

Akt/Protein Kinase B and Glycogen Synthase Kinase-3 β Signaling Pathway Regulates Cell Migration through the NFAT1 Transcription Factor

Merav Yoeli-Lerner, Y. Rebecca Chin, Christopher K. Hansen, and Alex Toker

Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Abstract

The phosphoinositide 3-kinase (PI3K) pathway regulates a multitude of cellular processes. Deregulation of PI3K signaling is often observed in human cancers. A major effector of PI3K is Akt/protein kinase B (PKB). Recent studies have pointed to distinct roles of Akt/PKB isoforms in cancer cell signaling. Studies have shown that Akt1 (PKB α) can attenuate breast cancer cell motility, whereas Akt2 (PKB β) enhances this phenotype. Here, we have evaluated the mechanism by which Akt1 blocks the migration of breast cancer cells through the transcription factor NFAT. A major effector of Akt/PKB is glycogen synthase kinase-3 β (GSK-3 β), also a NFAT kinase. Inhibition of GSK-3 β using short hairpin RNA or a selective inhibitor potently blocks breast cancer cell migration concomitant with a reduction in NFAT activity. GSK-3 β -mediated inhibition of NFAT activity is due to proteasomal degradation. Experiments using GSK-3 β mutants, which are unresponsive to Akt/PKB, reveal that inhibition of cell migration by Akt/PKB is mediated by GSK-3 β . These effects are recapitulated at the levels of NFAT degradation by the proteasome. Our studies show that activation of Akt/PKB leads to inactivation of the effector GSK-3 β and the outcome of this signaling event is degradation of NFAT by the proteasome and subsequent inhibition of cell migration. (Mol Cancer Res 2009;7(3):425–32)

Introduction

The phosphoinositide 3-kinase (PI3K) signaling pathway controls a variety of biological functions including cell survival, proliferation, and migration. Genetic lesions in the PI3K pathway are invariably found in all human cancers either at

the levels of PI3K itself or upstream regulators and downstream effectors. To the extent that the PI3K pathway is a major determinant of cancer progression, it is now known that the frequency of PI3K mutations in cancer is second only to Ras (1). A major effector of the PI3K signal in all cells types is the serine/threonine kinase Akt, also known as protein kinase B (PKB). Amplifications in one or more of the three mammalian Akt/PKB isoforms [Akt1 (PKB α), Akt2 (PKB β), and Akt3 (PKB γ)] as well as mutations in Akt1 are frequently found in human cancers (2). The signaling mechanisms leading to Akt/PKB activation by PI3K, and subsequent activation of downstream secondary signaling cascades leading to physiologic responses, have been characterized in considerable detail (reviewed in ref. 3). At the level of cell growth, much is known about the function of Akt/PKB in mediating cell proliferation through regulation of cell cycle progression and cell survival through regulation of proapoptotic and antiapoptotic transcription factors. Many of these mechanisms have been shown to be causal for cancer progression using mouse models.

A separate but equally important phenotype in carcinoma progression is invasive migration, a process that eventually leads to metastatic dissemination of tumor cells to distant organs. Recent studies from several laboratories have shown that Akt/PKB isoforms have opposing functions at modulating invasive migration of breast cancer cells both *in vitro* and *in vivo* (reviewed in ref. 4). Our laboratory showed that Akt1 attenuates invasive migration of breast cancer cells *in vitro* (5). The Brugge laboratory also showed that whereas Akt1 attenuates cell migration in two- and three-dimensional cell cultures, the Akt2 isoform promotes migration in a growth factor-dependent manner (6), consistent with earlier studies *in vivo* (7). The Bissell laboratory reported similar results for Akt1 (8). Distinct mechanisms were reported to explain the distinct functions of Akt/PKB isoforms in the regulation of invasive migration, including differential regulation of the extracellular signal-regulated kinase pathway (6) and tuberous sclerosis complex 2 (8). Our own studies revealed that Akt1 attenuates the activity of the transcription factor NFAT (5), which we showed previously to function as a promigratory and proinvasive transcription factor in breast cancer cells (9). Other studies have shown that activation of NFAT is concomitant with the induction of genes that promote these phenotypes, such as autotaxin and cyclooxygenase-2 (COX-2; refs. 10, 11). More recently, several independent studies have confirmed the distinct functions of Akt/PKB isoforms in modulating cancer cell invasion *in vivo* using both Akt/PKB-null mice and activated transgenes (12, 13).

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Requests for reprints: Alex Toker, Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, RN-237, Boston, MA 02215. Phone: 617-6678-8535; Fax: 617-667-3616. E-mail: atoker@bidmc.harvard.edu

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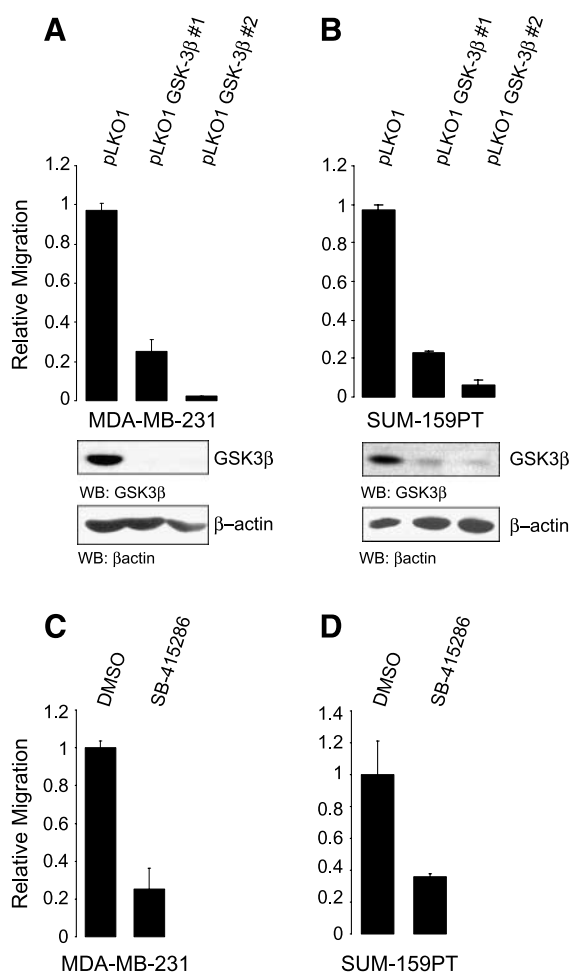


FIGURE 1. GSK-3 β regulates cell migration. MDA-MB-231 (**A**) and SUM-159-PT (**B**) cells were infected with lentiviruses directing expression of two distinct shRNA sequences against human GSK-3 β (GSK-3 β 1 and GSK-3 β 2). Twenty-four hours after infection, cells were selected for a further 48 h with puromycin and then subjected to chemotaxis assays. Cell lysates from each infection were immunoblotted with anti-GSK-3 β and control anti-actin. MDA-MB-231 (**C**) and SUM-159-PT (**D**) cells treated for 16 h with the GSK-3 β inhibitor SB-415286 (25 μ mol/L) or DMSO as control were subjected to chemotaxis assays. Representative of three independent experiments done in triplicates. Bars, SD.

The NFAT transcription factor, originally identified and characterized in immune cells, is ubiquitously expressed and functions in a variety of biological settings including in the endothelium, skeletal muscle, cardiac function, and neuronal signaling (14, 15). The classic NFAT activation pathway requires an influx of calcium, which activates the phosphatase calcineurin, and in turn dephosphorylates cytosolic NFAT unmasking a nuclear localization sequence leading to nuclear translocation. Nuclear export of NFAT is mediated by both nuclear and cytosolic NFAT kinases. One of the first identified NFAT kinases is glycogen synthase kinase-3 β (GSK-3 β ; ref. 16).

GSK-3 β was originally identified as a negative regulator of glycogen synthase in insulin-responsive tissues (17). GSK-3 is ubiquitously expressed and is basally catalytically active in cells. Following stimulation with agonists, inactivation of GSK-3 occurs through phosphorylation of several residues

and this is mediated by variety of upstream kinases. In this regard, one of the most studied GSK-3 β upstream kinases is Akt/PKB (18, 19). Two isoforms of GSK-3 exist in humans, GSK-3 α and GSK-3 β . *In vivo* studies using GSK-3 β -null mice have revealed that GSK-3 α cannot compensate for the loss of GSK-3 β , again suggesting isoform specificity between these two kinases (20). GSK-3 β activity is implicated in many pathophysiologic processes including cancer (reviewed in ref. 21). A major mechanism in the pathophysiology of cancer mediated by GSK-3 β is the Wnt signaling axis. In unstimulated cells, GSK-3 β facilitates ubiquitination and degradation of β -catenin through phosphorylation of axin and adenomatous polyposis coli-associated protein (22-24). Interestingly, mutations of β -catenin at the residues phosphorylated by GSK-3 β have been found in numerous cancers (25). A direct link among Akt/PKB, GSK-3 β , and NFAT is informed by the fact that GSK-3 β is an Akt/PKB substrate and NFAT is a GSK-3 β substrate. To this end, studies have shown that phosphorylation of NFAT2 by GSK-3 β is associated with reduced NFAT1 activity as well as nuclear export (16). Here, we provide a mechanistic link among Akt/PKB, GSK-3 β , and NFAT signaling to the phenotype of breast cancer cell migration and show that signaling through Akt/PKB leads to inactivation of NFAT and that this occurs in a GSK-3 β -dependent manner.

Results

GSK-3 β Regulates Cell Migration

To begin to investigate the role of GSK-3 β in cell migration, we first used MDA-MB-231 and SUM-159-PT cells, both highly migratory and invasive breast cancer cell lines, which we previously showed express NFAT and where signaling through Akt/PKB attenuates invasive migration (5, 9). Cells were infected with lentiviruses directing expression of two distinct different GSK-3 β short hairpin RNA (shRNA) sequences, GSK-3 β 1 and GSK-3 β 2 or virus control pLKO1. Cells were then selected with puromycin and analyzed for GSK-3 β expression. In both conditions, GSK-3 β expression was either eliminated or dramatically reduced compared with control cells (Fig. 1A and B). We next assessed the effect of GSK-3 β silencing on cell motility using Transwell chemotaxis assays. In both cell lines, infection with either GSK-3 β 1 or GSK-3 β 2 resulted in a potent inhibition of cell migration compared with cells infected with control virus (Fig. 1A and B). To determine whether GSK-3 β enzymatic activity is responsible for the effects on migration seen with shRNA, which could be due a loss of a scaffolding function of the protein and not just loss enzymatic activity per se, we also treated cells with SB-415286, a GSK-3 chemical inhibitor. SB-415286 is a potent cell-permeable maleimide compound that is widely used as a selective GSK-3 inhibitor (26). Serum-starved MDA-MB-231 and SUM-159-PT cells were treated with SB-415286 or DMSO control, and the ability of cells to migrate was evaluated as above. As predicted, enzymatic inhibition of GSK-3 activity recapitulated the results with shRNA and almost completely abolished the ability of treated cells to migrate when compared with control cells (Fig. 1C and D). Thus, GSK-3 β

activity is required for the highly migratory capacity of two distinct breast cancer cell lines, and reduction of GSK-3 β activity results in a near complete loss of the motile phenotype.

Signaling through Akt/PKB and GSK-3 β Regulates Cell Migration

We next sought to implicate Akt/PKB in the modulation of invasive migration through GSK-3 β . Because GSK-3 β is a well-characterized Akt/PKB substrate, we took advantage of a mutant of GSK-3 β , which is unresponsive to Akt/PKB, GSK-3 β .S9A. Phosphorylation of GSK-3 β at Ser⁹ by Akt/PKB effectively inhibits the high basal and constitutive activity of GSK-3 β (19). Because we and others have shown that Akt1 blunts invasive migration of breast cancer cells (5, 6, 8), we devised an experiment to determine whether one mechanism that accounts for this phenotype is signaling through GSK-3 β . As shown previously, introduction of a conditionally active Myr.Akt/PKB allele potently blocked breast cancer cell migration in both MDA-MB-231 and SUM-159-PT cells (Fig. 2A and B). Expression of the Akt/PKB unresponsive GSK-3 β .S9A mutant had little or no effect on cell migration. However, coexpression of the GSK-3 β .S9A mutant along with Myr.Akt/PKB either partially (Fig. 2A) or completely (Fig. 2B) rescued the inhibition of cell migration induced by

activated Akt/PKB. We also evaluated the ability of a physiologic ligand of the Akt/PKB and GSK-3 β pathway to control cell migration. We showed previously that stimulation of cells with insulin-like growth factor-I, which potently activates Akt/PKB, results in inhibition of cell migration, an effect that could be reversed with Akt1 shRNA (5). If this effect is mediated through Akt/PKB and GSK-3 β , then a GSK-3 β mutant resistant to Akt/PKB phosphorylation should phenocopy Akt1 shRNA. Consistent with this notion, SUM-159-PT cells stimulated with insulin-like growth factor-I showed a marked inhibition of cell migration, and this was completely rescued in cells expressing the GSK-3 β S9A mutant, which cannot be inhibited by Akt/PKB (Fig. 2C). Thus, with either genetic (Myr.Akt/PKB) or physiologic (insulin-like growth factor-I) means, inactivation of GSK-3 β by the Akt/PKB pathway regulates cell migration. Taken together, the interpretation here is that GSK-3 β activity is required for the high basal migratory phenotype of MDA-MB-231 and SUM-159-PT breast cancer cells, and inhibition of GSK-3 β activity either by shRNA (Fig. 1) or physiologically by Akt/PKB (Fig. 2) blocks this phenotype.

NFAT1 Activity Is Regulated by GSK-3 β

We next sought to determine the mechanism by which attenuation of GSK-3 β activity blocks breast cancer cell

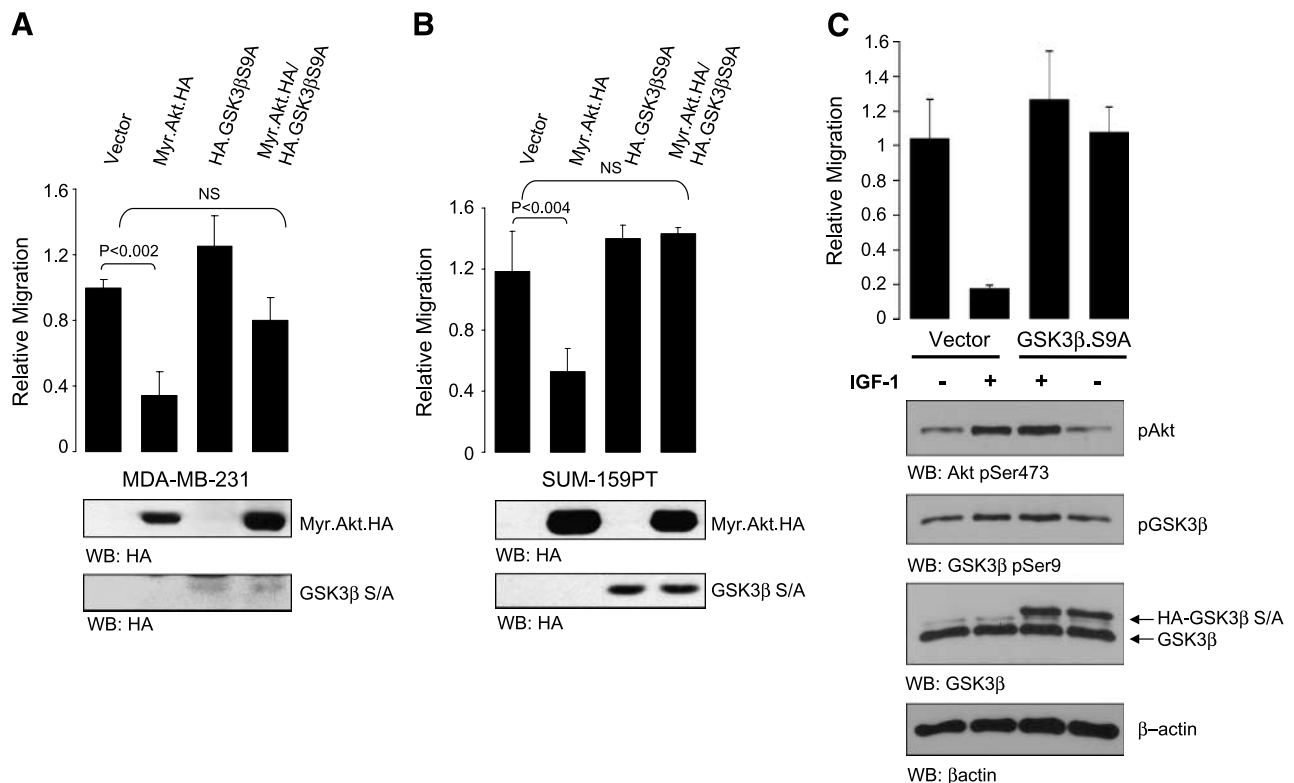


FIGURE 2. Akt1 blocks invasion through GSK-3 β . MDA-MB-231 (**A**) and SUM-159-PT (**B**) cells were transfected with either vector control, constitutively active Akt1 (Myr.Akt.HA) along with vector control or mutant GSK-3 β (HA.GSK-3 β .S9A), or mutant GSK-3 β alone. Sixteen hours after transfection, the ability of the cells to migrate was assessed by a chemomigration assays. A portion of the transfected cells were immunoblotted with anti-HA. **C.** SUM-159-PT cells were transfected with either vector control or mutant GSK-3 β (HA.GSK-3 β .S9A). Cells were then serum starved for 19 h and then stimulated with insulin-like growth factor-I for 18 h (100 ng/mL) followed by chemomigration assays. Representative of three independent experiments done in triplicate. Bars, SD, with *P* values. NS, not significant.

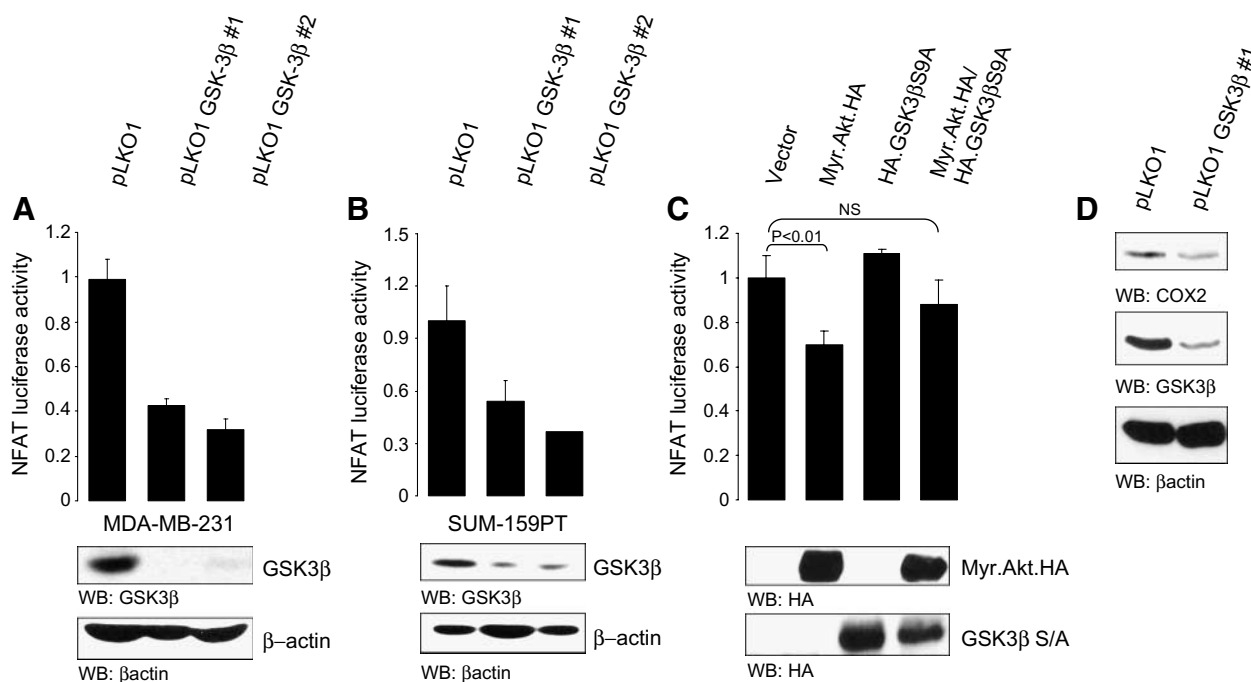


FIGURE 3. GSK-3 β regulates NFAT1 transcriptional activity. MDA-MB-231 (**A**) and SUM-159-PT (**B**) cells were infected with lentiviruses expressing shRNA against GSK-3 β and selected as in the legend to Fig. 1. Cells were transfected with interleukin-2-luciferase reporter plasmid and pCS2-(*n*)- β -gal immediately after selection. Sixteen hours after transfection, cells were lysed and luciferase assays were done. Lysates were immunoblotted with anti-GSK-3 β and anti-actin. **C.** SUM-159-PT cells were transfected with either vector control, constitutively active Akt (Myr.Akt.HA), Myr.Akt.HA and mutant GSK-3 β (HA.GSK-3 β .S9A), or GSK-3 β mutant alone together with the interleukin-2-luciferase reporter plasmid and pCS2-(*n*)- β -gal. Sixteen hours after transfection, luciferase assays were done. **D.** MDA-MB-231 cells were infected with lentivirus expressing shRNA against GSK-3 β and selected with puromycin (1 μ g/mL) for 48 h and then lysed and immunoblotted with anti-COX-2, anti-GSK-3 β , or anti-actin. All assays were done in triplicate. Bars, SD, with *P* values.

migration. We focused our attention on the transcription factor NFAT because we showed previously that NFAT is a proinvasive migration factor (9) and because NFAT activity is potently blocked by positive signaling through Akt/PKB, to the extent that, for example, constitutively active Akt/PKB completely abrogates NFAT transcriptional activity (5). To begin to investigate the model that GSK-3 β promotes enhanced migration through effects on NFAT activity, we first used shRNA. Consistent with the results on cell migration, lentiviral infection of both MDA-MB-231 and SUM-159-PT cells with two distinct shRNA sequences targeting GSK-3 β significantly decreased NFAT transcriptional activity as measured using a NFAT-luciferase reporter (Fig. 3A and B). As already shown, this was accompanied by a substantial reduction in GSK-3 β protein.

To provide a causal and direct demonstration of the effects of GSK-3 β on NFAT activity, we once again made use of the GSK-3 β .S9A mutant, which is Akt/PKB unresponsive. Again, and as published previously (5), expression of constitutively active Myr.Akt/PKB significantly reduced NFAT activity as measured in reporter assays (Fig. 3C). Expression of the GSK-3 β .S9A mutant had little or no effect on NFAT activity. In contrast, coexpression of GSK-3 β .S9A with Myr.Akt/PKB rescued the inhibition of NFAT activity induced by activated Akt/PKB. Finally, we used a distinct reporter of NFAT activity, COX-2. We and others have shown that COX-2 is a NFAT-induced gene in breast cancer cells (11). Consistent with the

luciferase reporter assays, depletion of GSK-3 β with shRNA also reduced total levels of COX-2 protein (Fig. 3D). These results essentially phenocopy the migration assays in Fig. 2. Again, the interpretation of these results is that phosphorylation of GSK-3 β by Akt/PKB at Ser⁹, which inhibits GSK-3 β activity, is required for the down-regulation of NFAT activity. Thus, the model here is that, under physiologic conditions, the high basal migratory capacity of these breast cancer cells is maintained by constitutive GSK-3 β activity and that removal of this activity by either Akt/PKB acting on GSK-3 β through Ser⁹ phosphorylation or artificially using shRNA reverses the phenotype.

NFAT1 Stability Is Regulated by the Akt/PKB and GSK-3 β Pathway

Finally, we investigated the mechanism by which the Akt/PKB and GSK-3 β signaling axis attenuates NFAT activity. Consistent with the results in Fig. 3A and B, shRNA-mediated silencing of GSK-3 β in MDA-MB-231 cells results in a marked reduction of NFAT protein under steady-state conditions (Fig. 4A). This effectively explains the diminution of NFAT activity under the same conditions.

We also evaluated the half-life of NFAT1 protein in cells treated with the GSK-3 β inhibitor SB-415286. Cells were serum-starved and treated with the protein synthesis inhibitor cycloheximide to prevent *de novo* NFAT1 protein synthesis and

then treated over a period of 6 h with the GSK-3 β inhibitor. The total levels of NFAT1 and control actin were measured by immunoblotting. There were little or no detectable changes in total NFAT1 protein during the first 3 h of drug administration (Fig. 4B). However, by 4 h, the levels of NFAT1 had declined to near undetectable levels, whereas levels of control actin were unchanged. As we have shown previously that NFAT is subject to proteasomal degradation by the Akt/PKB pathway, we next investigated if GSK-3 β is part of this mechanism. Again, exposure of cells to SB-415286 resulted in a marked reduction of NFAT1 protein, but this was completely reversed to control levels when cells were cotreated with the proteasome inhibitor ALLN (Fig. 4C). To provide genetic evidence for the regulation of NFAT1 proteasomal degradation downstream of GSK-3 β , we again used the GSK-3 β .S9A mutant. Expression of constitutively active Myr.Akt/PKB led to marked reduction in total levels of NFAT1 (Fig. 4D). Again, this was rescued to near control levels when cells were cotransfected with the Akt/PKB-resistant GSK-3 β .S9A mutant (Fig. 4D). Thus, signaling through Akt/PKB blocks NFAT1 activity and it does so by promoting the proteasomal degradation of the transcription factor. GSK-3 β is an intermediary in this pathway and specifically mediates the effects of Akt/PKB on NFAT. The net effect of this mechanism is attenuated cell migration.

Discussion

Hyperactivation of the PI3K pathway invariably leads to malignant transformation and tumor progression in humans. This can occur as a result of genetic alterations at the level of PI3K itself as well as upstream regulators such as Ras, PTEN, and PDK-1. Similarly, amplifications and mutations in downstream effectors such as Akt/PKB, HDM2, S6 kinase, and tuberous sclerosis complex 2 are often observed and have been shown in various *in vivo* models to promote tumorigenesis (1). For this very reason, numerous small-molecule inhibitors targeting one or more kinases in this pathway are currently being evaluated in clinical trials as cancer therapeutics. The Akt/PKB family of kinases have been shown to play a major role in PI3K signaling both at the level of signal relay and malignant transformation in a variety of settings. However, recent studies concerning the specific function of individual Akt/PKB isoforms and their effects on carcinoma invasive migration have shown that whereas the Akt2 isoform can promote tumor cell invasion and migration *in vitro* and *in vivo*, curiously the Akt1 isoform does not phenocopy this response (4). In fact, studies from our laboratory and others have shown that Akt1 can actually function as an inhibitor of breast cancer cell invasive migration (5, 6, 8), an observation originally made by the Muller laboratory, which showed that, in a mouse model

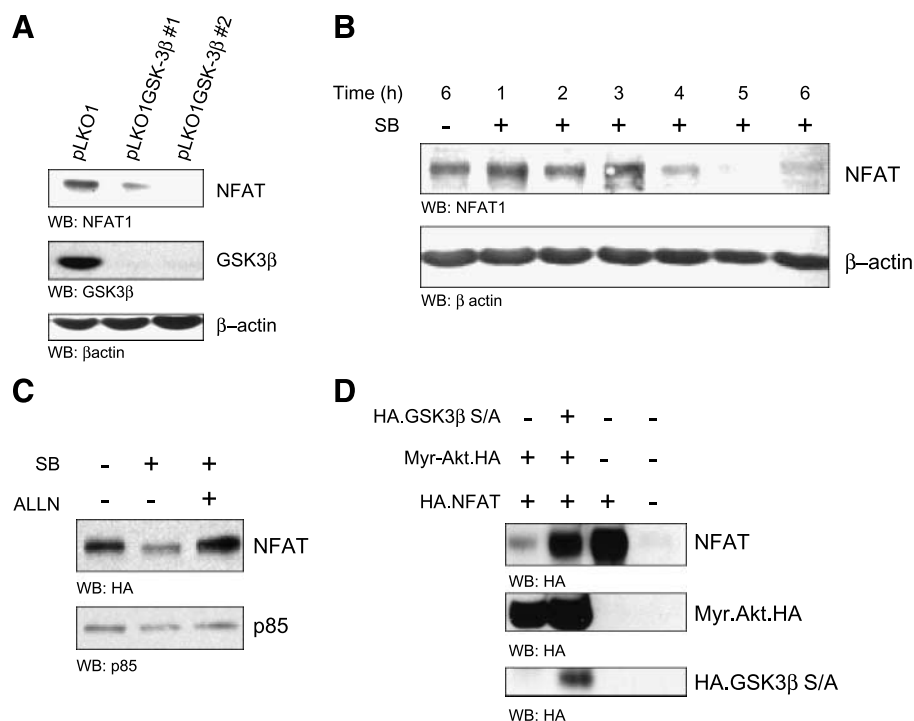


FIGURE 4. GSK-3 β stabilizes NFAT. **A.** MDA-MB-231 cells were infected with lentiviruses directing expression of two distinct sequences targeting human GSK-3 β . Cells were selected in the presence of 1 μ g/mL puromycin for 48 h, lysed and separated by SDS-PAGE, and immunoblotted with anti-NFAT, anti-GSK-3 β , and anti-actin. **B.** MDA-MB-231 cells were serum starved for 1 h and then treated with cycloheximide (100 μ g/mL) together with the SB-415286 (25 μ mol/L) over time. Total cell lysates were immunoblotted with anti-NFAT and anti-actin. **C.** SUM-159-PT.N1 clone 16 cells harboring tetracycline-inducible HA.NFAT were induced with doxycycline (0.5 μ g/mL) for 16 h in the presence of SB-415286 (25 μ mol/L) or DMSO control and with ALLN (50 μ mol/L). Cells were lysed and immunoblotted with anti-HA and anti-p85 as control. **D.** SUM-159-PT cells were transiently transfected with HA.NFAT, constitutively active Akt (Myr.Akt.HA), and GSK-3 β mutant (HA.GSK-3 β .S9A). Twenty-four hours following transfection, cells were lysed and lysates were immunoblotted with anti-HA, anti-Akt, and anti-GSK-3 β . All experiments were done in triplicate.

of ErbB2/*Neu*-driven mammary tumorigenesis, expression of activated Akt1 can promote proliferation but also leads to a reduction in the number of metastatic lesions (12). It is also noteworthy that the function of Akt isoforms in modulating invasive migration differs depending on the cell type. For example, whereas Akt1 inhibits or does not promote migration in breast epithelial cell lines, Akt1 clearly functions to promote motility, at least *in vitro*, in fibroblasts (27). Thus, the genetic background of distinct cell types is likely to govern the specific mechanisms that Akt isoforms use to modulate this phenotype.

In our recent study, we showed that the ability of Akt1 to block breast cancer cell migration is due in part to the inactivation of a crucial proinvasive migration transcription factor, NFAT1 (5). NFAT1 can promote this phenotype through the induction of genes that can enhance the motility and invasiveness of cells, including autotaxin/ENPP2 and COX-2 (10, 11). The ability of Akt1 to inactivate NFAT1 is due to the proteasomal degradation of the transcription factor, mediated by the E3 ubiquitin ligase HDM2, also an Akt/PKB substrate. In the present study, we have extended our investigation to delineate role of GSK-3 β in modulating cell migration in response to Akt1 signaling and the contribution of NFAT in this response. Using specific shRNAs as well as the chemical inhibitor SB-415286, we show that inhibition of GSK-3 β in two distinct breast cancer cells lines results in a marked reduction of cell migration. We then used a genetic rescue approach to provide a causal demonstration that the inactivation of GSK-3 β by Akt/PKB is responsible for the inhibition of cell migration. This was possible through the use of a GSK-3 β mutant, S9A, which is unresponsive to the Akt/PKB signal and therefore cannot be inactivated by phosphorylation. Indeed, this GSK-3 β mutant effectively rescued the ability of Akt/PKB to blunt cell migration (Fig. 2). Thus, the first conclusion to be drawn from these studies is that signaling through Akt/PKB leads to inactivation of GSK-3 β , and this results in inhibition of cell migration *in vitro*, an effect that is phenocopied by loss of GSK-3 β activity using pharmacologic or genetic means. It is, however, worth noting that there exist other mechanisms by which Akt can blunt cell migration in a manner that is independent of GSK-3 β . This is highlighted by the fact that, in certain experiments, expression of Myr.Akt/PKB in the context of activated GSK-3 β .SA does not elicit a complete rescue of migration (Fig. 2A). In this context, other studies have already shown that, for example, Akt1 can attenuate migration through extracellular signal-regulated kinase and tuberous sclerosis complex 2 signaling (6, 8).

Previous studies have investigated the role of GSK-3 β in modulating cell migration in distinct models, albeit with different results. For example, inactivation of GSK-3 β by Akt/PKB was shown to regulate the recycling of integrins, thus promoting cell migration in fibroblasts (28). Similarly, genetic or pharmacologic inactivation of GSK-3 β was shown to lead to a less of epithelial architecture and induction of a more mesenchymal phenotype of nontumorigenic breast epithelial cells, suggestive of a role for GSK-3 β in preventing the acquisition of a motile phenotype (29). In contrast, other studies using tumorigenic cell lines have shown that GSK-3 β is required for maintaining cell motility and invasion *in vitro*, consistent with our own studies. For example, GSK-3 β has

been shown to cooperate with h-prune to promote cell migration through modulation and phosphorylation of focal adhesion kinase (30). Similarly, in human keratinocytes, inhibition of GSK-3 β results in attenuated levels of the small GTPase Rac at lamellapodia, thus inhibiting wound closure (31). Moreover, paxillin has been identified as a GSK-3 β substrate by a dual extracellular signal-regulated kinase/GSK-3 β mechanism, and this was shown to play an important role in cytoskeletal rearrangement of various cell lines (32). Thus, depending on the cell origin and precise genetic background, GSK-3 β can play both positive and negative roles in modulating cell migration and invasion, and in many cases, this is modulated through the Akt/PKB pathway. In the context of aggressive breast cancer cells, GSK-3 β clearly functions as an enhancer of the motile phenotype, whereas, in other cell types of the surrounding stroma such as fibroblasts, GSK-3 β may function in an opposing manner.

We next turned our attention to the mechanism by which inactivation of GSK-3 β can attenuate cell migration in the Akt/PKB pathway. Because we recently showed that Akt1 can blunt cell migration in a NFAT1-dependent manner and because GSK-3 β has been identified as a NFAT kinase, we asked the most logical question: Does GSK-3 β inactivation lead to inactivation of NFAT, and does this then explain the consequence of GSK-3 β inhibition on cell migration? As predicted, inhibition of GSK-3 β by chemical or genetic means also potently blocked NFAT transcriptional activity in cells, and again we were able to show that this was specifically mediated by Akt/PKB signaling through the use of the GSK-3 β mutant, which is Akt/PKB unresponsive (Fig. 3). To extend this observation, we showed that the inactivation of NFAT activity by loss of GSK-3 β is due to proteasomal degradation of the transcription factor, a result that essentially recapitulates what is observed in cells expressing activated Akt/PKB, where NFAT1 is also degraded by the proteasome (ref. 5; Fig. 4D). Although we showed that HDM2 is in part required for the proteasomal degradation of NFAT1 by the Akt/PKB pathways, whether GSK-3 β functions in the same or a distinct but parallel pathway remains to be determined.

The ability of GSK-3 β to control ubiquitination and protein stability of its substrates through phosphorylation has been studied in some detail, again revealing differences on the mechanism depending on the pathway in question. For example, in the Wnt pathway, GSK-3 β phosphorylates axin and increases its stability (23). In contrast, GSK-3 β phosphorylation of β -catenin induces its ubiquitin and subsequent proteolysis. Although the detailed mechanism remains to be determined, GSK-3 β signaling to NFAT is associated with stabilization, as loss of GSK-3 β activity leads to a robust degradation of NFAT1 by the proteasome.

Given that opposing functions for Akt/PKB and GSK-3 β in modulating cell migration have been reported and shown to be dependent on the cellular context, it is perhaps not surprising to find that contrasting functions for GSK-3 β in the regulation of NFAT1 activity are also evident. In the original report where GSK-3 β was identified as a NFAT kinase, it was shown that GSK-3 β inactivates the transcription factor by promoting nuclear export (16). Thus, inactivation of GSK-3 β would be expected to retain NFAT in the nucleus and be reflected by

increased transcriptional activity. These results were obtained in COS cells. However, in our own studies in breast cancer cells, GSK-3 β appears to function in an opposing manner, because inactivation of GSK-3 β results in a loss of transcriptional activity, which would not be predicted to be associated with nuclear retention. Instead, it appears that GSK-3 β is required to maintain active NFAT1 in the nucleus, presumably through phosphorylation, and that loss of phosphorylation at one or more sites promotes proteasomal degradation. Considering the function of Akt/PKB in this response, our studies are consistent to those of Patra et al., who showed that expression of activated Akt/PKB results in cytoplasmic accumulation of NFAT and therefore inhibition of transcriptional activity (33). Regardless of the mechanisms, these findings highlight the importance of addressing specific mechanisms of signaling to phenotypic responses, such as migration, which are often found to be opposing in distinct cell types.

To date, numerous other NFAT upstream kinases have been identified, which affect NFAT cellular localization and in turn transcriptional activity. For example, casein kinase 1 was recently shown to phosphorylate NFAT1 in T cells and promote NFAT1 nuclear export in a manner dependent on subsequent GSK-3 β phosphorylation of the transcription factor (34). One extrapolation from these findings is that casein kinase 1 phosphorylates NFAT1, thus promoting nuclear export as well providing a priming site for subsequent GSK-3 β -mediated phosphorylation. This latter event may promote nuclear export of NFAT1 and also protect it from proteasomal degradation, a predominantly cytoplasmic event (5). Whether this or other mechanisms operate to control NFAT localization, activity and stability have yet to be determined.

In summary, our studies have added a new layer in the mechanism by which Akt1 functions as an inhibitor of breast cancer cell migration, specifically by inactivation of GSK-3 β leading to destabilization of NFAT. This has important consequences for the multitude of genes that are induced by NFAT in cancer cells, which are likely to affect phenotypes such as cell motility, survival, and proliferation.

Materials and Methods

Antibodies and Reagents

Anti-HA antibody was purified from the 12CA5 hybridoma in house. Anti-NFAT and COX-2 were from Santa Cruz Biotechnology. Anti-GSK-3 β and Akt pSer⁴⁷³ were from Cell Signaling Technology. Anti- β -actin antibody, cycloheximide, insulin, and hydrocortisone were from Sigma-Aldrich. SB-415286 was from BioMol International. Matrigel was from Fisher Scientific. Doxycycline was from Clontech. ALLN was from Calbiochem. Murine HA-tagged NFAT and interleukin-2-luciferase reporter plasmids have been described (9, 35, 36). The wild-type GSK and mutant GSK.S9A plasmids have also been described (37).

Production of shRNA Lentivirus

To silence the expression of GSK-3 β , the following oligonucleotides were cloned into the pLKO1 lentiviral vector: sequence 1 forward 5'-CCGGGAAGTCAGCTATACAGACTCTCGAGAGTGTCTGTATAGCTGACTTCTTTTG-3'

and reverse 5'-AATTCAAAAAGAAGTCAGCTATACAGACTCTCGAGAGTGTCTGTATAGCTGACTTC-3' and sequence 2 forward 5'-CCGGGAAGCTAGATCACTGTAACTCGAGTGTACAGTGTACTAGCTTTCTTTTG-3' and reverse 5'-AATTCAAAAAGAAAGCTAGATCACTGTAACACTCGAGTGTACAGTGTACTAGCTTTC-3'. Viruses were generated by cotransfecting the cloned pLKO1 plasmids with packaging plasmid pHR'8.2deltaR at an 8:1 ratio and envelope plasmid pCMVSV-G into HEK-293T cells. Virus was collected 48 h post-transfection, passed through a 45 μ m filter, and kept at -80°C.

Cell Lines

MDA-MB-231, HEK-293T, and NIH 3T3 cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C. The estrogen-independent human breast cancer SUM-159-PT cell line has been described (38). SUM-159-PT cells with inducible NFAT1 expression (SUM-159.N1) have been described (5, 11). NFAT1 expression was induced with 1 μ g/mL doxycycline for 16 h.

Immunoblotting

Cells were lysed in SDS sample buffer and lysates were resolved by SDS-PAGE and immunoblotted with the appropriate antibodies.

Migration Assays

Migration assays were carried out essentially as described previously using Transwell chambers (Corning) with 8 μ m pore membranes (5). Cells were treated to enhance or down-regulate specific signaling pathways by cotransfection with the relevant expression plasmids and a pCS2-(n)- β -gal reporter, by infection of lentiviruses expressing shRNA, or by treatment with chemical inhibitors. Cells were harvested, resuspended in serum-free medium containing 0.1% bovine serum albumin, added in triplicates to Transwell chambers, and allowed to migrate toward NIH 3T3 cell conditioned medium for 1.5 to 16 h at 37°C. Cells that had migrated to the lower surface of the membrane were fixed and stained with either X-gal or crystal violet.

Luciferase Assays

Cells were infected with a lentivirus expressing shRNA against the GSK-3 β sequence. The infected cells were selected in a medium containing puromycin for 2 days. Cells were then transiently cotransfected with NFAT1 reporter plasmids, pCS2-(n)- β -gal. Twenty-four hours after transfection, cells were analyzed for luciferase and β -gal activity using the luciferase assay system (Promega) and galacton-plus (Tropix) and measured on a luminometer. Luciferase activity was normalized against β -gal.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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