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Blockade of JAK2 Activity Suppressed Accumulation of β-Catenin in Leukemic Cells

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ABSTRACT

The Wnt/β-catenin pathway has been implicated in leukemogenesis. We found β-catenin abnormally accumulated in both human acute T cell leukemia Jurkat cells and human erythroleukemia HEL cells. β-Catenin can be significantly down-regulated by the Janus kinase 2 specific inhibitor AG490 in these two cells. AG490 also reduces the luciferase activity of a reporter plasmid driven by LEF/β-catenin promoter. Similar results were observed in HEL cells infected with lentivirus containing shRNA against JAK2 gene. After treatment with 50 μM AG490 or shRNA, the mRNA expression levels of β-catenin, APC, Axin, β-Trcp, GSK3α, and GSK3β were up-regulated within 12–16 h. However, only the protein levels of GSK3β and β-Trcp were found to have increased relative to untreated cells. Knockdown experiments revealed that the AG490-induced inhibition of β-catenin can be attenuated by shRNA targeting β-TrCP. Taken together; these results suggest that β-Trcp plays a key role in the cross-talk between JAK/STAT and Wnt/β-catenin signaling in leukemia cells. J. Cell. Biochem. 111: 402–411, 2010.

KEY WORDS: JAK2; β-CATENIN; β-TrCP; LEUKEMIA

Wnt signaling plays a key role in the regulation of cell fate decisions during development including cell proliferation, cell morphogenesis, cell motility, adhesion, and cell survival [Wodarz and Nusse, 1998; Howe and Brown, 2004; Willert and Jones, 2006; Paul and Dey, 2008]. Regulation of this pathway seems to be mostly mediated by a post-translation mechanism. In the absence of Wnt signaling, a pool of non-adhesion associated soluble β-catenin binds to the adenomatous polyposis coli (APC) axin tumor suppressor complex, where it is phosphorylated by glycogen synthase kinase 3β (GSK3β) and is then targeted for proteasomal degradation. In response to Wnt signaling, β-catenin can escape the degradation complex and move to the nucleus, where it forms a complex with TCF/LEF transcription factors and drives the transcription of the Wnt responsive genes, in particular cyclin D1, c-jun, and c-myc [Quasnichka et al., 2006; Giles et al., 2003].

The Wnt system is notably perturbed in a number of cancers. Mutations in various components of the pathway can frequently be found in cancers including breast, colon, hepatic, pancreatic, lung, prostate, gastrointestinal, ovarian, medulloblastoma, and melanoma [Giles et al., 2003]. Truncation or loss of the APC protein, which

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occurs in colorectal cancer, results in the accumulation of β-catenin and increases the expression of c-Myc and cyclin D1 [Bienz and Clevers, 2000; Kikuchi, 2003]. Deregulation of this Wnt/β-catenin pathway has also been implicated in leukemogenesis. During a blast crisis of chronic myelogenous leukemia (CML), Wnt/β-catenin signaling is activated resulting in increased expression of BCR-ABL [Jamieson et al., 2004]. A high level of the β-catenin protein expression has been found in patients with acute myeloid leukemia (AML) and there is constitutive mRNA expression of Wnt-1 and Wnt-2b in the AML blasts [Simon et al., 2005]. Moreover, accumulation of β-catenin protein and the TCF and LEF target gene products, such as *c-myc* and cyclin D1, has been found to be induced by AML1-ETO, PML-RARα, and PLZF-RARα fusion proteins [Müller-Tidow et al., 2004]. Recently, many investigators have provided compelling evidence that Wnt/β-catenin signaling is crucial for the maintenance of CML stem cells [Zhao et al., 2007; Hu et al., 2009].

The mechanism of action of the aberrant Wnt signaling pathway in leukemia cells remains unclear. Many signaling pathways and their status have been reported to interact with Wnt in a range of different cells. For example androgen receptor and Kank protein have been reported to interact with β-catenin and mediate its translocation into nucleus in OS-RC-2 cells [Chesire and Isaacs, 2002; Wang et al., 2006a,b]. Transforming growth factor-β stimulates cyclin D1 expression through activation of β-catenin signaling in chondrocytes [Li et al., 2006]. Integrin-linked kinase (ILK) has been reported as inhibiting GSK3 activity, stabilizing β-catenin, and improving the expression of β-catenin target genes [Tan et al., 2001; Oloumi et al., 2006]. Conversely, activation of the tumor suppressor p53 induces a faster degradation of β-catenin [Levina et al., 2004]. It is very possible that other pathways also regulate the Wnt signaling pathway in leukemia cells and contributed to its abnormal activation.

We therefore investigated the possible regulation mechanisms of β-catenin by treating leukemic cells with various inhibitors. We surprisingly found that the JAK2-specific inhibitor tyrphostin AG490 is able to diminish the accumulation of β-catenin in Jurkat and HEL cells. The cross-talk between these two pathways was then further studied. This study may shed new light on the mechanisms of β-catenin accumulation in leukemia cells.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

Human acute T cell leukemia Jurkat, human erythroleukemia HEL, CML K562, human leukemic monocyte lymphoma U937, and human acute promyelocytic leukemia HL-60 cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), 100 U/ml of penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (GIBCO) in 25 cm^2 cell culture flasks (Orange Scientific). Human kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO). All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere. Exponentially growing cells were used for all experiments. DMSO concentration was kept under 0.5% in all experiments.

Peripheral blood was obtained from healthy adult volunteers. After density gradient centrifugation in a Ficoll-Hypaque solution (1.077 g/ml, Sigma Chemical Co.) at 400*g* for 30 min, the mononuclear cells (PBMNC) were resuspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum.

The mouse anti-APC, anti-β-TrCP, anti-GSK3α/β and anti-Actin antibodies and the rabbit anti-Axin, anti-JAK2, and anti-β-catenin antibodies were purchased from Upstate Biotechnologies (Lake Placid, NY). The mouse anti-phospho-GSK3α/β (Ser21/9), rabbit anti-IMP (CRD-BP) and anti-IκB antibodies were purchased from Cell Signaling Technology (Danvers, MA) and Abcam (Cambridge, MA). The goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase or fluorescein (FITC) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). The AG490 and sodium orthovanadate were obtained from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO).

IMMUNOBLOTTING

β-Catenin, β-TrCP, GSK3-α/β, phospho-GSK3α/β, APC, Axin, JAK2, CRD-BP, and IκB were detected by immunoblotting and actin was employed as the loading control. Briefly, treated Jurkat or HEL cells were resuspended in lysis buffer (14.5 mM KCl, 5 mM MgCl, 10 mM HEPES pH 7.2, 1 μM EGTA, 0.2% NP-40, 0.2 μM PMSF, 0.1% aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin) and centrifuged to remove nuclei and cellular debris. The supernatants were then mixed with an equal amount of sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 10% glycerol), boiled and separated by SDS–PAGE on a 7.5% or 10% polyacrylamide gel. After transferred to PVDF membranes, the blots were blocked by PBS containing 0.05% Tween-20 and 3–5% non-fat dried milk for 1 h at room temperature. The blots were probed overnight at 4°C with an appropriate dilution of the primary antibodies. After washing with PBST, the horseradish peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG was applied to the membrane for 1–1.5 h at room temperature. The signals were then visualized with an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA) and the intensity of the bands quantified by the Quantity One program (Bio-Rad Laboratories, Hercules, CA) with normalization against actin.

ESTABLISHING THE pGL4-TOP REPORTER PLASMID

In order to obtain the Wnt/β-catenin reporter plasmids, primers were designed to amplify the 265 bp DNA fragment that contains three copies of the LEF/TCF binding sites from the TOPFLASH plasmid (Upstate Biotechnologies). All primers were designed to have *Bgl* II and *Kpn* I enzyme site at 5′ end in order to facilitate insertion into pGL4.30 plasmid (Promega). Amplification of the TOP element was performed using Pfu DNA polymerase. Finally, the new construct was called the pGL4-TOP reporter plasmid. To test the luciferase activity of this plasmid, pGL4-TOP plasmid was transfected into HEK293 cells by Lipofectamin™ 2000 (Invitrogen, St. Louis, MO) according to the manufacturer's instruction. The transfected cells were then incubated in culture medium containing hygromycin B (500 μg/mL) for 2 weeks. Finally, the HEK293-TOP

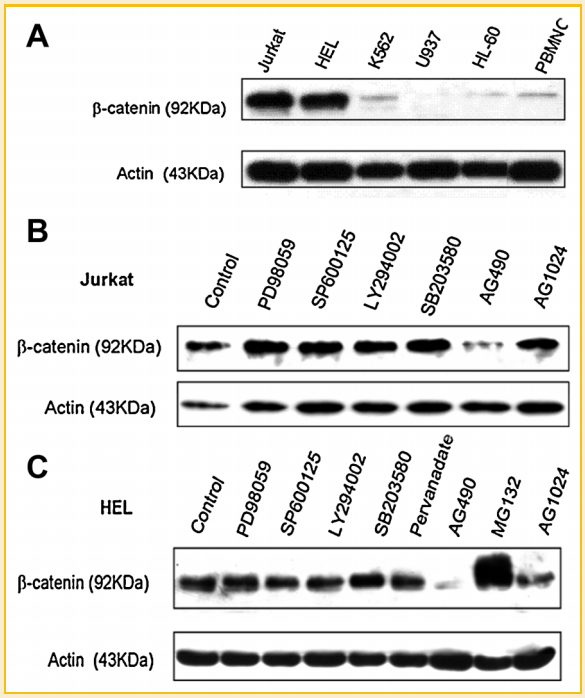

Fig. 1. Accumulation of β-catenin is reduced by treatment with the JAK2 inhibitor AG490 in both Jurkat and HEL cells. A: Total cell lysates from the indicated leukemic cell lines and normal PBMNCs were collected and the intracellular β-catenin protein level was detected by Western blotting. The Jurkat cells (B) and HEL cells (C) in serum free medium for 24 h were treated with specific inhibitors targeting various different signaling pathways. Cells were then harvested, lysed and assayed using anti-β-catenin antibody. Actin was used as the loading control.

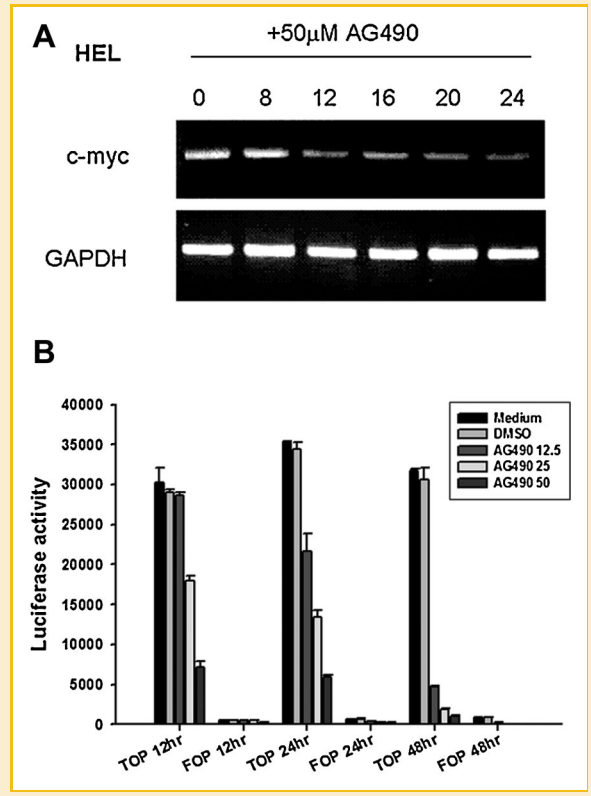

Fig. 2. AG490 suppresses both the JAK2-STAT responsive gene c-Myc and luciferase activity driven by the LEF/β-catenin promoter. A: The HEL cells were treated with 50 μM AG490 in serum free medium for indicated time. After treatment, the mRNA was extracted and subjected to RT-PCR in order to detect the mRNA level of the JAK2-STAT responsive c-Myc gene. The PCR product was analyzed by electrophoresis on a 2.5% agarose gel. GAPDH was used as an internal control. B: The luciferase activity decreased with time and as the dosage of AG490 increased.

cells were stimulated with recombinant Wnt3a (R&D) and luciferase activity was detected using a luciferase assay system kit (Promega).

TRANSFECTION AND LUCIFERASE REPORTER ASSAY

β-Catenin/Tcf transcriptional activity was determined by luciferase reporter assay. The pGL4-TOP plasmid was transfected into Jurkat cells by electroporation. Briefly, 200 μl of cell suspension and 10 μg of plasmid were combined in a Bio-Rad 0.4 cm electroporation cuvette on ice for 10 min. Electroporation was done with a Gene Pulser II (Bio-Rad) at 340 V and 960 mF. Electroporated cells were then incubated on ice for 10 min before transfer into medium containing hygromycin B (500 μg/mL); they were allowed to grow for 2 weeks. Next, the transfected cells were treated with 50 μM of AG490 in serum free medium for the indicated times. The control was treated with 0.05% DMSO. Finally, 20 μg of cytosolic extract was collected from each experimental and control group of cells in order to detect luciferase activity using a luciferase assay system kit (Promega).

REVERSE TRANSCRIPTION (RT)-PCR

Total RNA was extracted from 5×10^6 HEL or Jurkat cells using 1 ml TRIzol® RNA reagent (Invitrogen) according to the manufacturer's instructions. Between 0.1 and 5 μg total RNA was subjected to reverse transcription using 0.5 μg of random hexamer primers (0.5 μg/μl). The aliquot was then made up to a final volume of 11 μl with DEPC water and heated at 70°C for 10 min to denature the RNA. After addition of 4 μl of 5 × RT buffer, 2 μl dNTP (10 mM), 1 μl RiboLock™ RNase inhibitor, and 1 μl M-MuLV RT, the mixture was incubated at 42°C for 1 h. After the reaction, the cDNA product was heated to 70°C for 10 min and then kept on ice.

The cDNA was then amplified by PCR using Taq polymerase (Fernentas, Lithuania) and specific primers, for example, those for *c-Myc* (forward primer: 5′-TACCCTCTCAACGACAGCAGCT-3′; reverse primer: 5′- CTTGACATTCTCC TCGGTGTCC-3′). After 25 cycles with an annealing temperature of 60°C for *c-Myc*, the PCR product was subjected to electrophoresis on a 2.5% agarose gel and stained with ethidium bromide. The gels were then scanned and band density was calculated. The primers and annealing temperatures for the other genes, which treated in a similar way, are shown in Supplement Table I.

QUANTITATIVE RT-PCR

The cDNA of each sample was sent to the Microarray & Gene Expression Analysis Core Facility of the National Yang-Ming University VGH Genome Research Center (VYMGC) to perform Real-time quantitative PCR (TaqMan® Gene Expression Assays). The whole process followed the manufacturer's standard procedure (Applied Biosystems).

VSV-G PSEUDOTYPED LENTIVIRUS-shRNA SYSTEM

All the RNAi plasmids and vectors were obtained from the National RNAi Core Facility, Academia Sinica, Taiwan. We prepared the plasmid DNA from The RNAi Consortium (TRC) library to generate the lentivector constructs and then packed the viral-vector expression constructs into VSV-G pseudotyped viral particles for transient transfection. In brief, 293T cells were seeded at an appropriate concentration in a six-well plate. The lentivirus vectors containing pLKO.1-shRNA plus two other plasmids, VSV-G and pCMVΔR8.91, were mixed and co-transfected into 293T cells for virus generation. VSV-G. (pMD.G) encodes the envelope protein and pCMVΔ R8.91 encodes a HIV-1 Gag and polymerase (RT) expression (both from Genomic Research Center, Academia Sinica). The pLKO.1-shLuc was used as the control plasmid. Twelve hours later, the transfection medium was removed and replaced with fresh medium. At 24–36 h post-transfection, lentivirus particles are released into the supernatant. After this time point, the lentivirus-containing supernatant was harvested and filtered through a 0.22 μm filter.

For lentiviral infection, an appropriate number of HEL cells was seeded in a 24-well plate and incubated in RPMI culture medium with protamine sulfate (8 μg/ml) for 15 min. The lentiviral supernatant was then added and the cells incubated for a further 24 h. At this point cells stably expressing the lentivector construct were selected by the addition of an appropriate concentration of puromycin. Fresh RPMI medium with puromycin was used to replace the old medium every three days. The cells were harvested at a later stage for further study.

STATISTICAL ANALYSIS

Each result was expressed as a mean ± SE. Comparison of each group of data was analyzed by two-tailed Student's *t*-test with the level of statistical significance set at a *P*-value of ≤ 0.05.

RESULTS

β-CATENIN ACCUMULATION IN ACUTE T CELL LEUKEMIA JURKAT CELLS AND HUMAN ERYTHROLEUKEMIA HEL CELLS

Although a previous study has reported that β-catenin is highly expressed in most leukemic cell lines [Mai et al., 2007], we confirmed this by determining the level of β-catenin protein in five leukemia cell lines by Western blotting. As shown in Figure 1A, a high level of β-catenin accumulation was observed in both Jurkat and HEL cells whereas the level of β-catenin in K562 cells, U937

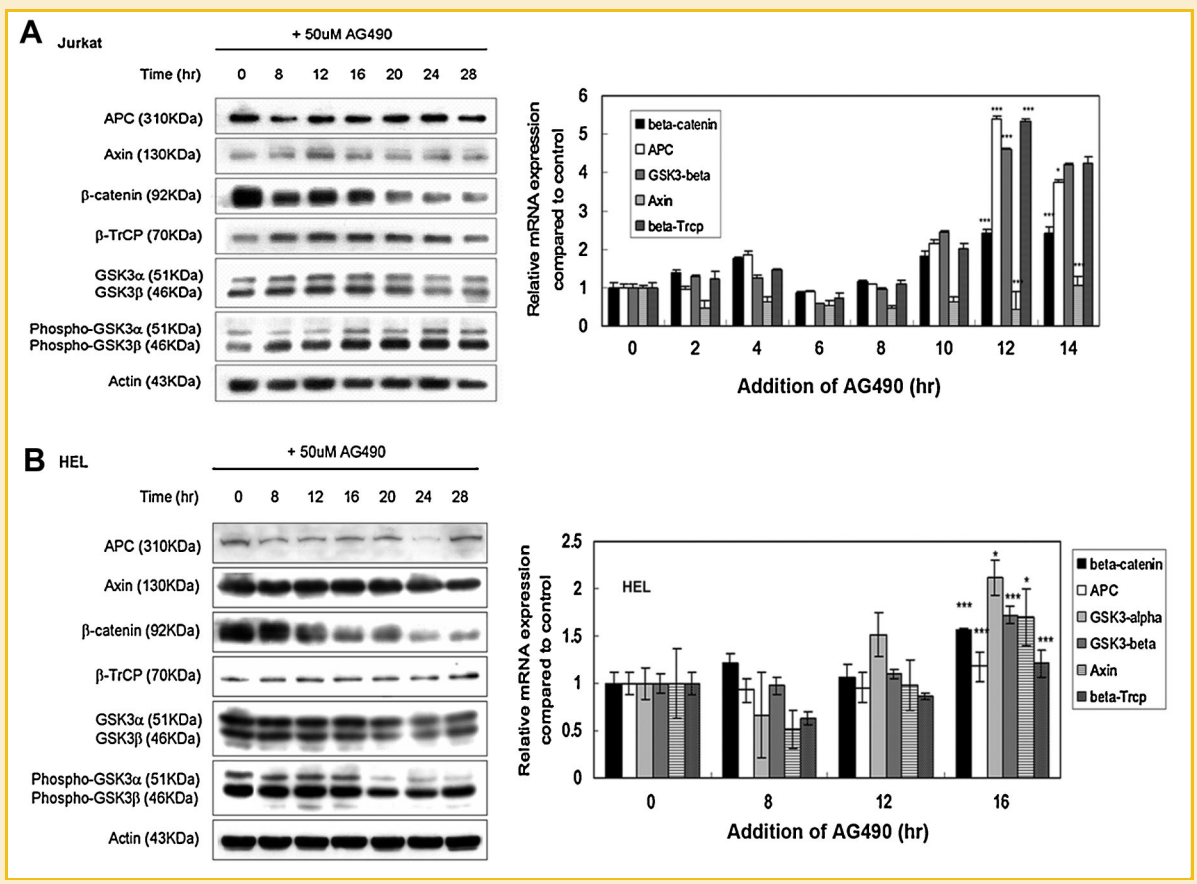

Fig. 3. Effect of AG490 on expression of β-catenin, APC, Axin, β-TrCP, and GSK3α/β in Jurkat and HEL cells. The Jurkat (A) and HEL (B) cells were exposed to 50 μM AG490 for the indicated times. Then, cell lysates and mRNA were collected and subjected to SDS–PAGE (left) and quantitative RT-PCR (right). The RT-PCR value was normalized against the housekeeping gene GAPDH. Data represent the average and standard error of two experiments. (Two-tailed Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ with respect to the 0 h control).

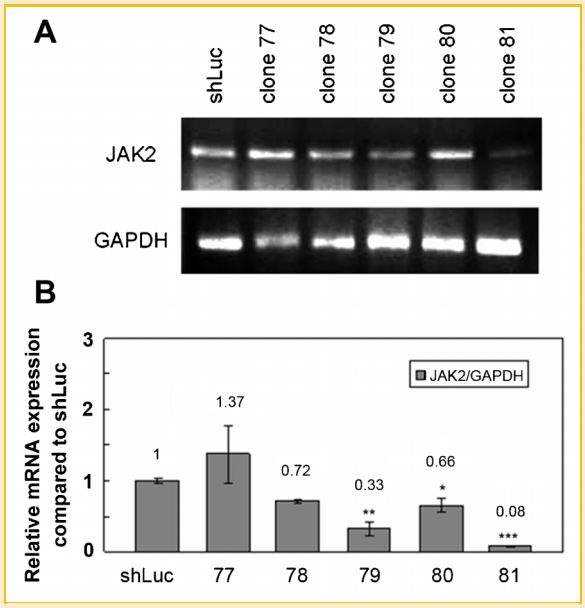

Fig. 4. JAK2 expression was knockdown by different shRNA clones in HEL cells. Lentivirus containing shRNA against five different regions of the JAK2 gene were infected to HEL cells. The mRNA expression of JAK2 in the knocked-down HEL cells was examined by RT-PCR (A) and the ratio of JAK2/GAPDH for each clone was quantified (B). PLK0.1-shLuc was used as a negative control. Each histogram indicates mean ± SD of three quantifications ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ with respect to shLuc).

cells, HL-60 cells and normal adult PBMNC cells showed relatively little difference. This suggests that Wnt/β-catenin signaling is aberrant in both Jurkat and HEL cells. These two cells were then employed to investigate aberrant Wnt/β-catenin signaling in leukemia.

THE JAK2-SPECIFIC INHIBITOR AG490 REDUCES β-CATENIN ACCUMULATION IN BOTH JURKAT AND HEL CELL LINES

To investigate whether other signaling pathways are also involved in Wnt/β-catenin signaling and cause β-catenin accumulation, we challenged Jurkat and HEL cells with a series of specific inhibitors against various signaling pathways. These include the MEK/MAPK inhibitor PD98059, the JNK inhibitor SP600125, the PI3K inhibitor LY294002, the p38 MAPK inhibitor SB203580, the JAK2 inhibitor AG490, the IGF-1 receptor inhibitor AG1024, the tyrosine phosphatase inhibitor pervanadate, and the proteasome inhibitor MG132. To avoid unexpected interference from the minor serum proteins, the cells were incubated in serum-free medium plus indicated concentration of drug. The results from these investigations showed that the accumulation of β-catenin was significant reversed by AG490 in both Jurkat and HEL cells (Fig. 1B,C). An increase in the amount of β-catenin after MG132 treatment was detected and this acted as a positive control since β-catenin is known to be degraded by the proteosome.

SUPPRESSION OF BOTH THE JAK2-STAT RESPONSIVE GENE c-Myc AND β-CATENIN/Tcf RESPONSIVE LUCIFERASE ACTIVITY BY AG490

It is known that JAK2 induces *c-Myc* expression through activation of STAT [Xie et al., 2002]. To understand whether 50 μM AG490 is able to efficiently suppress the JAK2/STAT pathway, we monitored the expression of *c-Myc* after treatment with 50 μM AG490. Figure 2A showed that mRNA expression of *c-Myc* in HEL cells decreased after 12 h of AG490 treatment. This time-dependent decrease of *c-Myc* can be observed up till 48 h of treatment (Supplementary Material S2). Next, we examined the effect of AG490 on β-catenin/Tcf-dependent transcriptional activity using the TOPflash/FOPflash reporter assay. We established a pGL4-TOP reporter plasmid containing three copies of the LEF/TCF binding sites from TOPFLASH plasmid and a hygromycin resistance marker. A FOPFLASH plasmid with mutant TCF promoter was also established. After transfection of the pGL4-TOP or pGL4-FOP reporter plasmid into Jurkat cells, the luciferase activity was measured following AG490 treatment for 48 h. After 12–48 h of AG490 treatment, the TOP luciferase activity showed both dose and time dependent decreases, whereas the transcription activity of FOP was very low and negligible (Fig. 2B). In addition, we also examined whether AG490 affect the β-catenin translocation from cytoplasm into nucleus. We found that β-catenin in both cytoplasmic and nuclear compartments was reduced by AG490 (Supplementary Material S3). This indicated that, in parallel with the effective suppression of the JAK2 pathway, AG490 not only diminished the amount of β-catenin but also suppressed the Wnt signaling pathway.

AN AG490 INDUCED INCREASE IN β-TrCP AND GSK-3α/β AT BOTH mRNA AND PROTEIN LEVEL

To understand how JAK2 inhibits the accumulation of β-catenin, we observed the changes in β-catenin and β-catenin related proteins by immunoblotting. The intensity of each band was quantified, normalized against actin, and shown as ratio of untreated control (Supplementary Material S4). In the Jurkat cells, β-catenin protein was down-regulated after 8 h of treatment; meanwhile, β-transducin repeat-containing protein (β-TrCP) was up-regulated and reached a maximum in 12–20 h (Fig. 3A). This increase in β-TrCP was also observed at the mRNA level. The other β-catenin destruction protein, Axin, and APC remained little changed over 28 h of treatment, in spite of the fact that the mRNA level of APC increased over 12–14 h. GSK-3α and GSK-3β protein levels had increased at 8 h but then had decreased after 20 h of 50 μM AG490 treatment. If normalized with their actin control, GSK-3α/β proteins still increased after 20 h. Their mRNA levels had also increased after 12 h. Taken together, we found the mRNA expression levels of β-catenin, APC, β-Trcp, and GSK-3α/β were up-regulated at 12–16 h. However, only the protein levels of GSK-3α/β and β-Trcp showed an increase compared to untreated cells. This suggests that inhibitory effect of AG490 on β-catenin must result from protein degradation but not involve the level of mRNA present in the cells. Furthermore, it would seem that the β-TrCP and GSK-3α/β proteins may be involved in the mechanism associated with β-catenin degradation.

Leukemia HEL cells possess a V617F mutation that constitutively activated JAK2. We therefore examined whether AG490 has the same effect in HEL cells as in Jurkat cells. As shown in Figure 3B, similar changes in APC, Axin, β-catenin, and β-TrCP

were observed. AG490 reduced the β-catenin concentration significantly at 12–20 h, but the increase in β-TrCP protein was found to occur much later at 28 h. However, GSK-3αβ showed little changed over this period. As observed in Jurkat cells, the mRNA expression of these genes in HEL cells was up-regulated after 12–14 h of treatment. AG490 activates the expression of β-catenin-related genes, but only β-TrCP is increased in protein level. To rule out the possibility of a DMSO effect, we repeated this experiment using 0.5% DMSO and the results showed no protein or mRNA changes in these genes (data not shown). Taken together, it would seem that the JAK2 blockade suppressed the Wnt/β-catenin pathway in HEL cells in a similar manner to that which occurred in Jurkat cells. However, HEL has constitutive JAK2 activity and therefore AG490 might not be as effective as in Jurkat cells.

KNOCKDOWN OF JAK2 PRODUCES SIMILAR RESULTS TO AG490 TREATMENT

Since AG490 might target more than one kinase, we repeated the above experiments using the more specific shRNA knockdown technique. JAK2 expression in HEL cells is much higher than in Jurkat cells, therefore we employed the HEL cells as the model system. We obtained five shRNAs that targeted different sequences within the JAK2 gene from a TRC lentivirus library. The HEL cells were infected with lentivirus carrying these shRNA. As shown in Figure 4A,B, clone 81 showed a >90% of knockdown efficiency for JAK2 expression in HEL cells, but clones 78, 79, and 80 were only able to give knockdowns of between 28% and 60%. We then compared the mRNA expression and protein level of β-catenin and β-catenin related proteins between clones 78 and 81.

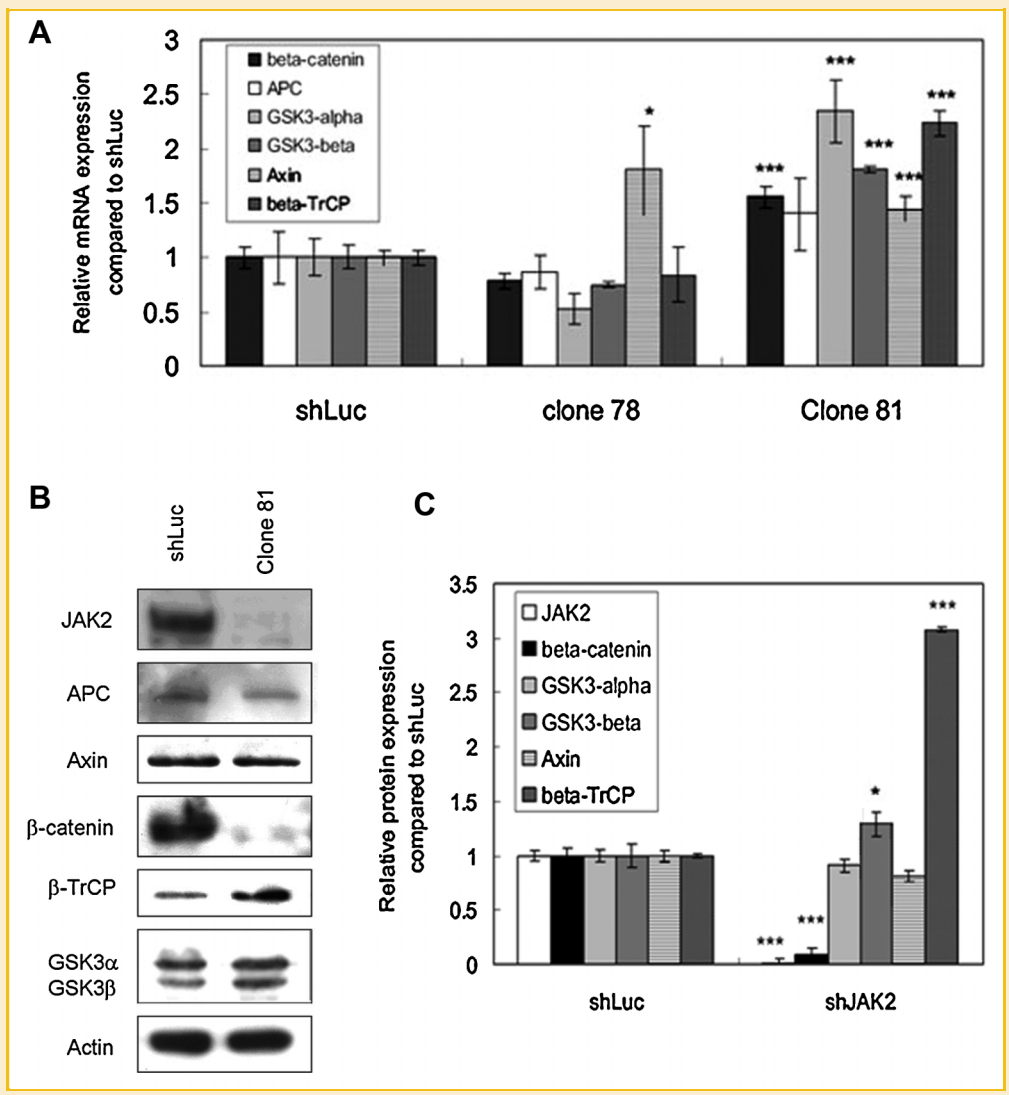

Fig. 5. Expression of β-catenin and β-catenin related proteins in the JAK2 knockdown clones. A: The mRNA expression of the various β-catenin related genes in HEL cells with different knockdown efficiencies was detected by quantitative RT-PCR. B: Total cell lysate in HEL cells with or without knockdown of the JAK2 gene were subjected to Western blotting to observe any changes in the APC, Axin, β-catenin, β-TrCP, GSK3, and Phospho-GSK3 protein levels. C: The results were normalized against actin and the vector control.

Figure 5A shows that β-catenin, APC, GSK3-α, GSK3-β, Axin, and β-TrCP were up-regulated in clone 81, which is similar to the situation in cells treated with AG490. These up-regulation events were absent in clone 78 except for Axin. In Figure 5B,C the expression of β-catenin and β-catenin related proteins in clone 81 show a similar pattern to AG490 treatment. These results confirm that AG490's effect on β-catenin is a resulted of its anti-JAK2 activity.

β-TrCP ACTIVATION INDUCED BY JAK2 BLOCKADE IS ACCOMPANIED BY IκB DEGRADATION AND NF-κB TRANSLOCATION

It is well known that β-TrCP can degrade IκB and facilitate NF-κB translocation into nucleus [Wang et al., 2004]. We therefore explored whether β-TrCP activity also increased under AG490 treatment. As shown in Figure 6A, there was an increase in β-TrCP that was accompanied by a reduction in IκB. Immunofluorescence showed that the activated NF-κB translocated from the cytosol into the nucleus on AG490 treatment (Fig. 6B). This demonstrates that treatment with AG490 is able to elevate not only amount of β-TrCP but also the activity of β-TrCP.

PARTIAL KNOCKDOWN OF β-TrCP ATTENUATES AG490-INDUCED β-CATENIN DEGRADATION

If β-TrCP mediates β-catenin degradation during JAK2 blockade, knockdown of this protein should be able to reverse this effect. We again challenged HEL cells with a specific shRNA against β-TrCP1 but it is difficult to completely shut down this gene. As shown in Figure 7A, clone 41 can knockdown about 50% of the expression of β-TrCP at both the protein and mRNA level. After treatment of clone 41 cells with 50 μM AG490 for 12–48 h, the amount of β-TrCP was found mild increase and the reduction in β-catenin was significantly

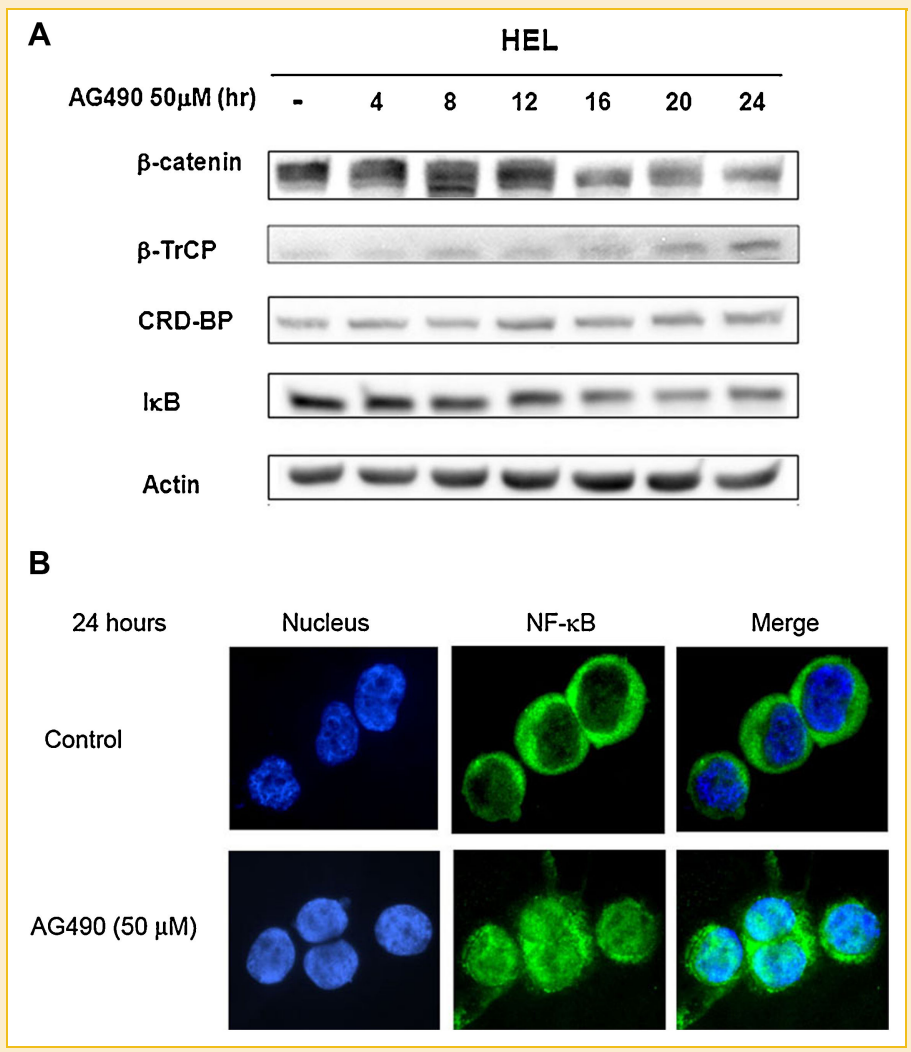

Fig. 6. AG490 induces I-κB degradation and NF-κB translocation. A: After treatment with 50 μM AG490 for 24 h, the changes in CRD-BP and I-κB protein level were detected by specific antibodies. B: The AG490-treated and untreated cells were cytospun onto a slide and NF-κB was detected using a specific antibody. FITC-conjugated secondary antibody was used to visualize the cellular localization of NF-κB. The nuclei were stained with DAPI (left panel). The merged pictures are shown in right panel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

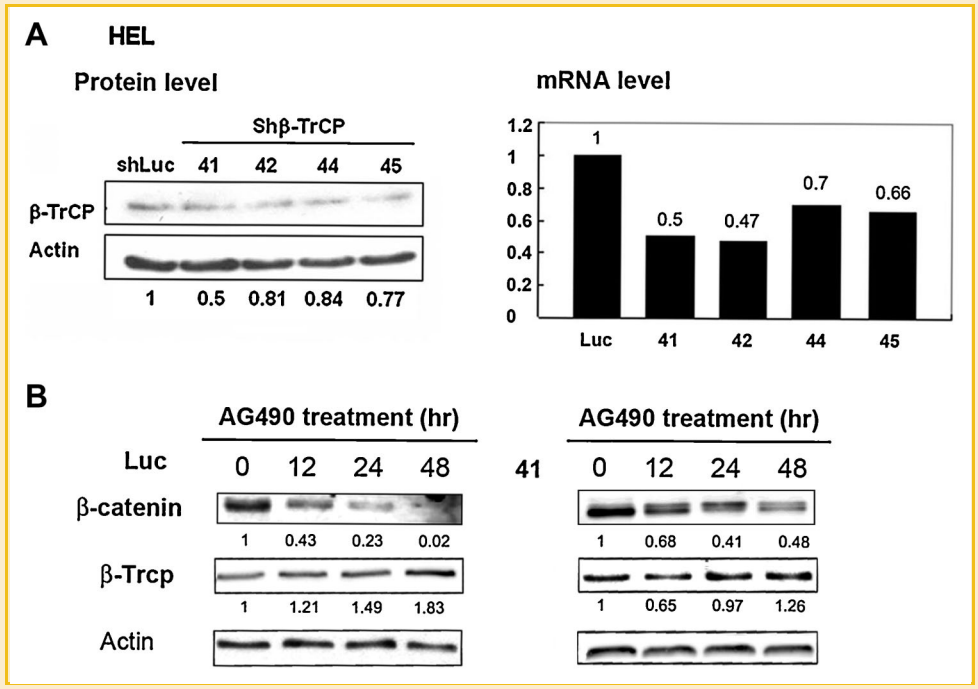

Fig. 7. Partial knockdown of β-Trcp attenuates AG490-induced β-catenin degradation. A: HEL cells were infected individually with four lentiviruses carrying shRNAs that targeting to different regions of β-TrCP. The knockdown efficiency for each clone was assessed by Western blotting and the quantified data are showed in right panel. B: The β-Trcp partial knock-down clone 41 was established as the best. Clone 41 was treated with AG490 for 48 h. The changes in β-catenin and β-Trcp were detected by Western blotting. The intensity of the β-catenin band was quantified and normalized against actin. Three independent experiments were performed and a representative result is shown.

reversed. These results support the hypothesis that β-TrCP plays a key role in β-catenin degradation. However, we cannot rule out the possibility that the other mechanism involves in this pathway.

DISCUSSION

In this study, we have demonstrated a novel role in human leukemia cells for JAK2 as a regulator of the canonical Wnt/β-catenin signaling pathway. We found β-catenin signaling can be significantly blocked by pretreatment with the JAK2 specific inhibitor AG490. This result was also confirmed by knockdown of JAK2 by shRNA. We also showed that at least one of the mechanisms involved is mediated via β-TrCP E3 ubiquitin ligases. β-TrCP can recognize phospho-β-catenin and degrades β-catenin via the Ebi F-box protein and Siah1 E3 ligase [Liu et al., 1999, 2001; Fuchs et al., 2004]. We showed that both the protein level and functioning of β-TrCP are increased on JAK2 blockade. Partial knockdown of β-TrCP attenuated the AG490 induced β-catanin degradation. This suggests that JAK2/STAT blockade induces β-TrCP activation, which in turn leads to β-catenin degradation. The present experiments provide an insight into mechanisms involved in cross-talk between the JAK2/STAT and WNT pathways.

The effect of AG490 on β-catenin has been previously reported in adhesion cell lines. Neria et al. [2007] observed that AG490 increased the total and nuclear amount of β-catenin in bovine aorta endothelial cells (BAEC) and that β-catenin may protect endothelial cells (EC) from detachment-induced death. Kawada et al. [2006] showed that AG490 did not alter the total amount of β-catenin but reduced nuclear accumulation of β-catenin in the SW480 human colon cancer cell line. Our results differ from these reports and showed that accumulation of β-catenin in a non-adherent leukemia cell line was reduced by AG490. We also found that cell proliferation was slowed after inhibition of the Wnt/β-catenin signaling (Supplementary Material S1). Since β-catenin acts not only as a transcriptional activator but also as a cadherin-associated protein in the adherens junction [Howard et al., 2003; Gu et al., 2008], we suggest that β-catenin plays more complicated role in adherent cells compared to leukemic cells.

The relationship between β-catenin and β-Trcp is interesting. It has been found that β-catenin is able to specifically bind to the ZBP1 promoter via a conserved β-catenin/TCF4 response element and that this activates expression of this gene in breast cancer cells [Gu et al., 2008]. Noubissi et al. [2006] reported that CRD-BP (also known as ZBP1) mediates stabilization of β-TrCP1 mRNA in response to β-catenin signaling. Nonetheless, β-TrCP is known to be a negative regulator of Wnt/β-catenin signaling and a positive regulator of NF-κB signaling [Wang et al., 2004]. Although these findings are based on various different types of cells, they suggest that these three proteins are involved in a feedback loop. In HEL cells, we also found that amount of CRD-BP protein increased when there was a JAK2 blockade (Fig. 6A). It is possible that the low efficiency of silencing of β-TrCP resulted from this feedback loop involving β-catenin, CRD-BP, and β-Trcp. In clinical colorectal cancer patients, however, an increased β-TrCP level has been reported to be associated with β-catenin activation and an inhibition of apoptosis [Ougolkov et al.,

2004]. Whether leukemic cells are able to maintain a subtle balance in these three proteins remains to be investigated.

Using quantitative RT-PCR, we found the mRNA expression of β-catenin and of most β-catenin related genes was induced by AG490, but only the β-Trcp and GSK-3 proteins showed a significant increase in their cellular level (Figs. 3 and 5). The inconsistency between mRNA expression levels and protein expression levels might be a result of the rapid degradation of some of the β-catenin destruction complex proteins. In addition, blockade of JAK2 activity by AG490 did induce an increase in GSK3α/β in Jurkat cells, but it decrease GSK3α/β in HEL cells (Fig. 3A,B). Nevertheless, if we compared the JAK2-knockdown clone of HEL cell with the vector control, the increase was evident (Fig. 5B). We believe that AG490 treatment combines both JAK2 inhibition and serum starvation effect. The inconsistency might result from serum starvation-induced apoptosis. However, we cannot rule out the possibility that the mutant JAK2 transduces its signal via a different pathway.

The role of GSK-3α/β in our experiment system remains controversial. It has been previously shown that phosphorylation at serine 9 near the N-terminus of GSK-3β mimics the prephosphorylation of the enzyme's substrate. This pseudosubstrate binds to the catalytic core and inhibits activity [Ilouz et al., 2008]. After treatment with AG490, although GSK3α/β did show an increase in Jurkat cells, the level of the inactive form (phospho-GSK3β) also increased over the same period (Fig. 3A). The situation was different in HEL cells (Fig. 3B). We can hardly find a clear relationship between GSK-3 phosphorylation with β-catenin degradation. It remains possible that the activity of GSK3α/β cannot be determined simply by measuring the total amount of protein and the total amount of the protein's phosphorylated form.

The present studies provide a set of new insights into the regulation of the Wnt pathway and show that AG490 can be used a negative regulator of canonical Wnt signaling in leukemic cells. Thus this drug could be very useful as a way of inhibiting aberrant Wnt signaling. New drugs targeting the interactions between JAK2/STAT signaling and Wnt/β-catenin might be developed that are able to suppress aberrant Wnt signaling and, as a result, inhibit the proliferation of leukemia cells.

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