of stem cells and progenitor cells, and also in the inhibition of goblet cell differentiation [12]. Notch signaling activation leads to Hes1 upregulation, and Atoh1/Hath1/Math1 down-regulation. Atoh1 bHLH transcription factor induces differentiation to the goblet cells [13]. Hath1 expression also influences the biological properties of colon cancer cells. This factor was found to be down-regulated in most colon cancers. Moreover, the forced expression of Hath1 in colon cancer cells diminished the proliferative potential of the cells and led to increased MUC2 expression. Hath1 is involved in regulating MUC2 gene expression in colorectal cancers and possibly in regulating other biological properties of mucinous cancers and signet ring carcinomas [14]. Hath1 rodent homologue Math1 is also required for development of secretory cells, including goblet cells, in the embryonic mouse intestine.

The majority of colon cancers are nonmucinous cancers,however, mucinous cancers and signet ring carcinomas make up 10% to 20% of all colorectal cancers, with a characteristic set of clinical/pathologic features and distinct molecular genetic attributes. Mucinous adenocarcinoma of the colon may have a goblet-cell phenotype (MUC2-positive), and may display a different mechanism of development from non-mucinous carcinoma [15]. In the present study, we investigated the effect of DAPT on proliferation and invasion of colorectal cancer cells LS174T, which contain mucus granules, produce significant amounts of secretory mucin, and have a resemblance to goblet cells[16]. We found that down-regulation of Notch signal using a gamma-secretase inhibitor inhibited the proliferation and invasion of LS174T cells. Thus, our results further provide in vitro evidence in support of using gamma-secretase inhibitors as a new target therapy in colorectal cancer cells, including MUC2-positive cancer cells. Therefore, gamma-secretase inhibitors will be applied as anti-cancer drugs for human cancer, such as mucinous cancer. Treatment of colon cancer cell lines SW480 and DLD-1 by DAPT significantly enhanced taxane-induced mitotic arrest and apoptosis both in vitro and in vivo, although the inhibitors themselves did not have proapoptotic effect [17]. Similarly, treatment of colon cancer HCT116 cells with another gamma-secretase inhibitor GSI34 significantly sensitized the cells to Oxaliplatin- and 5-fluorouracil-induced apoptosis and growth inhibition[3].However, if the same results can be obtained in cancer patients, more researches are necessary. In our study, blocking of Notch signaling by DAPT could down-regulate its downstream target gene Hes1, while enhancing the expression of Math1 and MUC2 in LS174T cells. It maybe related to the crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. In the intestine, the Wnt signaling pathway has been implicated in the regulation of the proliferation or differentiation balance. Indeed, it was shown that Hath1 expression is repressed at the protein level in some human colon cancer tissues in which the Wnt signal is activated, strongly suggesting that this mechanism might be involved in growth promotion as well as in the maintenance of the undifferentiated state of the cancer cells [18].Meanwhile, given that the inactivation of Notch signaling even under Wnt-active conditions results in Math1 protein expression and the differentiation of some progenitors into secretory lineages [19].Furthermore, as significant similarities are likely to exist between the behavior of intestinal epithelial progenitor cells and that of cancer progenitor cells, advances in this field will also provide significant insight into how the Wnt and Notch pathways coordinately drive oncogenic potential and what might be novel targets for improved treatments for intestinal cancers [20].In conclusion, we demonstrated that blocking of Notch signaling by DAPT could inhibit the proliferation and invasion of human colorectal cancer LS174T cells and suggested that gamma-secretase inhibitors may provide a targeted therapy for MUC2-positive colorectal tumors.

Acknowledgment.This work was supported by grants from the Science and Technology Foundation of Southeast University, China (No. KJ2010438).

References

[1] KATOH M, KATOH M. Notch signaling in gastrointestinal tract (review).Int J Oncol 2007;30:247–251.

[2] FRE S, HUYGHE M, MOURIKIS P, ROBINE S, LOUVARD D, et al.Notch signals control the fate of immature progenitor cells in the intestine. Nature 2005;435:964–968. doi:10.1038/nature03589

[3] MENG RD, SHELTON CC, LI YM, QIN LX, NOTTERMAN D, et al.Gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. Cancer Res 2009;69:573–582. doi:10.1158/0008-5472.CAN-08-2088

[4] MILANO J, MCKAY J, DAGENAIS C, FOSTER-BROWN L, POGNAN F, et al.Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci 2004;82:341–358. doi:10.1093/toxsci/kfh254

[5] WONG GT, MANFRA D, POULET FM, ZHANG Q, JOSIEN H,et al.Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. J Biol Chem 2004;279:12876–12882. doi:10.1074/jbc.M311652200

[6] JENSEN J, PEDERSEN EE, GAQLANTE P, HALD J, HELLER RS, et al.Control of endodermal endocrine development by Hes-1. Nat Genet 2000;24:36–44. doi:10.1038/71657

[7] LEOW CC, ROMERO MS, ROSS S, POLAKIS P, GAO WQ.HATHL, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. Cancer Res 2004;64:6050–6057.

[8] TOZAWA E, AJIOKA Y, WATANABE H, NISHIKURA K, MUKAI G, et al.Mucin expression, p53 overexpression, and peritumoral lymphocytic infiltration of advanced colorectal carcinoma with mucus component: is mucinous carcinoma a distinct histological entity? Pathol Res Pract 2007;203:567–574. doi:10.1016/j.prp.2007.04.013

[9] OKUDAIRA K, KAKAR S, CUN L, CHOI E, WU DECAMILLISs R, et al.MUC2 gene promoter methylation in mucinous and non-mucinous colorectal cancer tissues.Int J Oncol 2010;36:765–775.

[10] LIVAK KJ, SCHMITTGEN TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.

[11] QIAO L, WONG BC.Role of Notch signaling in colorectal cancer. Carcinogenesis 2009;30:1979–1986. doi:10.1093/carcin/bgp236

[12] BOSSUYT W, KAZANJIAN A, DE GEEST N, VAN KELST S, DE HERTOGH G, et al.Atonal homolog 1 is a tumor suppressor gene.PLoS Biol 2009;7:e39. doi:10.1371/journal.pbio.1000039

[13] KAZANJIAN A, NOAH T, BROWN D, BURKART J, SHROYER NF.Atonal homolog 1 is required for growth and differentiation effects of notch/gamma-secretase inhibitors on normal and cancerous intestinal epithelial cells. Gastroenterology 2010;139:918–928. doi:10.1053/j.gastro.2010.05.081

[14] LEOW CC, POLAKIS P, GAO WQ.A role for Hath1, a bHLH transcription factor, in colon adenocarcinoma.Ann N Y Acad Sci 2005;1059:174–183. doi:10.1196/annals.1339.048

[15] MELIS M, HERNANDEZ J, SIEGEL EM, MCLOUGHLIN JM, LY QP, et al.Gene expression profiling of colorectal mucinous adenocarcinomas.Dis Colon Rectum 2010;53:936–943.

[16] TOM BH, RUTZKY LP, JAKSTYS MM, OYASU R, KAYE CI, et al. Human colonic adenocarcinoma cells. I. Establishment and description of a new line. In Vitro 1976;12:180–191.

[17] AKIOSHI T, NAKAMURA M, YANAI K, NAGAI S, WADA J, et al. Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. Gastroenterology 2008;134:131–144. doi:10.1053/j.gastro.2007.10.008

[18] PANNEQUIN J, BONNANS C, DELAUNAY N, RYAN J, BOURGAUX JF,et al.The wnt target jagged-1 mediates the activation of notch signaling by progastrin in human colorectal cancer cells. Cancer Res 2009;69:6065–6073. doi:10.1158/0008-5472.CAN-08-2409

[19] NAKAMURA T, TSUCHIYA K, WATANABE M.CROSSTALKk Between Wnt and Notch signaling in intestinal epithelial cell fate decision.J Gastroenterol 2007;42:705–710. doi:10.1007/s00535-007-2087-z

[20] Radtke F, Clevers H. Self-renewal and cancer of the gut: two sides of a coin. Science. 2005;307:1904–1909. doi:10.1126/science.1104815