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Inhibition of GSK-3 β promotes survival and proliferation of megakaryocytic cells through a β -catenin-independent pathway

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Abstract

The interaction of thrombopoietin (TPO) with its receptor c-Mpl initiates intracellular signals that are critical for megakaryopoiesis. Previously we and others have shown that TPO activates PI3K and Akt and that this pathway is important for megakaryocyte growth. Here, we investigate the importance of the Akt substrate glycogen synthase kinase (GSK)-3 β in TPO signaling. GSK-3 β is phosphorylated and inhibited by Akt as part of the PI3K pathway. GSK-3 β can also be inhibited by Wnt signaling through a distinct mechanism, leading to reduced phosphorylation and accumulation of the transcription factor β -catenin. Therefore, we asked if TPO and Wnt3a can both inhibit GSK-3 β in megakaryocytic cells, and if they can act synergistically to promote cell growth. Although both TPO and specific chemical inhibitors of GSK-3 β result in increased survival and proliferation in a megakaryocytic cell line model, treatment with Wnt3a failed to increase cell growth either in the absence or presence of TPO, despite inducing high levels of β -catenin. Similarly, expression of a constitutively active version of β -catenin did not increase cell growth either in the absence or presence of TPO, suggesting that the effects of GSK-3 β inhibition downstream of TPO signaling are distinct from those induced by Wnt3a and independent of β -catenin. The growth promoting effects of TPO are not mediated by either of the two known GSK-3 β targets, cyclin D or HIF-1 α . We conclude that GSK-3 β is phosphorylated and inhibited by TPO-induced Akt, promoting survival and proliferation in megakaryocytic cells through a pathway that does not involve β -catenin.

Keywords

GSK-3 β ; thrombopoietin; β -catenin; megakaryocyte; Wnt3a

Introduction

Thrombopoietin (TPO) is the primary cytokine responsible for platelet production [1–5]. TPO binds to the proto-oncogene receptor c-Mpl on megakaryocytic cells and promotes cell survival, proliferation and maturation through the activation of intracellular signaling pathways (reviewed [6]. c-Mpl does not have intrinsic kinase activity, but instead utilizes the cytoplasmic kinase Jak2 to initiate downstream signals, including signal transducer and activator of

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transcription (STAT), mitogen activated protein kinase (MAPK) and other kinases. Previously we and others demonstrated that TPO also activates phosphoinositol-3-kinase (PI3K) and its downstream signaling kinase Akt, and that this pathway is important for megakaryocyte growth [7–9]. Akt has many known effectors, and the contributions of these complex networks to TPO signaling are just beginning to be understood. For example, Forkhead family transcription factors are phosphorylated by Akt, sequestering them in the cytoplasm where they are unable to activate their target genes, many of which are involved in growth arrest and apoptosis [10–13]. Recently it has been shown that TPO-induced phosphorylation of Forkhead family members leads to reduced expression of p27 and enhanced cell growth [14,15]. Similarly, TPO induced-Akt promotes phosphorylation of Bad, inhibiting its function in apoptosis [16].

Glycogen synthase kinase (GSK)-3 β is also a known substrate of Akt [17–19]. GSK-3 β is active under resting conditions in many cell types; stimulation of Akt leads to phosphorylation of GSK-3 β on Ser9, resulting in inhibition of its kinase activity [20]. GSK-3 β also plays a critical role in transducing Wnt signaling to mediate transcription of Wnt target genes. In contrast to its regulation by Ser9 phosphorylation, Wnt signaling negatively regulates GSK-3 β activity by disrupting a protein complex that juxtaposes GSK-3 β with its substrate in this pathway, β -catenin. Therefore, in this context the inability of GSK-3 β to phosphorylate β -catenin is not due to inhibition of its kinase activity, but rather secondary to the lack of association with substrate.

While Wnt and β -catenin signaling regulate survival and proliferation of hematopoietic stem cells (HSCs) [21,22], the role of this pathway in megakaryopoiesis is unknown. In addition, although TPO is important for the maintenance and expansion of HSCs (reviewed [23]), the importance of GSK-3 β inhibition for TPO signaling or the possibility of synergy between the TPO and Wnt pathways has not been explored. In this work, we investigate the contribution of GSK-3 β to TPO signaling in a megakaryocytic cell line and ask whether TPO and Wnt3a can synergize through the stabilization of β -catenin. Using a TPO-dependent megakaryocytic cell line (UT-7/TPO), we found that GSK-3 β is phosphorylated and inhibited by TPO and this requires PI3K activity. Although inhibition of GSK-3 β using TPO or chemical inhibitors of the kinase promotes survival and proliferation of UT-7/TPO cells, our work demonstrates that cell growth requires targets of GSK-3 β that are distinct from β -catenin. Furthermore, we did not observe any synergy between TPO and β -catenin in promoting growth in UT-7/TPO cells. Therefore, despite the fact that both the TPO and Wnt pathways utilize inhibition of GSK-3 β to transmit their signal, only TPO has growth promoting properties in these cells.

Materials and Methods

Reagents

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. 6-bromoindirubin-3'-oxime (BIO) was purchased from Calbiochem (San Diego, CA). Wnt3a was prepared as described [21]; each batch was titered for potency based on the ability to activate a Wnt-specific promoter driving luciferase (TOP-Flash). Antibodies to phosphorylated Akt (Ser473) and phosphorylated GSK-3 β (Ser9) were from Cell Signaling Technologies (Beverly, MA). Antibodies to GSK-3 β , β -catenin and HIF-1 α were from BD Bioscience (San Diego, CA). Antibodies to α -tubulin were from Sigma, and antibodies to β -actin, LaminB, Cyclin D1 and Cyclin D3 were from Santa Cruz (Santa Cruz, CA).

Cells

UT-7/TPO cells are a previously characterized cytokine-dependent human megakaryocytic cell line [24]. For routine culture cells were maintained in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin,

and 10 ng/ml of recombinant human (rh)TPO (a gift from Zymogenetics, Inc., Seattle, WA) at 37 °C and 5% CO₂.

RT-PCR

Total RNA was extracted from UT-7/TPO cells using the RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Valencia, CA). cDNA was synthesized with MuLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Subsequently, RT-PCR was carried out in a 50 µl volume containing 2 µl of cDNA reaction sample, equivalent to 100 ng of template total RNA, and 0.05 U/µl AmpliTaq Gold (Applied Biosystems, Foster City, CA). The amplification reaction was set for denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec for 35 cycles followed by a final extension at 72°C for 10 min. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a loading control. The following nucleotide sequences were used for primers: Frizzled (Fzd)1 forward, 5'-ccgagctcaagttcttctg-3'; Fzd1 reverse, 5'-gggaactctcgcacttgag-3'; Fzd3 forward, 5'-gaagcaaagcagggagtg-3'; Fzd3 reverse, 5'-atgctgccgtgaggtagct-3'; Fzd5 forward, 5'-acatggaacgattccgtac-3'; Fzd5 reverse, 5'-ggccatgccaaagaataga-3'; Sequences for GAPDH primers are available upon request.

Western blotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (20mM Tris/HCl, pH 8.0, 150mM NaCl, 1mM EDTA, 1% Nonidet P-40 (NP-40), 10µg/l aprotinin, 10µg/ml leupeptin, 2mM Na₃VO₄, 1mM DTT, 1mM phenylmethylsulfonyl fluoride (PMSF), and 20mM NaF) according to standard protocols. For fractionation of nuclear and cytoplasmic proteins, cells were first lysed in hypotonic buffer (10mM HEPES, pH 7.9, 0.5% NP-40, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1mM PMSF) and nuclei collected by centrifugation at 2000g for 10 min at 4°C. The supernatant was collected as the cytoplasmic extract and clarified by centrifugation at 14,000g for 20 min at 4°C. The nuclear pellet was resuspended in hypertonic buffer (20mM HEPES, pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF) and centrifuged at 10,000g for 20 minutes at 4°C; the supernatant was collected as nuclear extract. Protein concentrations were determined colorimetrically (Bio-Rad, DC protein assay). Proteins were size fractionated by SDS-PAGE and transferred electrophoretically to PDVF membranes (Bio-Rad). Membranes were blocked in Tris-buffered saline containing Tween 20 (10mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween 20)(TBS-T) and 5% non-fat dried milk for 1 hr, probed in the blocking solution containing the appropriate dilution of the antibody overnight at 4°C. Secondary detection was with the appropriate horseradish peroxidase-conjugated antibodies followed by chemiluminescence (LumiGlo, Cell Signaling Technologies) and exposure to autoradiographic film. If blots were reprobbed with additional antibodies, they were first stripped using Re-Blot Plus (Chemicon, Temecula, CA) according to the manufacturer's protocol. Densitometric analysis of scanned images was performed using ImageJ (www.rsbweb.nih.gov/ij/).

MTT assays

UT-7/TPO cells were cultured with 100 µl of IMDM containing 0.5% BSA with or without 10 ng/ml rhTPO and GSK-3β inhibitor in 96 well plates at 37°C. After 24 or 36 hr of culture, 20 µl of 5 mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added and incubated for 3 hr at 37°C followed by adding 100 µl of lysis buffer (20% SDS, 40% dimethylformamide and 2% acetic acid, pH 5). After 6 hr of incubation at 37°C, when the dark green MTT formazan had completely dissolved, absorbance at 570 nm was measured using a microplate reader (Bio-Rad). Statistical significance of observed differences was determined using the Mann-Whitney U test (StatView, Adept Scientific, Bethesda, MD).

Annexin V assays

Annexin V-FITC was purchased from BD Bioscience. UT-7/TPO cells were washed 3 times with PBS and then cultured in IMDM containing 0.5% BSA with or without 10 ng/ml rhTPO and BIO or DMSO for 24 hr at 37°C. At the end of culture, cells were resuspended in Annexin-binding buffer containing Annexin V-FITC and PI according to manufacturer's protocol. Apoptotic cells were quantitated by flow cytometry using a FACSCalibur/Cell Quest system (BD Biosciences, Franklin Lakes, NJ).

BrdU and cell cycle analysis

BrdU assays were performed using the BrdU Flow Kit (BD Pharmingen, San Diego, CA). Briefly, UT-7/TPO cells were washed 3 times with PBS and then cultured in IMDM containing 0.5% BSA with or without 10 ng/ml rhTPO and BIO or DMSO for 24 hr at 37°C. At the end of culture, cells were pulse-labeled with BrdU for 30 minutes, then fixed and stained with anti-BrdU antibody and PI according to the manufacturer's protocol. S and G2/M phase cells were identified according to their DNA and BrdU content and quantitated by flow cytometry. For cell cycle analysis based on DNA content alone, HIV-CA- β -catenin-IRES or pHIV-Neo-IRES-GFP-transfected cells were starved for 24 hr and then fixed with 0.5% paraformaldehyde in PBS, permeabilized with 0.1% NP-40, and incubated with PI to label DNA. The fraction of cells in G1, S and G2 phases was determined by DNA content and quantitated by flow cytometry.

Expression of constitutively active β -catenin

The previously described constitutively active β -catenin gene [22] was inserted into HIV-IRES-GFP vector to generate HIV-CA- β -catenin-IRES-GFP. pHIV-Neo-IRES-GFP was the generous gift of Dr. Y. Soda (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Transient transfection of UT-7/TPO cells was performed using Nucleofection (Amaxa, Cologne, Germany, buffer R/program T24) according to the manufacturer's directions.

Results

TPO induces phosphorylation of GSK-3 β

Previously, we had shown that TPO induces phosphorylation of Akt [7]; therefore, we asked if TPO also induced phosphorylation and inhibition of the Akt target GSK-3 β . UT-7/TPO cells were starved for 24 hr and stimulated with 100 ng/ml recombinant human TPO (rhTPO). Cells were lysed either in the absence of TPO or after 5–60 min of TPO stimulation and cell lysates were analyzed by Western blotting using phospho-specific antibodies for Akt and GSK-3 β . As expected, treatment with TPO induced serine phosphorylation of Akt on Ser473, as well as GSK-3 β on Ser9 (Figure 1A). As one marker of GSK-3 β activity, we asked if TPO stimulation resulted in the accumulation of the GSK-3 β target, β -catenin. We found that TPO stimulation led to a modest accumulation of β -catenin, an effect that was maximal after 30–60 min (Figure 1A). Inhibition of GSK-3 β in resting cells using the chemical inhibitors lithium chloride or BIO led to a significantly greater accumulation of β -catenin, which was especially marked in cells treated with BIO (Figure 1B). Although lithium chloride induced phosphorylation of GSK-3 β on Ser9 similar to what is seen following TPO stimulation, the unrelated inhibitor BIO did not alter GSK-3 β phosphorylation, confirming a different mechanism of kinase inhibition. Therefore, these experiments show that TPO treatment results in phosphorylation and inhibition of GSK-3 β , but only a modest accumulation of β -catenin in comparison to inhibition of GSK-3 β using the chemical inhibitor BIO.

Inhibition of GSK-3 β leads to accumulation of β -catenin in the nucleus

When phosphorylated by GSK-3 β on its amino-terminus, β -catenin is targeted for degradation. If this phosphorylation is blocked (for example by pharmacological inhibition of GSK-3 β with BIO or lithium chloride, or by mutation of key serine and threonine residues) β -catenin is not longer degraded and consequently accumulates, thus allowing it to enter the nucleus where it can activate transcription in concert with the Lef/Tcf family of DNA binding proteins. Thus, we asked if inhibition of GSK-3 β results in accumulation of β -catenin to the nucleus in megakaryocytic cells. UT-7/TPO cells were treated with BIO or vehicle (DMSO) in the absence of TPO for 12 hr, and nuclear and cytoplasmic fractions were prepared. Western blotting revealed that when cells are treated with BIO the amount of β -catenin present in nuclear fractions increases (Figure 2). Densitometric analysis reveals that although total cellular β -catenin is increased, the ratio of cytoplasmic to nuclear β -catenin is unchanged (ratio of cytoplasmic:nuclear β -catenin 0.32 in response to vehicle, 0.44 in response to BIO), findings that are consistent with previous studies. These data indicate that treatment of UT-7/TPO cells with inhibitors of GSK-3 β results in accumulation of β -catenin in the cell including its presence in the nucleus, where it likely functions to promote transcription of genes important for cell growth.

Treatment of UT-7/TPO cells with Wnt3a results in accumulation of β -catenin

Inhibition of GSK-3 β by BIO results in a more marked accumulation of β -catenin than is seen following treatment with TPO (Figure 1B). We hypothesized that TPO signaling only inhibits one pool of GSK-3 β in UT-7/TPO cells, whereas a separate pool could be acted on by another pathway, such as the Wnt pathway. To test this, we first confirmed that UT-7/TPO cells express receptors for Wnt proteins and that Wnt3a is active in these cells. Total RNA was prepared from UT-7/TPO cells and was analyzed for expression of known Wnt receptors by RT-PCR. We found that UT-7/TPO cells express transcripts for the Wnt receptors Fzd-1, Fzd-3, and Fzd-5 (Supplemental Figure 1). To confirm that Wnt3a is active in UT-7/TPO cells, we stimulated cells with TPO or Wnt3a and compared the ability of these treatments to induce phosphorylation of GSK-3 β and accumulation of β -catenin. While Wnt3a has no effects on the phosphorylation of GSK-3 β in UT-7/TPO cells, it strongly induces the accumulation of β -catenin (Figure 3A). As with BIO, the increase in total cellular β -catenin induced by Wnt3a results in increased nuclear β -catenin (Figure 3b). Densitometry indicates that the relative distribution of cytoplasmic and nuclear β -catenin is not changed. Therefore, these results suggest there are at least two pathways in UT-7/TPO cells through which GSK-3 β can be inhibited, leading to differing degrees of β -catenin accumulation and potentially different downstream effects.

Inhibition of GSK-3 β through BIO or lithium chloride promotes TPO-independent cell survival and growth

To determine the functional consequences of GSK-3 β inhibition by TPO, we asked if inhibition of GSK-3 β alone, using chemical inhibitors in the absence of TPO, could mimic the favorable TPO effects on survival and proliferation of UT-7/TPO cells. We treated UT-7/TPO cells with or without TPO and with increasing doses of BIO for 24 hr and then performed Annexin V and BrdU/cell cycle assays. We found that in the absence of TPO, 0.5 μ M BIO significantly reduced the fraction of apoptotic cells ($p < 0.05$) (Figure 4A). In addition, in the absence of TPO or at suboptimal TPO levels, the addition of 0.5 μ M BIO to the culture significantly increased the proportion of cells in S and G2/M phases of the cell cycle ($p < 0.05$) (Figure 4B). No additional effect of BIO on cell proliferation was seen at maximal doses of TPO. This suggests that in the presence of TPO, the GSK-3 β signals associated with cell growth are already inhibited and further inhibition does not provide additional effects. These results indicate that inhibition of GSK-3 β promotes both decreased apoptosis and increased cell cycling.

Inhibition of GSK-3 β through Wnt3a signaling has no effect on cell growth in UT-7/TPO cells

Because BIO had a prominent effect on promoting cell survival and growth in the absence of TPO and increased accumulation of β -catenin, we hypothesized that Wnt3a should also promote cell growth in these megakaryocytic cells and possibly synergize with TPO. We starved UT-7/TPO cells as above and treated them with or without TPO and with either chemical inhibitors of GSK-3 β or purified Wnt3a, and then asked if these treatments could promote cell growth in MTT assays. Whereas in the absence of TPO, lithium chloride and BIO significantly increased cell growth ($p < 0.05$), the effects of GSK-3 β inhibition were less prominent in cultures supplemented with TPO (Figure 5). At the highest concentrations of inhibitors tested (20 mM lithium chloride, 1 μ M BIO), evidence of nonspecific toxicity was present and likely explains reduced effects in these cultures. Strikingly, although both lithium chloride and BIO showed activity in promoting cell growth, Wnt3a did not have any growth promoting effect on UT-7/TPO cells. These findings suggest that TPO-induced GSK-3 β is promoting cell growth through a β -catenin-independent pathway, and that TPO and Wnt3a do not synergize in promoting cell growth in UT-7/TPO cells. Results shown are from assays performed at 24 hr; similar results were also obtained at 36 hr (not shown).

Expression of a constitutively active β -catenin (CA- β -catenin) does not prevent apoptosis nor increase proliferation in UT-7/TPO cells

If both TPO and BIO treatment mediate survival and proliferation in megakaryocytic cells through a β -catenin-independent pathway, then we anticipated that constitutive activation of β -catenin would not be sufficient to support cell growth in UT-7/TPO cells. Therefore, we utilized a mutant of β -catenin bearing alanine substitutions at serine/threonine phosphorylation sites in the N-terminus thus producing a non-phosphorylatable and consequently a constitutively active (CA) form of β -catenin. Inclusion of an internal ribosome entry site after the CA- β -catenin gene followed by GFP allows us to specifically track transfected cells. We expressed the constitutively active mutant (HIV-CA- β -catenin-IRES-GFP) or the control empty vector (pHIV-Neo-IRES-GFP) in UT-7/TPO cells. As shown in Figure 6A, transfected cells showed strong expression of exogenous β -catenin relative to control cells. However, CA- β -catenin expressing UT-7/TPO cells, as detected by flow cytometry of GFP-positive cells, did not exhibit increased (Figure 6B, compare the S+G2/M fraction C). In fact, the fraction of apoptotic cells was increased in cells expressing this construct relative to cells transfected with control vector (compare the sub-G1 fraction A). These data, combined with the lack of growth in Wnt3a, indicates that β -catenin does not mediate cell growth in UT-7/TPO cells in response to TPO- or BIO-induced inhibition of GSK-3 β .

GSK-3 β targets HIF-1 α and cyclin do not mediate TPO-induced cell growth in UT-7/TPO cells

Because β -catenin does not stimulate cell growth in UT-7/TPO cells, we investigated several additional proteins that are phosphorylated by GSK-3 β and hence are candidates to regulate cell growth in megakaryocytic cells. Two such proteins are HIF-1 α and cyclin D. We have previously demonstrated that HIF-1 α is stimulated by TPO and promotes growth in primitive hematopoietic cells [25]. In addition, HIF-1 α is phosphorylated by GSK-3 β , and this phosphorylation reduces its accumulation [26]. Therefore, we examined if inhibition of GSK-3 β using TPO or BIO can promote the accumulation of HIF-1 α in the nucleus of UT-7/TPO cells. As expected and shown in Figure 7, TPO stimulation results in increased nuclear levels of HIF-1 α ; in contrast BIO does not result in detectable induction of HIF-1 α levels, as the modest increase present is also seen in the vehicle control. Therefore, inhibition of GSK-3 β is not responsible for the accumulation of HIF-1 α following TPO stimulation, and accumulation of HIF-1 α does not mediate the growth of UT-7/TPO cells in response to inhibition of GSK-3 β . We also examined cyclin D1 and cyclin D3 as potential targets of GSK-3 β that might mediate growth in UT-7/TPO cells. Both cyclins D1 and D3 are known to

regulate cell cycling in megakaryocytic cells [27–29], and it has been previously shown that active GSK-3 β can phosphorylate cyclin D1 and target it for nuclear export and degradation [30,31]. Therefore we treated UT-7/TPO cells with TPO or BIO and determined the nuclear levels of cyclin D. As shown in Figure 8, TPO induced cytoplasmic accumulation of both cyclins D1 and D3. However, no such accumulation was noted in cells treated with BIO. Therefore, TPO- and BIO-induced inhibition of GSK-3 β promotes cell growth in UT-7/TPO cells that is mediated by a β -catenin-independent pathway, and the targets of GSK-3 β in this pathway do not appear to be either HIF-1 α or cyclin D. The key targets of GSK-3 β regulating cell growth are still unknown, and it will likely require a more unbiased approach to identify the targets responsible for the growth promoting effects of GSK-3 β inhibition in megakaryocytic cells.

Discussion

Inhibition of GSK-3 β promotes survival and proliferation in megakaryocytic cells, but not through the action of β -catenin. Wnt3a, while active in UT-7/TPO cells as evidenced by significant accumulation of β -catenin, did not detectably enhance megakaryocytic cell growth, either with or without TPO, suggesting that the effectors of GSK-3 β involved in TPO signaling are distinct from those involved in Wnt3a signaling and that these two pathways are not synergistic. Given prior studies on the importance of Wnt signaling in hematopoietic and other stem cells [21,22,32–35], it is somewhat surprising that neither Wnt3a nor constitutively active β -catenin showed any ability to augment cell growth in UT-7/TPO cells, which are derived from a human megakaryoblastic leukemia. We also did not find any evidence that Wnt3a promotes cell growth in primary immature mouse megakaryocytes (data not shown). It is possible that β -catenin has other functions besides proliferation in megakaryocytic cells or that additional signals are required for its activity. In addition, it is possible that β -catenin acts primarily on a more primitive precursor or the hematopoietic stem cell itself. Nevertheless, it is clear that GSK-3 β substrates other than β -catenin are responsible for the enhanced cell growth that we observed in the presence of BIO and lithium chloride and may be involved in TPO signaling. We did examine two proteins that are known to be responsive to TPO and have been shown to be targets by GSK-3 β in other settings; however inhibition of GSK-3 β did not significantly increase protein levels of cyclin D1 or HIF-1 α in UT-7/TPO cells and therefore analysis of additional candidates is necessary. Additional candidates for proteins that are known to be targeted by GSK-3 β and could potentially regulate cell cycling or apoptosis in megakaryocytic cells include NF κ B, p21, Bcl3, c-jun, c-myc and Notch, among others [36–41], but testing of individual candidates when multiple candidates are possible, as shown in this work, can be of limited value. We have performed microarray studies to assist us in the discovery of novel GSK-3 β pathways for future investigation.

Conclusions

TPO signaling results in the inhibition of GSK-3 β , whose downstream substrates participate in the regulation of cell survival and proliferation. The function of GSK-3 β in megakaryocytic cells has not been previously explored, and given its role in cellular growth, this pathway may be a potential cancer target or important off-target consideration. Several agents that inhibit GSK-3 β either directly or indirectly, including lithium chloride and enzastaurin, are currently in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. Kuter DJ, Beeler DL, Rosenberg RD. *Proc Natl Acad Sci U S A* 1994;91:11104. [PubMed: 7972018]
2. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS, et al. *Nature* 1994;369:568. [PubMed: 8202159]
3. Kaushansky K. *Stem Cells* 1994;12:91. [PubMed: 7696972]
4. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ, et al. *Nature* 1994;369:533. [PubMed: 8202154]
5. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, Methia N, Breton-Gorius J, Cosman D, Vainchenker W. *Nature* 1994;369:571. [PubMed: 8202160]
6. Geddis AE, Linden HM, Kaushansky K. *Cytokine Growth Factor Rev* 2002;13:61. [PubMed: 11750880]
7. Geddis AE, Fox NE, Kaushansky K. *J Biol Chem* 2001;276:34473. [PubMed: 11418622]
8. Miyakawa Y, Rojnuckarin P, Habib T, Kaushansky K. *J Biol Chem* 2001;276:2494. [PubMed: 11054408]
9. Sattler M, Salgia R, Durstin MA, Prasad KV, Griffin JD. *J Cell Physiol* 1997;171:28. [PubMed: 9119889]
10. Kops GJ, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffey PJ, Lam EW, Burgering BM. *Mol Cell Biol* 2002;22:2025. [PubMed: 11884591]
11. Kashii Y, Uchida M, Kirito K, Tanaka M, Nishijima K, Toshima M, Ando T, Koizumi K, Endoh T, Sawada K, Momoi M, Miura Y, Ozawa K, Komatsu N. *Blood* 2000;96:941. [PubMed: 10910908]
12. Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, Coffey PJ. *Mol Cell Biol* 2000;20:9138. [PubMed: 11094066]
13. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffey PJ. *J Cell Biol* 2002;156:531. [PubMed: 11815629]
14. Nakao T, Geddis AE, Fox NE, Kaushansky K. *Cell Cycle* 2007;7. [PubMed: 18196966]
15. Tanaka M, Kirito K, Kashii Y, Uchida M, Watanabe T, Endo H, Endoh T, Sawada K, Ozawa K, Komatsu N. *J Biol Chem* 2001;276:15082. [PubMed: 11278373]
16. Abe M, Suzuki K, Inagaki O, Sassa S, Shikama H. *Leukemia* 2002;16:1500. [PubMed: 12145691]
17. Cross DA, Alessi DR, Vandenhede JR, McDowell HE, Hundal HS, Cohen P. *Biochem J* 1994;303(Pt 1):21. [PubMed: 7945242]
18. Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. *Proc Natl Acad Sci U S A* 1998;95:11211. [PubMed: 9736715]
19. Manoukian AS, Woodgett JR. *Adv Cancer Res* 2002;84:203. [PubMed: 11883528]
20. Stambolic V, Woodgett JR. *Biochem J* 1994;303(Pt 3):701. [PubMed: 7980435]
21. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR 3rd, Nüsse R. *Nature* 2003;423:448. [PubMed: 12717451]
22. Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nüsse R, Weissman IL. *Nature* 2003;423:409. [PubMed: 12717450]
23. Kaushansky K. *Ann N Y Acad Sci* 2005;1044:139. [PubMed: 15958707]
24. Komatsu N, Kunitama M, Yamada M, Hagiwara T, Kato T, Miyazaki H, Eguchi M, Yamamoto M, Miura Y. *Blood* 1996;87:4552. [PubMed: 8639823]
25. Kirito K, Fox N, Komatsu N, Kaushansky K. *Blood* 2005;105:4258. [PubMed: 15705785]
26. Mottet D, Dumont V, Deccache Y, Demazy C, Ninane N, Raes M, Michiels C. *J Biol Chem* 2003;278:31277. [PubMed: 12764143]
27. Magne S, Caron S, Charon M, Rouyez MC, Dusanter-Fourt I. *Mol Cell Biol* 2003;23:8934. [PubMed: 14645506]

28. Matsumura I, Tanaka H, Kawasaki A, Odajima J, Daino H, Hashimoto K, Wakao H, Nakajima K, Kato T, Miyazaki H, Kanakura Y. *J Biol Chem* 2000;275:5553. [PubMed: 10681535]
29. Wang Z, Zhang Y, Lu J, Sun S, Ravid K. *Blood* 1999;93:4208. [PubMed: 10361118]
30. Diehl JA, Cheng M, Roussel MF, Sherr CJ. *Genes Dev* 1998;12:3499. [PubMed: 9832503]
31. Alt JR, Cleveland JL, Hannink M, Diehl JA. *Genes Dev* 2000;14:3102. [PubMed: 11124803]
32. Feng Z, Srivastava AS, Mishra R, Carrier E. *Biochem Biophys Res Commun* 2004;324:1333. [PubMed: 15504360]
33. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. *Nat Med* 2004;10:55. [PubMed: 14702635]
34. Naito AT, Akazawa H, Takano H, Minamino T, Nagai T, Aburatani H, Komuro I. *Circ Res* 2005;97:144. [PubMed: 15994435]
35. Trowbridge JJ, Xenocostas A, Moon RT, Bhatia M. *Nat Med* 2006;12:89. [PubMed: 16341242]
36. Nikolakaki E, Coffey PJ, Hemelsoet R, Woodgett JR, Defize LH. *Oncogene* 1993;8:833. [PubMed: 8384354]
37. Pulverer BJ, Fisher C, Vousden K, Littlewood T, Evan G, Woodgett JR. *Oncogene* 1994;9:59. [PubMed: 8302604]
38. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. *Nature* 2000;406:86. [PubMed: 10894547]
39. Espinosa L, Ingles-Esteve J, Aguilera C, Bigas A. *J Biol Chem* 2003;278:32227. [PubMed: 12794074]
40. Viatour P, Dejardin E, Warnier M, Lair F, Claudio E, Bureau F, Marine JC, Merville MP, Maurer U, Green D, Piette J, Siebenlist U, Bours V, Chariot A. *Mol Cell* 2004;16:35. [PubMed: 15469820]
41. Rossig L, Badorff C, Holzmann Y, Zeiher AM, Dimmeler S. *J Biol Chem* 2002;277:9684. [PubMed: 11779850]

Figure 1. TPO signaling inhibits GSK-3β and stimulates accumulation of total cellular β-catenin
 A) UT-7/TPO cells were starved for 24 hr and stimulated with 100 ng/ml recombinant human TPO (rhTPO). At the indicated time points, whole cell lysates were prepared and analyzed by Western blotting with anti-phospho Akt (pAkt), anti-phospho GSK-3β (pGSK-3β), anti-GSK-3β (total), and anti-β-catenin antibodies. Actin was probed as loading control. Results from densitometric analysis, normalized for loading and expressed relative to time 0, are shown graphically. B) UT-7/TPO cells were washed with PBS and then treated with TPO, GSK-3β inhibitors (10 mM LiCl, 0.5 μM MBIO) or their vehicles (dH₂O, DMSO) for 12 hr. Whole cell lysates were prepared and analyzed by Western blotting with anti-pGSK-3β, β-catenin and actin antibodies. Results from densitometric analysis, normalized for loading, are shown numerically below the respective blot.

Figure 2. Inhibition of GSK-3 β using BIO results in the accumulation of nuclear β -catenin in UT-7/TPO cells

UT-7/TPO cells were washed and then treated with BIO or its vehicle DMSO for 12 hr. Cells were lysed and fractionated for cytoplasmic and nuclear extracts followed by Western blotting with anti- β -catenin antibody. α -tubulin and LaminB were probed as markers for the purity of the cytoplasmic and nuclear fractions, respectively.

Figure 3. Wnt3a is active in UT-/TPO cells and induces accumulation of β-catenin

A) UT-7/TPO cells were washed and then stimulated with 100 ng/ml rhTPO, Wnt3a or their vehicles (DMSO or CHAPS) for 12 hr. At the end of stimulation, whole cell lysates were prepared and analyzed by Western blotting using anti-pGSK-3β and anti-β-catenin antibodies. Actin was probed for a loading control. Results from densitometric analysis normalized for loading are shown numerically below the respective blot. B) UT-7/TPO cells were washed and then stimulated with Wnt3a or vehicle for 12 hours. Cytoplasmic and nuclear extracts were prepared and localization of β-catenin was analyzed using Western blotting. α-tubulin and LaminB were probed as loading controls.

Figure 4. Inhibition of GSK-3 β blocks apoptosis and supports proliferation in UT-7/TPO cells

UT-7/TPO cells were washed and then cultured with DMSO or BIO in the presence or absence of TPO for 24 hr. At the end of culture analyses of apoptosis (Annexin V) and cell cycling (BrdU) were performed. (A) Annexin V assay. Cells were incubated with Annexin V-FITC in Annexin-binding buffer and analyzed by flow cytometry. Each bar indicates the average percentage of Annexin V-positive cells $\pm$ S.E. calculated from 4 individual sets of experiments. (B) Cell cycle assay using BrdU. Cells were pulse labeled with BrdU for 30 min and stained with anti-BrdU antibody conjugated to FITC and the DNA dye propidium iodide (PI), followed by flow cytometric analysis. Each bar indicates the average percentage of cells within the S G2/M peaks of the cell cycle $\pm$ S.E. calculated from 4 individual sets of experiments. Compared to DMSO, 0.5 μ M BIO significantly increases cell cycling in cells cultured with 0, 0.1 ng/ml, and 0.5 ng/ml TPO but not in cells cultured with 10 ng/ml (maximal) TPO. Bars marked with * indicate a statistically significant difference ($p < 0.05$).

Figure 5. Inhibition of GSK-3 β signaling by Wnt3a does not promote growth in UT-7/TPO cells
 UT-7/TPO cells were washed and plated at the density of 2×10^4 /well and then cultured with indicated concentration of LiCl, BIO or Wnt3a in the presence or absence of TPO for 24 hr. At the end of culture, MTT assays were performed as described in Materials and Methods to compare the overall growth of cells during the duration of the experiment under the various conditions. MTT incorporation for each condition was normalized to control wells cultured with 100 ng/ml of TPO alone. Each bar represents the average $\pm$ S.E. calculated from triplicate cultures. As compared to DMSO, treatment with lithium chloride and BIO results in significantly increased MTT activity in the absence of TPO. Bars marked with * indicate a statistically significant difference ($p < 0.05$).

Figure 6. Expression of a constitutively active β -catenin (CA- β -catenin) does not prevent apoptosis nor increase proliferation in UT-7/TPO cells

(A) UT-7/TPO cells were transfected with a lentiviral vector expressing constitutively active- β -catenin (HIV-CA- β -catenin-IRES-GFP) or the control vector (pHIV-Neo-IRES-GFP). At the indicated time point after transfection, whole cell lysates were prepared and analyzed by Western blotting with anti- β -catenin antibody. Actin was probed as a loading control. (B) 48 hr after transfection, UT-7/TPO cells expressing CA- β -catenin or the control vector were washed of TPO and starved for 24 hr, after which they were fixed and DNA was stained with propidium iodide. The cell cycle profiles of the transfected, GFP-positive cells were analyzed by flow cytometry. The fractions labeled a, b and c represent sub G1 (apoptotic), G0/G1 and S+G2/M populations, respectively. The percentage of cells within each fraction was calculated using CellQuest software. The results shown are representative of 3 independent experiments.

Figure 7. HIF-1α expression is not specifically induced by inhibition of GSK-3β

UT-7/TPO cells were starved for 24 hr and then stimulated with 100 ng/ml rhTPO or washed followed by treatment with BIO or its vehicle DMSO. At indicated time points, cells were lysed to fractionate nuclear and cytoplasmic extracts. Nuclear extracts were analyzed by Western blotting with anti-HIF-1α antibody. The blot was reprobed for β-catenin and LaminB as controls for the fractionation. Although HIF-1α levels are increased following treatment with BIO, DMSO appears to have the same effect suggesting that this is not specific. Results from densitometric analysis, normalized for loading, are shown numerically below the respective blot.

Figure 8. Cyclin D1 and D3 expression is induced by TPO but not by BIO or DMSO treatment
Cytoplasmic extracts prepared simultaneously with nuclear extracts (Suppl 2) were analyzed for cyclin D1 and D3 expression by Western blotting. The blot was reprobed for β-catenin and α-tubulin as loading controls. Results from densitometric analysis, normalized for loading, are shown numerically.