Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 334 (2005) 1365–1373
BBRC
www.elsevier.com/locate/ybbrc

Deregulated GSK3β activity in colorectal cancer: Its association with tumor cell survival and proliferation ☆

Abbas Shakoori [a,b,1], Andrei Ougolkov [a,d,1], Zhi Wei Yu [a], Bin Zhang [a], Mohammad H. Modarressi [c], Daniel D. Billadeau [d], Masayoshi Mai [a], Yutaka Takahashi [a], Toshinari Minamoto [a,b,*]

[a] Divisions of Diagnostic Molecular Oncology and Surgical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan
[b] Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan
[c] Department of Medical Genetics, Tehran University of Medical Sciences, Keshavarz Blvd, Tehran, Iran
[d] Division of Oncology Research, Department of Immunology, Mayo Clinic College of Medicine, MN 55905, USA

Received 27 June 2005
Available online 19 July 2005

Abstract

Glycogen synthase kinase 3β (GSK3β) reportedly has opposing roles, repressing Wnt/β-catenin signaling on the one hand but maintaining cell survival and proliferation through the NF-κB pathway on the other. The present investigation was undertaken to clarify the roles of GSK3β in human cancer. In colon cancer cell lines and colorectal cancer patients, levels of GSK3β expression and amounts of its active form were higher in tumor cells than in their normal counterparts; these findings were independent of nuclear accumulation of β-catenin oncoprotein in the tumor cells. Inhibition of GSK3β activity by phosphorylation was defective in colorectal cancers but preserved in non-neoplastic cells and tissues. Strikingly, inhibition of GSK3β activity by chemical inhibitors and its expression by RNA interference targeting GSK3β induced apoptosis and attenuated proliferation of colon cancer cells in vitro. Our findings demonstrate an unrecognized role of GSK3β in tumor cell survival and proliferation other than its predicted role as a tumor suppressor, and warrant proposing this kinase as a potential therapeutic target in colorectal cancer.

Keywords: Glycogen synthase kinase 3β; Phosphorylation; Colorectal cancer; Cell survival; Proliferation; Apoptosis; Therapeutic target; Wnt signaling; β-catenin; NF-κB

Activation of the Wnt/β-catenin signaling pathway has emerged as an oncogenic pathway leading to colorectal cancer development [1,2]. In an earlier study, we identified distinct patterns of β-catenin activation in human colorectal cancer and demonstrated that activation of this oncoprotein in the tumor invasion front reliably identifies a subset of colon cancer patients who are susceptible to tumor recurrence and have a less favorable survival rate [3]. These clinical observations establish the importance of molecular mechanism(s) underlying the distinct patterns of β-catenin activation. Among regulators of the Wnt signaling pathway [2] is glycogen synthase kinase 3β (GSK3β), which phosphorylates β-catenin, thereby recruiting it for ubiquitin-mediated degradation under physiological conditions [2,4,5]. Since GSK3β is a negative regulator of Wnt/β-catenin

☆ *Abbreviations:* APC, adenomatous polyposis coli; *CTNNB1*, β-catenin gene; GSK3β, glycogen synthase kinase 3β; NF-κB, nuclear factor-κB; NSAID(s), non-steroidal anti-inflammatory drug(s); NIDDM, non-insulin-dependent diabetes mellitus; PKB, protein kinase B; RNAi, RNA interference; siRNA, small interfering RNA.

* Corresponding author. Fax: +81 76 234 4523.
E-mail address: minamot@kenroku.kanazawa-u.ac.jp (T. Minamoto).

[1] These authors contributed equally to this study.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.041

signaling, clarifying whether GSK3β plays a part in the mechanism underlying the different patterns of oncogenic β-catenin activation is a matter of considerable interest.

GSK3β is a multifunctional serine/threonine kinase that regulates various cellular pathways, depending on its substrates for phosphorylation [4–6], and it is evident that regulation of Wnt/β-catenin signaling is only one of its diverse functions [4,5]. Since oncogenic transcription factors (e.g., c-Jun, c-Myc) and proto-oncoproteins (i.e., β-catenin, Gli proteins) are putative GSK3β substrates for phosphorylation-dependent inactivation [4], it is hypothesized that GSK3β interferes with cellular neoplastic transformation and tumor development, as exemplified by its activity in Wnt/β-catenin signaling [2]. However, only a few studies have addressed its role(s) in human cancer, and these studies have reported differing effects of GSK3β on cancer cells [7,8]. Using GSK3β deficient mouse embryonic fibroblasts, it was shown that GSK3β plays a crucial role in cell survival mediated by the nuclear factor-κB (NF-κB) pathway [9,10]. Interestingly, we have previously shown that the Wnt/β-catenin and NF-κB pathways were co-activated in colorectal cancer by dysregulation in the ubiquitin system [11]. Thus, these observations bring forward apparently opposing notions regarding the functions of GSK3β in neoplastic cells on the one hand, removing a neoplastic trigger by phosphorylation-dependent degradation of β-catenin oncoprotein in the ubiquitin system, and on the other, contributing to a cell proliferation and survival pathways by regulating NF-κB.

The present study was therefore undertaken to clarify the role of GSK3β in cancer by analyzing expression and activity of this kinase in colon cancer cell lines and clinical colorectal cancers and investigating its effects on cancer cells. Our results demonstrate that independently of its effects on Wnt/β-catenin signaling, GSK3β has a hitherto unrecognized pathologic role in cell survival and proliferation in colorectal cancer, and suggest the necessity of further studies to evaluate GSK3β as a potential therapeutic target for cancer treatment.

Materials and methods

Cell lines. Colon cancer cell lines (SW480, SW620, HT29, LoVo, HCT116, SW48, and RKO) and HEK293 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells of each type were grown and harvested for extraction of protein and DNA. Genetic alterations in all but one of the colon cancer cell lines have been documented (Table 1) [12].

Patients and tissue samples. Subjects comprised 20 patients who underwent surgical removal of colorectal cancer in our institute in the period 2002–2003. Clinical data, tumor stage at initial treatment according to the TNM classification [13], and presence of distant metastasis and recurrence are shown in Table 2. All patients agreed to enrollment in the study and each gave informed consent. The Institutional Review Board of Kanazawa University approved all protocols upon patients' agreement. A pair of normal and tumor tissue samples was obtained from each fresh surgical specimen and stored at −80 °C until use.

In the light of evidence that nuclear accumulation (NA) of β-catenin is an indicator of oncogenic activity [2], β-catenin activation in the primary tumor was determined by immunohistochemistry using the antibody to β-catenin (BD Biosciences) as described in our previous study [3]. Each instance of β-catenin expression in the primary tumor was classified into one of three distinct previously defined patterns [3]: membranous expression (M), imitating that found in normal crypts; diffuse NA (NAd), defined as carcinoma cells with β-catenin-positive nuclei distributed throughout the tumor; and NA only in the tumor invasion front (NAinv).

Western blotting analysis. Cellular protein was extracted from frozen surgical specimens and cell lines using lysis buffer (CelLytic-MT, Sigma–Aldrich, St. Louis, MO) in a mixture of protease and phosphatase inhibitors (both from Sigma–Aldrich). A 100 μg aliquot of protein extract was subjected to Western blotting analysis as described previously [11] to examine expression of the respective kinases using primary antibodies against GSK3β (diluted 1:2,500; BD Biosciences, Lexington, KY) and its fractions phosphorylated at the serine 9 residue (phospho-GSK3β^{Ser9}) (diluted 1:1,000; Cell Signaling Technology, Beverly, MA), and the tyrosine 216 (phospho-GSK3β^{Tyr216}) (diluted 1:1,000; BD Biosciences), respectively. As a representative of activated kinases upstream to GSK3β, protein kinase B (PKB, also termed Akt) activity was determined by immunoblotting with antibodies to phospho-Akt^{Thr308} and phospho-Akt^{Ser473} (both diluted 1:1,000) (Cell Signaling Technology). In each case, signals were developed using an enhanced chemiluminescent detection reagent (ECL, Amersham, Little Chalfont, UK).

In vitro kinase assay. GSK3β was immunoprecipitated from the cell lines and subjected to an in vitro kinase assay using a recombinant human β-catenin protein (generated in our laboratory) as a substrate according to the method described previously [14]. The phosphorylated fraction of β-catenin was detected by autoradiography.

Table 1
Comparison of colon cancer cell lines in regard to GSK3β phosphorylation and mutations in *APC*, *CTNNB1*, and K-*ras*

Cell lines	Mutation [12]			Phosphorylation			
	APC	*CTNNB1*[a]	K-*ras*[b]	GSK3β^{Ser9}	GSK3β^{Tyr216}	Akt^{Tyr308}	Akt^{Ser473}
SW480	+	−	12	−	+	+	+
SW620	+	−	12	−	+	+	−
HT29	+	−	−	−	+	+	−
LoVo	+	−	13	−	+	+	+
HCT116	−	45	13	−	+	+	−
SW48	−	33	−	−	+	Trace	−
RKO	NA	−	−	−	+	Trace	+

+, present; −, absent or undetectable; NA, data not available.
[a] 45, 33: mutation in codon 45 or 33.
[b] 12, 13: mutation in codon 12 or 13.

Table 2
Clinical data and molecular characteristics of all cases of colorectal cancer

Patient ID	Age/sex	Site of tumor	TNM/ stage at surgery	Metastasis/ recurrence[a]	GSK3β expression	GSK3β phosphorylation		β-Catenin activation	Phospho-Akt^{Thr308}	Phospho-Akt^{Ser473}	K-*ras*[b] mutation
						GSK3β^{Ser9}	GSK3β^{Tyr216}				
31	44/F	R	T_2 N_0 M_0/II	NE	N < T	−	N = T	NAd	−	−	—
59	74/M	Tr	T_2 N_1 M_0/IIIA	NE	N < T	−	N < T	NAinv	+	+	12
61	83/M	S	T_2 N_0 M_0/II	NE	N < T	+	N = T	NAd	−	−	—
68	62/F	Tr	T_2 N_1 M_0/IIIA	NE	N < T	Trace	N < T	NAd	Trace	+	—
70	79/M	R	T_2 N_1 M_0/IIIA	NE	N < T	−	N < T	NAinv	−	−	—
71	55/M	A	T_2 N_0 M_0/II	NE	N < T	−	N < T	NAinv	−	+	—
72	93/M	S	T_2 N_0 M_0/II	NE	N < T	−	N < T	—	+	+	13
73	80/M	C	T_2 N_0 M_0/II	NE	N < T	Trace	N = T	—	Trace	+	—
74	70/M	S	T_2 N_0 M_1/IV	Peritoneum	N < T	+	N < T	NAinv	+	−	—
75	73/M	C	T_2 N_0 M_0/II	NE	N < T	−	N < T	—	Trace	+	—
76	61/M	R	T_2 N_0 M_0/II	NE	N < T	−	N = T	—	−	−	—
77	78/M	S	T_2 N_0 M_0/II	NE	N < T	−	N < T	NAd	Trace	+	—
78	71/F	R	T_2 N_1 M_0/IIIA	Liver	N < T	Trace	N < T	NAd	Trace	−	12
79	71/F	A	T_2 N_0 M_0/II	NE	N > T	+	N < T	—	+	+	12
80	61/M	Tr	T_3 N_2 M_1/IV	Liver, peritoneum	N < T	Trace	N < T	—	+	Trace	—
81	71/M	S	T_3 N_1 M_0/IIIA	NE	N < T	−	N < T	NAinv	−	+	—
82	81/F	A	T_2 N_2 M_1/IV	Liver, peritoneum	N < T	−	N < T	NAd	−	−	12
83	59/M	S	T_2 N_0 M_0/II	NE	N < T	Trace	N < T	—	Trace	+	—
84	54/F	R	T_2 N_1 M_0/IIIA	NE	N < T	−	N < T	NAinv	−	−	—
85	69/F	R	T_2 N_0 M_0/II	NE	N = T	−	N < T	NAinv	−	−	—

Abbreviations: F, female; M, male; A, ascending colon; C, cecum; R, rectum; S, sigmoid colon; Tr, transverse colon; N, non-neoplastic mucosa tissue; T, primary tumor; NE, not evident; −, absent; +, present; NAd, diffuse pattern of β-catenin activation in tumor; NAinv, β-catenin activation in tumor cells forming the invasion front.

[a] Metastasis/recurrence: Follow-up examination after surgery disclosed metastasis to distant organs in four patients.

[b] K-*ras* mutation: 12, mutation in codon 12; 13, mutation in codon 13.

Cytochemical and immunocytochemical staining. Cell lines grown on glass coverslips were subjected to immunofluorescence staining [15] to detect whole GSK3β and phospho-GSK3β^{Ser9} with the same primary antibodies (diluted 1:100) used for immunoblotting, respectively. Cy3-labeled goat anti-mouse IgG (H + L) and FITC-labeled goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch, Baltimore, MD) were diluted 1:200, respectively, to detect whole GSK3β and phospho-GSK3β^{Ser9}. Nuclei were counterstained with Hoechst 33342. The stained cells were observed under a fluorescence microscope (Olympus AX80, Tokyo, Japan) connected to a CCD digital camera (Olympus DP70).

Detection of mutations in GSK3β^{Ser9} and K-ras genes. Genomic DNA was extracted from colon cancer cell lines and pairs of normal and tumor tissues from surgical specimens by proteinase K digestion and treatment with phenol and chloroform. Exon 1 of the *GSK3β* gene was PCR-amplified (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 35 cycles) with a set of upstream primer 5′-ATTCGCGAAG AGAGTGATCAT-3′ and downstream primer 5′-CACTGCTAAC TTTCATGCTGC-3′. Mutation in GSK3β codon 9 (TCC) was analyzed by restriction fragment length polymorphism (PCR-RFLP) analysis using *Mnl*I (NEB, Beverly, MA), which cleaves a 110 base pair PCR product with wild-type codon 9 into two fragments of 52 and 58 base pairs but does not cleave a PCR product with any mutation in codon 9. Mutations in codons 12 and 13 of the K-*ras* gene were detected by mismatched primers-mediated PCR-RFLP analysis, as described in our previous report [3].

Treatment with GSK3β inhibitors. HEK293 and colon cancer cells seeded in a 96-well plate were treated with dimethyl sulfoxide (DMSO), or AR-A014418 (Calbiochem, San Diego, CA) and SB-216763 (Sigma–Aldrich), both known GSK3β inhibitors [16,17] dissolved in DMSO at the indicated concentrations. At designated times (24, 48, 72, and 96 h), relative numbers of viable cells were determined by measuring the optical density using a WST-8 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay kit (Cell Counting Kit-8, Wako, Osaka, Japan) by means of spectrophotometry using a microtiter-plate reader (Bio-Rad, Hercules, CA). The results were expressed as the mean optical density and standard deviations of the 6-well set for each AR-A014418 or SB-216763 dose.

RNA interference. Small interfering RNA (siRNA) specific to human GSK3β and negative control siRNA were purchased from Dharmacon (Lafayette, CO). The effect of siRNA transfection was optimized using TransIT-TKO Transfection Reagent (Mirus, Madison, WI) according to the manufacturer's protocol. The effect of siRNA on GSK3β expression was observed using Western blotting with an antibody that binds to both GSK3α and GSK3β (diluted 1:1,000; Upstate Biotechnology, Lake Placid, NY). At the optimal concentration, the effects of GSK3β siRNA transfection on cell viability at the designated times were investigated as described above.

Apoptosis and cell proliferation assays. Influences of the inhibitors and siRNA specific to GSK3β on cells were analyzed to determine changes in apoptosis and cell proliferation. After treatment with the GSK3β inhibitors, siRNA and their control reagents as described above, cells were immunostained with the antibody to proliferating cell nuclear antigen (PCNA; DakoCytomation, Glostrup, Denmark) to label proliferating cells and counterstained with Hoechst to detect apoptotic cells characterized by discrete fragmentation of nuclei. A total of 200 nuclei per field was counted and scored for apoptosis changes and PCNA-positive nuclei, respectively. The mean scores for apoptosis and PCNA-positive nuclei in all five fields were then calculated.

Results and discussion

Expression and activity of GSK3β in colorectal cancer

All colon cancer cells showed higher basal levels of GSK3β and its active form phospho-GSK3β^{Tyr216}, and no detectable phospho-GSK3β^{Ser9} (inactive form) than HEK293 cells in Western blotting analysis (Fig. 1A). An in vitro kinase assay demonstrated that GSK3β isolated from these cell lines phosphorylated β-catenin protein (Fig. 1B). In contrast to the findings in colon cancer cells, phospho-GSK3β^{Ser9} was detected in HEK293 cells, where we previously showed physiological regulation of a steady-state low level of β-catenin expression [11]. Immunocytochemically, GSK3β was localized in the cytoplasm of all cell lines analyzed, and phospho-GSK3β^{Ser9} was detected only in HEK293 cells (Fig. 2). The results indicated that high levels of GSK3β expression and activity were characteristic of all colon cancer cells where β-catenin was activated either

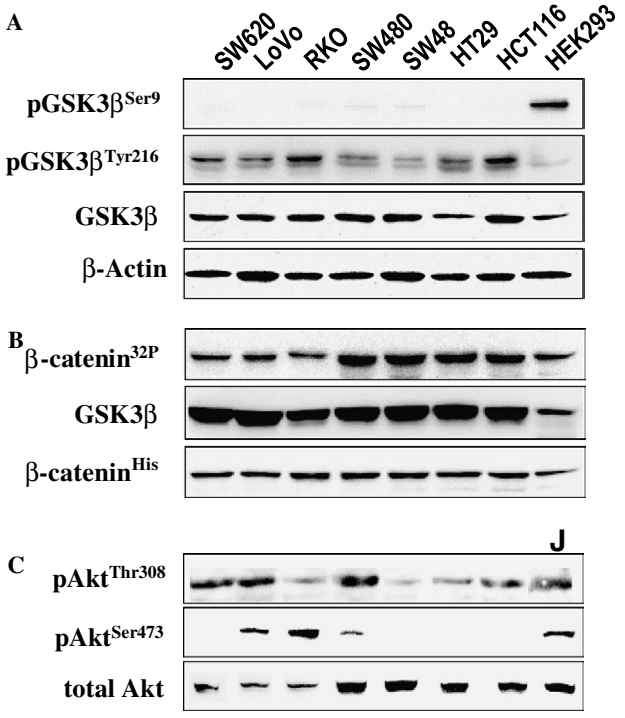

Fig. 1. Expression and activity of GSK3β (A,B) and Akt (C) in colon cancer cells, HEK293 and Jurkat (J) cells. (A) Fractions of phospho-GSK3β^{Ser9} (pGSK3β^{Ser9}: inactive form), phospho-GSK3β^{Tyr216} (pGSK3β^{Tyr216}: active form) and total GSK3β (GSK3β) were detected in protein extracts from the indicated colorectal cancer cell lines and HEK293 cells by Western immunoblotting analysis. The amount of protein extract of each sample was monitored by expression of β-actin. (B) Activity of GSK3β immunoprecipitated from each cell line extract was examined using an in vitro kinase assay with histidine-tagged recombinant β-catenin protein (β-catenin^{His}) as a substrate of this kinase. Phosphorylated β-catenin was detected by autoradiography (top). Immunoprecipitated GSK3β was detected by Western blotting (middle) and β-catenin^{His} (substrate) was stained with Coomassie blue (lower). (C) Expression of Akt and presence of its active forms phospho-Akt^{Thr308} (pAkt^{Thr308}) and phospho-Akt^{Ser473} (pAkt^{Ser473}) were determined by Western blotting in colon cancer cell lines. The protein extract from a lymphocyte cell line, Jurkat (J), was used as a positive control for detection of phospho-Akt^{Thr308} and phospho-Akt^{Ser473}, according to the manufacturer's directions for the antibodies to Akt and phospho-Akt. Results of these analyses are described in Table 1.

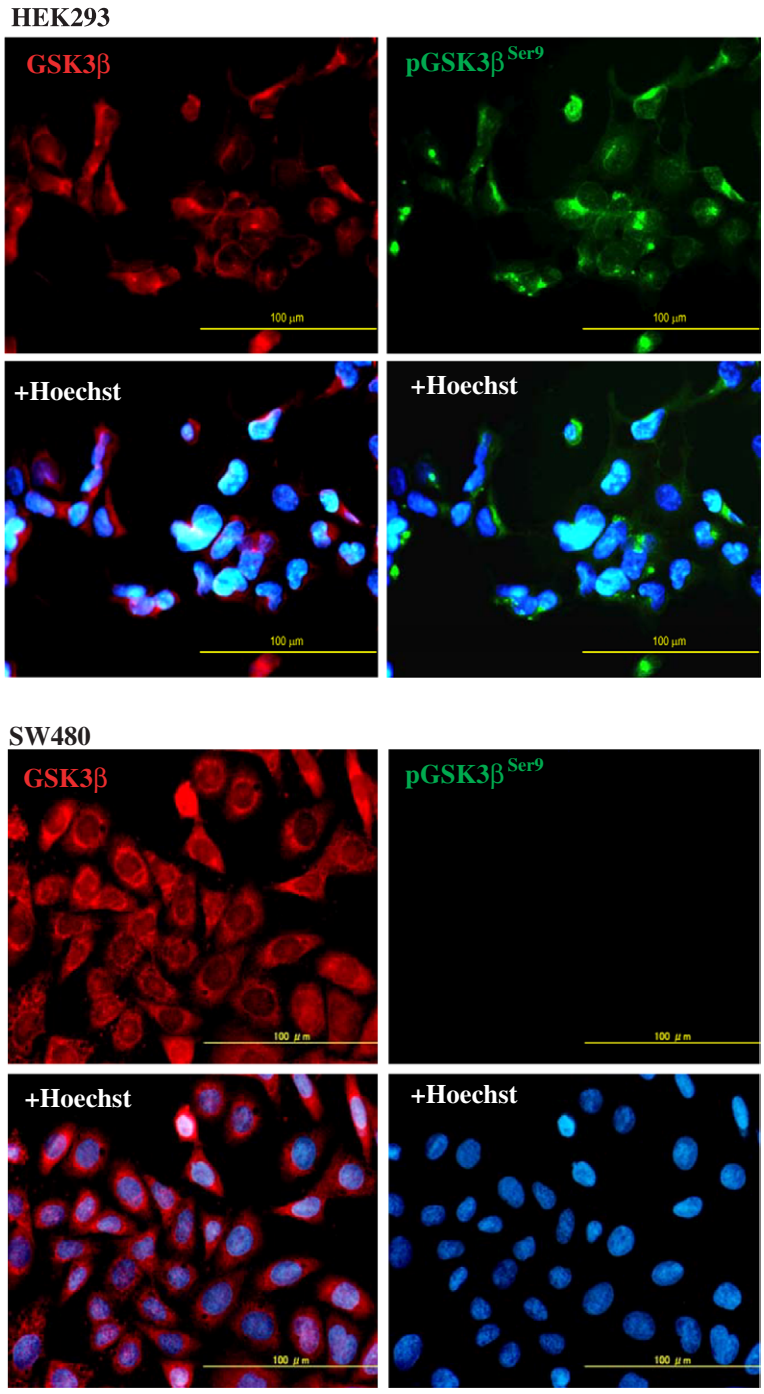

Fig. 2. Double immunofluorescence cytochemical staining of total GSK3β (probed with Cy3-labeled secondary antibody, red fluorescence) and phospho-GSK3β^{Ser9} (probed with FITC-labeled secondary antibody, green fluorescence) in HEK293 cells (upper four panels) and SW480 colon cancer cells (lower four panels). Nuclei were counterstained with Hoechst 33342 (blue fluorescence) and merged with respective fluorescent microscopic images for total GSK3β and phospho-GSK3β^{Ser9} (indicated as +Hoechst). The expression intensity of total GSK3β in cytoplasm was higher in SW480 than in HEK293 cells. Phospho-GSK3β^{Ser9} was found in the cytoplasm of HEK293 but not SW480 cells. The scale bar in each panel indicates 100 μm. All images in a gallery have the same magnification.

by mutations in *APC* (SW480, SW620, and LoVo) or *CTNNB1* (β-*catenin* gene) (HCT116, SW48) (Table 1).

Levels of GSK3β and the two phosphorylated forms were analyzed in pairs of normal and tumor tissues of colorectal cancer patients (Fig. 3). The levels of GSK3β and its active form phospho-GSK3β^{Tyr216} in the tumors were higher than in their normal counterparts in most cases. On the contrary, the inactive form phospho-GSK3β^{Ser9} was frequently detected in high levels in non-neoplastic tissues, but tumors in most cases con-

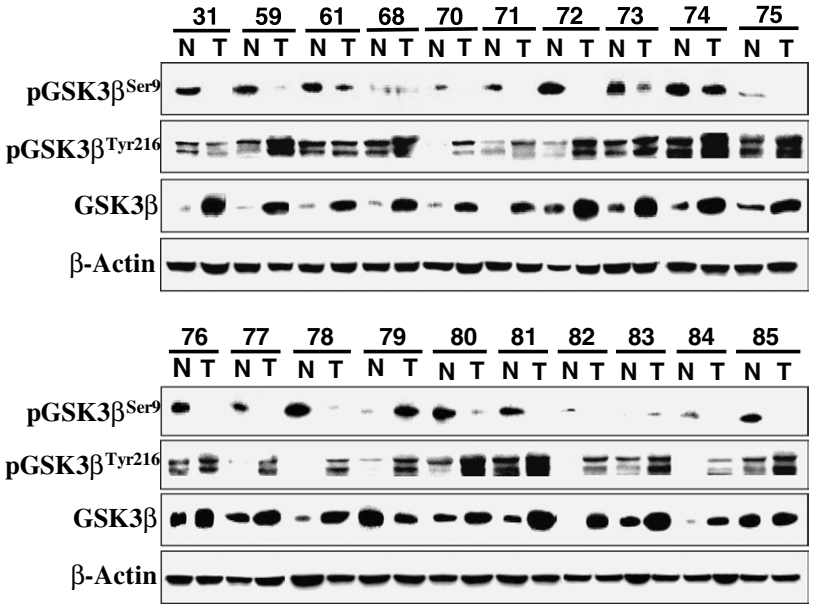

Fig. 3. Expression of GSK3β and presence of phospho-GSK3β^{Ser9} (pGSK3β^{Ser9}: inactive form) and phospho-GSK3β^{Tyr216} (pGSK3β^{Tyr216}: active form) were detected in protein extracts from primary tumor (T) and corresponding non-neoplastic mucosa tissue (N) sampled from the 20 colorectal cancer patients. The amount of protein extract in each sample was monitored by expression of β-actin. Identification numbers of patients in this study are indicated at the top of each panel. Presence and patterns (i.e., NAd and NAinv) of β-catenin activation in the tumors are described in Table 2. In most patients, levels of total GSK3β and its active form phospho-GSK3β^{Tyr216} in the primary tumors were higher than in corresponding normal tissues. Whereas the inactive form phospho-GSK3β^{Ser9} was frequently detected in non-neoplastic tissues, the primary tumors in most cases (other than patients No. 74 and 79) had trace or undetectable levels of the inactive form. As described in Table 2, expression of GSK3β or presence of phospho-GSK3β^{Ser9} or phospho-GSK3β^{Tyr216} was not correlated with presence or different patterns of β-catenin activation in the tumors.

tained only trace amounts or no detectable level of this form (Table 2). In light of the in vitro kinase assay referred to above, the major fraction of GSK3β in the tumor was considered active. β-Catenin activation was detected in the tumors of 13 (65%) patients—7 with the NAd pattern and 6 with the NAinv pattern. Similar to the observations in colon cancer cell lines, expression and activity of GSK3β in colorectal tumors showed no particular association with the presence or pattern of β-catenin activation (Table 2).

In contrast to the postulated function of GSK3β as a negative regulator of Wnt/β-catenin signaling [2,4], GSK3β is constitutively active and phosphorylation-dependent inhibition of its activity was defective in most colorectal cancers analyzed in the present study. It is well documented that the basal level of GSK3β activity in cells is normally high and that its physiological activity is maintained by inhibitory serine 9 phosphorylation of this kinase initiated by diverse stimuli [4–6]. The presence of an inactive form, phospho-GSK3β^{Ser9}, in HEK293 cells and normal tissues demonstrated here suggests a regulation of balanced expression of active and inactive forms of this kinase. Paradoxically, absence of phospho-GSK3β^{Ser9} in the tumors implies that expression and activity of GSK3β in colorectal cancer are no longer regulated physiologically, indicating that such expression and activity represent one of the biological characteristics of neoplastic cells as discussed below, and thus reflect a potentially non-physiological role for this kinase in colorectal cancer.

The first recognized function of GSK3β is to phosphorylate and inhibit glycogen synthase activity, resulting in a relative increase of glucose metabolism [4–6]. It is well known that tumors consistently rely on anaerobic pathways to convert glucose to ATP even in the presence of abundant oxygen, and that tumor cells maintain ATP production by increasing glucose influx to fuel the energy requirements of unrestricted proliferation [18]. The up-regulation of active GSK3β in most colorectal cancers shown here may contribute to high consumption of glucose by cancer cells through inhibition of glycogen synthesis.

The expression level or activity of GSK3β is not a determinant of the pattern (i.e., NAd and NAinv) of β-catenin activation in the tumors of colorectal cancer patients. This is partly in line with a report showing that no β-catenin accumulation was found in tissues of GSK3β-knockout mice [9]. Importantly, it in turn indicates that GSK3β may play a role in colorectal cancer other than its predicted role [4–6] as a tumor suppressor. There was no association between expression and activity of GSK3β and any clinical characteristics of colorectal cancer patients, including tumor stage and occurrence of metastasis (Table 2). These findings suggest that GSK3β may participate in colorectal cancer development through a pathway independent of Wnt signaling.

Putative upstream events in GSK3β activity

We next addressed possible upstream event(s) regulating expression or phosphorylation-dependent inactivation of GSK3β in colorectal cancer. The best characterized upstream kinase that inactivates GSK3β is PKB/Akt [19], which is known to be activated by phosphoinositide 3-kinase (PI3K)-dependent phosphorylation [20]. PI3K activation is downstream of both insulin [20] and ras signaling [21], and the latter signaling pathway is frequently accelerated by mutational activation of K-ras in colorectal cancer [21]. In view of these signaling network considerations, we analyzed active forms of PKB/Akt (phospho-Akt^{Thr308} and phospho-Akt^{Ser473}) (Fig. 1C) and mutations in the K-*ras* gene, and investigated whether either factor influences expression of GSK3β and its activity, as estimated on the basis of presence of the two phosphorylated forms. As shown in Tables 1 and 2, there was no correlation between GSK3β activity and PKB/Akt phosphorylation or mutational activation of K-ras in the colon cancer cell lines and 20 colorectal cancers (Fig. 1C, supplementary Fig. 1). The results suggest that upstream factors or mechanisms yet unknown, other than signaling mediated by the Ras or PKB/Akt pathway, increase the level of GSK3β or prevent its inactivation in colorectal cancer.

It has been reported that a mutation generated in codon 9 of the *GSK3*β gene, which prevents phosphorylation-dependent inactivation, results in constitutive activation of this kinase [22]. Although this would be an alternative mechanism that increases GSK3β activity, no mutation was detected in *GSK3β* gene codon 9 in colon cancer cell lines or clinical colorectal cancers (data not shown). Thus, a mutation that inhibits Ser 9 phosphorylation is not a putative mechanism for increased activity of GSK3β in colorectal cancer.

Inhibition of GSK3β expression and activity attenuates cell survival and proliferation and induces apoptosis

In view of our data showing that GSK3β is overexpressed and active in colorectal cancer, it is important to ascertain the functional role of this kinase in this type tumor. Therefore, we assessed the effects of inhibition of GSK3β activity and down-regulation of its expression on viability and proliferation of colon cancer cells in vitro. When colon cancer cell lines were treated with various concentrations of the GSK3β inhibitors AR-A014418 or SB-216763 [16,17], cell viability was reduced in a dose-dependent manner (Figs. 4A and B, supplementary Fig. 2). This effect was associated with increased apoptosis and a decreased fraction of proliferating cells labeled by PCNA (Figs. 4C and D). However, no or little effect of these inhibitors on cell survival rate or apoptosis was found in HEK293 cells (Fig. 4). It is of interest whether these pharmacological inhibitors alter

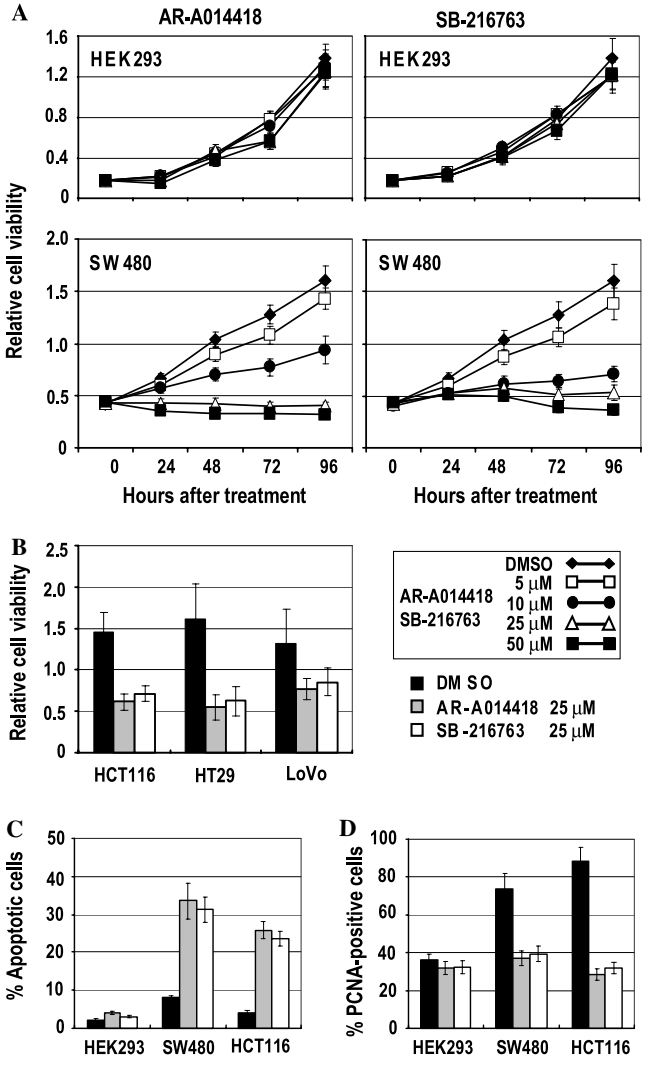

Fig. 4. Effects of treatment with different concentrations of small-molecule GSK3β inhibitors AR-A014418 and SB-216763 on in vitro survival (A,B), apoptosis (C), and proliferation (D) of HEK293 cells and colon cancer cell lines. (A) HEK293 and SW480 colon cancer cells were treated with DMSO (dimethyl sulfoxide) or indicated concentrations of GSK3β inhibitors (AR-A014418, SB-216763) for designated times. Relative cell viability (indicated on the *Y*-axis) was measured by the WST-8 assay at indicated time points (shown in the *X*-axis) as described under Materials and methods. Relative viabilities of the colon cancer cells but not HEK293 cells were inhibited in a dose-dependent fashion by treatment with either inhibitor. Concentrations of each GSK3β inhibitor (5, 10, 25, and 50 μM) used are indicated on the right-lower side of the panel. (B) GSK3β inhibitors decreased viability of colon cancer cells. Relative cell viabilities of colon cancer cells HCT116, HT29, and LoVo were measured by the WST-8 assay 96 h after treatment with DMSO or 25 mM of AR-A014418 or SB-216763. (C) GSK3β inhibitors induced apoptosis in colon cancer cells but not in HEK293 cells. As described under Materials and methods, apoptosis changes (% apoptotic cells) in the several types of cells were scored 96 h after treatment with DMSO or 25 μM of AR-A014418 or SB-216763. (D) GSK3β inhibitors attenuated cell proliferation in colon cancer cells but not in HEK293 cells. Proliferating cells in the cell lines, 96h after treatment with DMSO or 25 μM of AR-A014418 or SB-216763, were measured by scoring cells with PCNA-positive nuclei (% PCNA-positive cells) in all cells counted, as described under Materials and methods.

expression and phosphorylation of GSK3β in colon cancer cells. Levels of expression and phosphorylation at Ser9 or Tyr216 of GSK3β were monitored in colon cancer cells (SW480 and HCT116) at the different time points (24 and 48 h) after treatment with different doses (25 and 50 μM) of each inhibitor or DMSO. As represented in Supplementary Fig. 3, no alteration was found in the level of expression or amounts of the phosphorylated forms of GSK3β after treatment with GSK3β inhibitors. This finding is consistent with the reported mechanism of action of most pharmacological GSK3β inhibitors that act by competing with ATP in the ATP-binding site of the kinase [17].

In order to validate the effects of the GSK3 inhibitors on colorectal cancer cell proliferation and survival, we genetically depleted GSK3β using RNA interference (RNAi). Transfecting siRNA mixtures (25 nM each) specific to GSK3β decreased only the expression of GSK3β, but not the closely related GSK3α isoform, in the cells examined (Fig. 5A, supplementary Fig. 4). Strikingly, depletion of GSK3β in colon cancer cells, but not HEK293 cells, lead to decreased cell viability and an increase in apoptosis, (Figs. 5A and B, supplementary Fig. 4). Furthermore, knockdown of this kinase by RNAi decreased PCNA-positive cells, indicating inhibition of tumor cell proliferation (Fig. 5C). These results together indicate that both activity and expression of GSK3β are prerequisite to colorectal tumor cell survival and proliferation, thereby reflecting a potential pathologic role of this kinase in colorectal cancer.

The pathways that use GSK3β as a key regulator have been implicated in the development of human diseases other than cancer [5,16]. One such disease is non-insulin-dependent (type 2) diabetes mellitus (NIDDM), which is frequently associated with increased GSK3β activity [5,16]. The latter observation is in line with a reported association between NIDDM or insulin-resistance and risk of developing colorectal cancer [23,24]. Many studies link activation of GSK3β and neuropsychiatric disorders, exemplified by Alzheimer's disease, on the basis of GSK3β-mediated phosphorylation of tau, one of the hallmarks of this disease (reviewed in [16,25]). Interestingly, it is reported that risk of cancer development in psychiatric patients treated with lithium carbonate, a GSK3β inhibitor used for bipolar disorder [5], is significantly lower than in the general population, and that an inverse relationship has been observed between cancer morbidity and lithium dosage [26].

A number of GSK3β inhibitors have been generated primarily for development of new drugs for treatment of NIDDM and Alzheimer's disease [16,17], but never for treatment of neoplastic diseases [16]. In this study, however, we showed that either inhibition of GSKβ activity or down-regulation of its expression reduced the cell viability and proliferation and increased frequency of apoptosis in colon cancer cells. In this connection, it

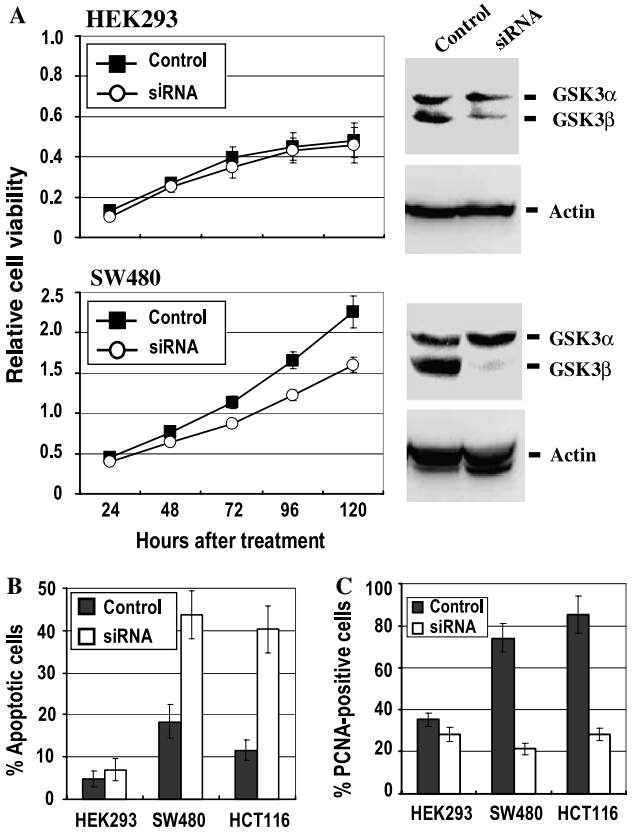

Fig. 5. Effect of RNAi targeting GSK3β on in vitro viability (A), apoptosis (B), and proliferation (C) of HEK293 cells and colon cancer cells. (A) Cells of each type were transfected with a mixture of siRNA duplexes (25 nM each) targeting GSK3β (siRNA) or negative control siRNA (25 nM) (control), and relative viabilities of these cells were determined by measuring the number of viable cells by the WST-8 assay at indicated time points, as shown in the two left panels. Effects of transfection with siRNA targeting GSK3β (siRNA) and negative control siRNA (control) on expression of GSK3α, GSK3β, and β-actin in HEK293 (right-upper panels) and SW480 cells (right-middle panels) were determined by Western immunoblotting with antibodies to GSK3 (both α and β) and β-actin, respectively, as indicated. With RNAi targeting of GSK3β, inhibition of cell proliferation was found in SW480 but not HEK293 cells, although specific reduction of GSK3β expression was observed in both cell lines. (B) RNAi targeting GSK3β induced apoptosis in colon cancer cells 96 h after transfection of the specific siRNA. (C) RNAi targeting GSK3β attenuated cell proliferation in colon cancer cells 96 h after transfection of the specific siRNA. Apoptosis changes and proliferating cells were scored as described under Materials and methods.

has been shown that GSK3β plays a role in cell survival by phosphorylating the p65 subunit of NF-κB in hepatocytes [9,10]. Moreover, a recent study [27] has demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs), the known cancer chemopreventive agents, induced phosphorylation of both β-catenin and GSK3β (serine 9 residue) prior to down-regulation of β-catenin/Tcf signaling. Taken together, our results and these considerations suggest that GSK3β could be a potential target for treatment and prevention of colorectal cancer. In addition, whether GSK3β is regulating NF-κB activity in colorectal cancer cells remains to be determined.

In all, our study demonstrates a previously unrecognized pathologic role of GSK3β in colorectal cancer, both by substantiating its activity in established colon cancer cells and clinical colorectal cancers and by pharmacologically and genetically modulating the activity and expression of this kinase in cancer cells. In addition to its promoting effect on cell survival and proliferation, GSK3β regulates a wide range of cellular processes including energy metabolism, transcription control, and cell fate determination, by modulating cellular regulatory proteins and transcription factors [4–6]. Therefore, investigating broader mechanisms underlying a potential oncogenic role of GSK3β may provide insights into molecular pathways leading to colorectal tumorigenesis, and support development of novel strategies for treatment and chemoprevention targeting this kinase and the molecular epidemiology of colorectal cancer.

Acknowledgments

We thank Dr. Hiroyasu Esumi (National Cancer Center Research Institute, Japan) for reviewing the manuscript, and Mr. Michael Meyer for editorial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Technology and Culture (to T.M.), from the Ministry of Health, Labour and Welfare (to T.M.), and from the Japan Society for the Promotion of Science (to T.M., A.O., and B.Z.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.07.041.

References

[1] J.R. Jass, V.L.J. Whitehall, J. Young, B.A. Leggett, Emerging concepts in colorectal neoplasia, Gastroenterology 123 (2003) 862–876.
[2] P. Polakis, The oncogenic activation of beta-catenin, Curr. Opin. Genet. Dev. 9 (1999) 15–21.
[3] A.V. Ougolkov, K. Yamashita, M. Mai, T. Minamoto, Oncogenic β-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer, Gastroenterology 122 (2002) 60–71.
[4] A.S. Manoukian, J.R. Woodgett, Role of glycogen synthase kinase-3 in cancer: regulation by Wnts and other signaling pathways, Adv. Cancer Res. 84 (2002) 203–229.
[5] B.W. Doble, J.R. Woodgett, GSK-3: tricks of the trade for a multi-tasking kinase, J. Cell Sci. 116 (2003) 1175–1186.
[6] R.S. Jope, G.V.W. Johnson, The glamour and gloom of glycogen synthase kinase-3, Trends Biochem. Sci. 29 (2004) 95–102.
[7] X. Liao, L. Zhang, J.B. Thrasher, J. Du, B. Li, Glycogen synthase kinase-3β suppression eliminates tumor necrosis factor-related apoptosis-inducing resistance in prostate cancer, Mol. Cancer Ther. 2 (2003) 1215–1222.
[8] M. Mazor, Y. Kawano, H. Zhu, J. Waxman, R.M. Kypta, Inhibition of glycogen synthase kinase-3 represses androgen receptor activity and prostate cancer cell growth, Oncogene 23 (2004) 7882–7892.
[9] K.P. Hoeflich, J. Luo, E.A. Rubie, M.S. Tsao, O. Jin, J.R. Woodgett, Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation, Nature 406 (2000) 86–90.
[10] R.F. Schwabe, D.A. Brenner, Role of glycogen synthase kinase-3 in TNF-α-induced NF-κB activation and apoptosis in hepatocytes, Am. J. Physiol. Gastrointest. Liver Physiol. 283 (2002) G204–G211.
[11] A. Ougolkov, B. Zhang, K. Yamashita, V. Bilim, M. Mai, S.Y. Fuchs, T. Minamoto, Associations among β-TrCP, an E3 ubiquitin ligase receptor, β-catenin and NF-κB in colorectal cancer, J. Natl. Cancer Inst. 96 (2004) 1161–1170.
[12] J. Gayet, X.P. Zhou, A. Duval, S. Rolland, J.-M. Hoang, P. Cottu, R. Hamelin, Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines, Oncogene 20 (2001) 5025–5032.
[13] L.K. Sobin, C. Wittekind (Eds.), UICC International Union Against Cancer. TNM Classification of Malignant Tumours, fifth ed., Wiley, New York, 1997, pp. 66–69.
[14] V. Adler, M.R. Pincus, T. Minamoto, S.Y. Fuchs, M.J. Bluth, P.W. Brandt-Rauf, F. Friedman, J.M. Chen, X.W. Wang, C.C. Harris, Z. Ronai, Conformation-dependent phosphorylation of p53, Proc. Natl. Acad. Sci. USA 94 (1997) 1686–1691.
[15] A. Shakoori, G. Fujii, S. Yoshimura, M. Kitamura, K. Nakayama, T. Ito, H. Ohno, N. Nakamura, Identification of a five-pass protein family localizing in the Golgi apparatus and the ER, Biochem. Biophys. Res. Commun. 312 (2003) 850–857.
[16] P. Cohen, M. Goedert, GSK3 inhibitors: development and therapeutic potential, Nat. Rev. Drug. Discov. 3 (2004) 479–487.
[17] L. Meijer, M.P. Flajolet, P. Greengard, Pharmacological inhibitors of glycogen synthase kinase 3, Trends Pharm. Sci. 25 (2004) 471–480.
[18] R.A. Gatenby, E.T. Gawlinski, The glycolytic phenotype in carcinogenesis and tumor invasion: insights through mathematical models, Cancer Res. 63 (2003) 3847–3854.
[19] M. Sharma, W.W. Chuang, Z. Sun, Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3β inhibition and nuclear β-catenin accumulation, J. Biol. Chem. 277 (2002) 30935–30941.
[20] D.A. Cross, D.R. Alessi, P. Cohen, M. Andjelkovich, B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, Nature 378 (1995) 785–789.
[21] A.A. Adjei, Blocking oncogenic ras signaling for cancer therapy, J. Natl. Cancer Inst. 93 (2001) 1062–1074.
[22] L. Wang, H.-K. Lin, Y.-C. Hu, S. Xie, L. Yang, C. Chang, Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen kinase 3β in prostate cells, J. Biol. Chem. 279 (2004) 32444–32452.
[23] F.B. Hu, J.E. Manson, S. Liu, D. Hunter, G.A. Colditz, K.B. Michels, F.E. Speizer, E. Giovannucci, Prospective study of adult onset diabetes mellitus (type 2) and risk of colorectal cancer in woman, J. Natl. Cancer Inst. 91 (1999) 542–547.
[24] D. Komninou, A. Ayonote, J.P. Richie Jr., B. Rigas, Insulin resistance and its contribution to colon carcinogenesis, Exp. Biol. Med. (Maywood) 228 (2003) 396–405.
[25] R.V. Bhat, S.L. Budd, GSK3β signalling: casting a wide net in Alzheimer's disease, Neurosignals 11 (2002) 251–261.
[26] Y. Cohen, A. Chetrit, Y. Cohen, P. Sirota, B. Modan, Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment, Med. Oncol. 15 (1998) 32–36.
[27] S. Dihlmann, S. Klein, M.v.K. Doeberitz, Reduction of β-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated β-catenin, Mol. Cancer Ther. 2 (2003) 509–516.