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Can a Two-Faced Kinase be Exploited for Osteosarcoma?

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There are 518 protein kinases in the human genome, many of which act as important nodes in signal transduction pathways (1). Members of this class of important regulatory enzymes are also, unsurprisingly, frequently mutated or dysregulated in cancer and many other diseases. They have thus become the target of therapeutic interventions, with many successes. What is often overlooked, however, is that there are fewer than 15 signaling pathways for transmitting extracellular information into the cell. This paucity of communication routes is responsible for extracting appropriate cellular responses to a myriad of external cues. If it sounds as though too many eggs are in a limited number of baskets, the situation is further exacerbated by sharing of several transduction components between pathways. The most egregious example is that of glycogen synthase kinase-3 (GSK-3), a protein kinase first identified as a regulator of glycogen synthesis (2). This innocuously named protein is anything but because it plays a central role in at least four of these signaling pathways—the Wnt, Notch, Hedgehog, and nuclear factor-κB (NF-κB) pathways—with important roles in at least six more—the ras/mitogen-associated protein kinase (RAS/MAPK), cyclic-AMP, transforming growth factor-β/activin (TGF-β), phosphatidylinositol -3-kinase (PI3K), jun kinase/stress- activated protein kinase (JNK/SAPK), and janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. There are two highly related isoforms of GSK-3 (termed α and β) encoded by distinct genes, but that is still a substantial responsibility assigned to a particular protein kinase begging the question of why and how pathways maintain the authenticity of their signals if relying on the same molecules (3). Only the cyclic GMP, p38 mitogen-activated protein kinase (p38 MAPK), Ca^{2+}, calmodulin, and Hippo pathways, and the intracellular DNA damage response and unfolded protein response pathways currently lack known roles for GSK-3.

In this issue of the Journal, Tang *et al.* (4) report that suppression of GSK-3β interferes with the growth and viability of osteosarcoma cells, likely by inhibition of NF-κB. This transcription factor plays a critical role in inflammatory responses and has been directly implicated in the promotion of tumorigenesis (5). An important connection between GSK-3 and NF-κB was revealed upon the generation of mice that were genetically engineered to lack GSK-3β (6). GSK-3β null embryos frequently died before birth of extensive apoptosis in the liver, a phenotype reminiscent of mice that were engineered to lack RelA, a subunit of NF-κB (7). Mouse embryo fibroblasts derived from the GSK-3β null mice displayed a

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substantial deficit in the ability of tumor necrosis factor alpha (TNF-α) to induce activation of NF-κB and its regulation of anti-apoptotic gene expression. (TNF-α-mediated induction of the caspase pathway was unaffected; hence, the hepatocytes were sensitized to programmed cell death.) Interestingly, mice engineered to lack GSK-3α are viable and NF-κB signaling is normal (8). The molecular mechanism by which GSK-3β selectively regulates and is required for NF-κB function remains unclear, but this kinase modifies the spectrum of genes regulated by NF-κB (9).

Both isoforms of GSK-3 are negatively regulated by serine phosphorylation (i.e., Serine 21 of GSK-3α and Serine 9 of GSK-3β), and this is one mechanism by which the cyclic-AMP and phosphatidylinositol-3- kinase pathways suppress GSK-3 activity. Tang *et al.* (4) observed that the level of inhibitory phosphorylation of GSK-3β at Serine 9 was low in several osteosarcoma lines compared with that in a normal osteoblast cell line, suggesting that GSK-3 activity was higher than normal, although this was not directly measured. They also found that β-catenin levels (a target of the Wnt pathway) were increased in some lines, but this finding is unlikely to be related to GSK-3β phosphorylation for several reasons. First, agonists that induce serine phosphorylation of GSK-3 do not typically affect β-catenin (10, 11), probably because the degree of protein kinase inactivation by this mechanism is approximately 50%, whereas more than 75% inhibition of total GSK-3 (both GSK-3α and β) activity is required for an effect on β-catenin phosphorylation and stability; the rate-limiting factor in promoting phosphorylation of β-catenin is the concentration of a scaffolding protein termed Axin, which is present at only 10% of the level of GSK-3α + GSK-3β (12). Second, there does not appear to be a relationship between the level of GSK-3β phosphorylation in the U2OS vs SAOS2 cells and β-catenin levels— likely because of activated Wnt signaling in the SAOS2 cells (13).

The authors next modulated GSK-3β activity by stably expressing a kinase-inactive mutant of the protein kinase (which inhibits both endogenous GSK-3α and GSK-3β) to suppress activity or a Serine 9 to Alanine mutant (S9A) to increase activity in U2OS osteosarcoma cells. The cell lines that expressed the inactivated mutant behaved like wild-type (vector control) cells and were unable to form tumors in nude mice. By contrast, expression of the activated GSK-3β mutant promoted tumor formation. Partial (approximately 50%) silencing of GSK-3β expression by small interfering RNA (siRNA) in transformed (tumorigenic) U2OS/MTX300 cells reduced the ability of these cells to form colonies *in vitro* and to form tumors in nude mice, supporting a role for GSK-3β in the promotion of tumor growth. Treatment of a variety of osteosarcoma lines with several different (isoform non-selective) GSK-3 inhibitors, including lithium, reduced cell proliferation, and increased caspase activation and apoptosis, as did short hairpin RNA to GSK-3β (which should be isoform selective, although the authors did not show that GSK-3α levels or activity were unaffected). GSK-3 inhibitors worked additively with three different chemotherapeutic agents (doxorubicin, methotrexate, and cisplatin) to induce cell death of the osteosarcoma cells *in vitro* and in the case of lithium in animal xenografts.

To investigate the mechanism by which GSK-3 inhibition interfered with osteosarcoma cell growth, the authors assessed localization and transcriptional activity of NF-κB and found that treatment of U2OS cells with lithium or GSK-3β siRNA reduced nuclear localization

and NF-κB-dependent luciferase expression. Direct inhibition of NF-κB by expression of a dominant negative IκB mutant or siRNA to the p65 subunit of NF-κB suppressed tumor cell growth, whereas silencing of IκB expression partially reversed the pro-apoptotic effects of lithium treatment. Finally, analysis of osteosarcoma samples from 74 patients suggested an association between poor outcome and phosphorylated GSK-3β levels, suggesting potential prognostic value.

Given these findings, is GSK-3β a useful biomarker and/or a viable therapeutic target in osteosarcoma? Setting aside the issue of extrapolation of osteosarcoma cell line data to actual patient tumor responses (a caveat noted by the authors), a number of questions remain. First, as pointed out in the article, GSK-3 acts as a potent tumor suppressor in several pathways, for example, by promoting degradation of β-catenin, a known oncogene. Hence, inhibition of GSK-3 activity might promote cancer. But regulation of NF-κB is largely GSK-3β-specific, and if this isoform were selectively targeted, residual GSK-3α activity would be sufficient to maintain control of β-catenin and other substrates. The problem is that the protein kinase domains of both GSK-3 isoforms are essentially identical, and all small-molecule inhibitors that have been tested are isoform equipotent. The only way to selectively target one isoform more than the other is by using siRNA-like approaches—which will be a challenge. Second, GSK-3 activity cannot be accurately determined by use of phospho-specific antibodies because this measurement is only relative—the stoichiometry of phosphorylation is very difficult to assess. Even the activity of fully phosphorylated GSK-3β is not completely inhibited, and there are other mechanisms that affect GSK-3 activity. Because the effects of GSK-3 inhibitors observed in the Tang *et al.* (4) study were additive, there are likely multiple mechanisms at play. Moreover, because the chemical inhibitors used target both GSK-3 isoforms, correlative studies are needed to directly determine total GSK-3 activity levels, rather than the surrogate indicator of GSK-3β phosphorylation.

GSK-3 has pleiotropic effects on many cellular processes. This study revealed that the combination of both GSK-3 and NF-κB inhibition with chemotherapeutics was most effective in controlling tumor growth. Such combinatorial approaches may have the advantage of efficacy and specificity by avoiding the need for more complete inhibition of any particular target. The further development of such combination therapies should triangulate to more precisely target a common tumor vulnerability while permitting normal cells to maintain function. This approach presents challenges in regulating the dosage of combined therapies, but it is likely to be required if we are to target proteins with promiscuous and often dichotomous cellular functions such as GSK-3 and NF-κB.

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