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Knockdown of apoptosis signal-regulating kinase 1 modulates basal glycogen synthase kinase-3β kinase activity and regulates cell migration

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ABSTRACT

GSK-3β is a basally active kinase. Axin forms a complex with GSK-3β and β-catenin; this complex promotes the GSK-3β-dependent phosphorylation of β-catenin, thereby inducing its degradation. However, the inhibition of GSK-3β provokes cell migration via the dysregulation of β-catenin. In this study, we determined that the level of apoptosis signal-regulating kinase 1 (ASK1) was lower in a metastatic breast cancer cell line, compared to that of non-metastatic cancer cell lines and the knockdown of ASK1 not only induces β-catenin activation via the inhibition of GSK-3β and collapsing the subsequent protein complex by regulating Axin dynamics, but also stimulates cell migration. Together, the blockage of the GSK-3β–β-catenin pathway resulting from the knockdown of ASK1 modulates the migration of breast cancer cells.

1. Introduction

Apoptosis signal-regulating kinase 1 (ASK1), a serine-threonine kinase, was initially discovered as a mitogen-activated protein kinase kinase kinase (MAPKKK) in the c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways [1]. ASK1 induces apoptotic cell death via a mitochondria-dependent pathway related to cytochrome c release and caspase 9 and caspase 3 activation [2]. ASK1 has also been implicated in various cellular events, including differentiation, proliferation and innate immunity [3].

The serine/threonine kinase glycogen synthase kinase-3 (GSK-3) was initially identified as a regulator of glycogen metabolism, and has been shown to regulate a variety of cellular processes, including apoptosis, differentiation, growth, motility and the epithelial-mesenchymal transition (EMT) [4]. GSK-3 stabilizes Axin via phosphorylation and maintains the formation of the Axin–GSK-3β–β-catenin destruction complex [5–8]. This complex promotes the GSK-3β-dependent phosphorylation of β-catenin, thereby inducing the degradation of β-catenin [6,8]. However, Wnt signaling disrupts the Axin–GSK-3β–β-catenin destruction complex and induces the abnormal accumulation of β-catenin [9,10]. Free β-catenin then translocates to the nucleus, where it interacts with T cell factor (TCF)/lymphoid enhancer-binding factor family members to induce the expression of specific genes associated with physiological events such as development, proliferation and migration [11].

Here, we report that not only basal GSK-3β activity but also the Axin–GSK-3β–β-catenin destruction complex regulated by ASK1 modulates β-catenin-mediated cancer cell migration in a GSK-3β-dependent manner.

2. Materials and methods

2.1. Cell culture

MCF7 GFP, ASK1 siRNA cells were maintained in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 g/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. RNAi

The ASK1 siRNA sequence, 5′-GCACUCCUUCAUCGAGCUAUU-3′ and the GFP siRNA sequence 5′-CCUACGCCACCAAUUUCGU-3′ siRNAs were synthesized by Bioneer (Daejeon, South Korea). pSUPER·retro·puro siRNA expression vector (Oligoengine, Inc.) was used to construct an ASK1 siRNA plasmid. MCF7 Cells were transiently transfected with a microporator (Digital Bio Technology, Seoul, South Korea).

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2.3. Co-immunoprecipitation analysis

Co-immunoprecipitation analysis was performed as previously described [12], with slight modifications. Cells were lysed with buffer (0.5% NP-40, 1 mM EDTA, 50 mM Tris–HCl, pH 8.0, 120 mM NaCl) and the lysates were subjected to 10 min of microcentrifugation at 12 000×*g*. The soluble fraction was then incubated for 2 h at 4 °C with the appropriate antibodies, after which the mixture was incubated for an additional 1 h at 4 °C with protein G-coupled Sepharose beads (Amersham Pharmacia Biotech, Uppsala). The resultant precipitates were subjected to SDS–PAGE and analyzed via immunoblotting.

2.4. Immunoblot analysis

In brief, cell lysates were subjected to SDS–PAGE and transferred to PVDF membranes. The PVDF membranes were then blocked with 5% non-fat milk in a washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and incubated with the indicated antibodies for 1 h at room temperature. The membranes were subsequently washed and incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Protein bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.5. Luciferase assay

Cells were transiently cotransfected using a microporator (Digital Bio Technology, South Korea) with a pTopflash and a control pβ-galactosidase report construct. After 36 h of incubation, the cells were treated for an additional 12 h with 10 mM LiCl. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega, WI) using a luminometer and a spectrophoto-meter, respectively.

2.6. Migration assay

Cell migration was evaluated using Transwell chambers supplied with filters containing polycarbonate membranes (8 μm pore size; Corning, NY). The cells were counted (1×10^4 cells) and then added to the upper chambers of 24-well transwell plates in serum-reduced media (DMEM with 0.5% FBS) and were cultured for 24 h with DMEM media containing 5% FBS in the lower well, and motile cells at the bottom of the filter were fixed with 4% formaldehyde and stained with 4′,6′-diamidino-2-phenylindole (DAPI). The number of migrating cells was quantified by counting the stained cells under a fluorescence microscope (×40 objective).

2.7. Cell proliferation assay

Cell proliferation assay using Brd-uridine incorporation was performed using the Cell Proliferation ELISA kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer's instruction. In brief, cells were seeded at a density of 10 000 cells per well, allowed to adhere for 24 h. Proliferation was quantified 36 h post-transfection.

3. Results

3.1. ASK1 is highly expressed in less metastatic breast cancer cell lines (MCF7, SKBR3, MDA468) rather than metastatic breast cancer cell line (MDA231)

To determine whether ASK1 is related with breast cancer metastasis, we examined the level of ASK1 in various breast cancer cell lines. As shown in Fig. 1, ASK1 was expressed at higher levels in non-metastatic breast cancer cell lines (MCF7, SKBR3, MDA468), rather than metastatic breast cancer cell line (MDA231). From this result, we knew that ASK1 is closely involved in breast cancer metastasis.

3.2. MCF7 ASK1 siRNA cells migrate rapidly as compared to MCF7 GFP control cells, as the result of GSK3 inhibition but not JNK or P38 inhibition

Next, to confirm the relativity of ASK1 in breast cancer metastasis, we manufactured MCF7 ASK1 siRNA cells and investigated cell migration and proliferation. The migration of MCF7 ASK1 siRNA cells was accelerated as compared with that of MCF7 GFP control cells (Fig. 2A and C). However, cell proliferation was not significantly altered, regardless of the presence of ASK1 (Supplementary Fig. 1). We attempted to determine which signaling disruption caused by ASK1 knockdown provokes this increased migration using JNK, p38, and GSK-3 inhibitors. In GSK-3 inhibitor-treated cells, but not JNK or p38 inhibitor-treated cells, increased migration levels were detected (Fig. 2B and D). Additionally, the characteristics of EMT, including the loss of E-cadherin, increased N-cadherin and snail, one of the EMT regulators, were also noted in MCF7 ASK1 siRNA cells (Supplementary Fig. 2). These data show that the ASK1-mediated regulation of GSK-3 activity, but not JNK or p38 activity, performs an important role in altering cell motility, rather than cell proliferation.

3.3. ASK1 inhibits β-catenin activity via the regulation of GSK-3 β activity

In order to determine whether the increased migration in MCF7 ASK1 siRNA cells is dependent on the activation of β-catenin via the inhibition of the GSK-3 signaling pathway, we evaluated the activity of β-catenin, a representative downstream target of GSK-3. Indeed, β-catenin activity was increased in the MCF7 ASK1 siRNA cells, as compared with MCF7 GFP control cells (Fig. 3A). Owing to the aberrant upregulation of β-catenin activity under conditions of absent ASK1 expression, we attempted to assay directly the activity of GSK-3β. As shown in Fig. 3B, the phosphorylation of Tyr 216 on GSK-3β necessary for its activation and the level of Axin, a scaffolding protein of the Wnt signaling pathway, were diminished in the MCF7 ASK1 siRNA cells, as compared with those in the MCF7 GFP control cells. Because basally active GSK-3β stabilizes Axin via phosphorylation [5–8], reduced levels of Axin are also associated with inhibited GSK-3β activity. These data imply that basal GSK-3 kinase activity is inhibited under reduced ASK1 conditions. As a consequence, β-catenin activity was increased. We inferred that ASK1 modulates not only β-catenin activity but also Axin

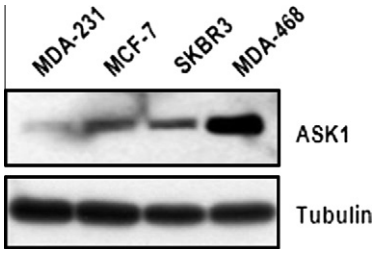

Fig. 1. ASK1 is highly expressed in less metastatic breast cancer cell lines (MCF7, SKBR3, MDA468) rather than metastatic breast cancer cell line (MDA231). Whole-cell extracts were prepared from MDA231, MCF7, SKBR3 and MDA468 cell lines and were analyzed via immunoblot analysis using anti-ASK1 and anti-tubulin antibodies.

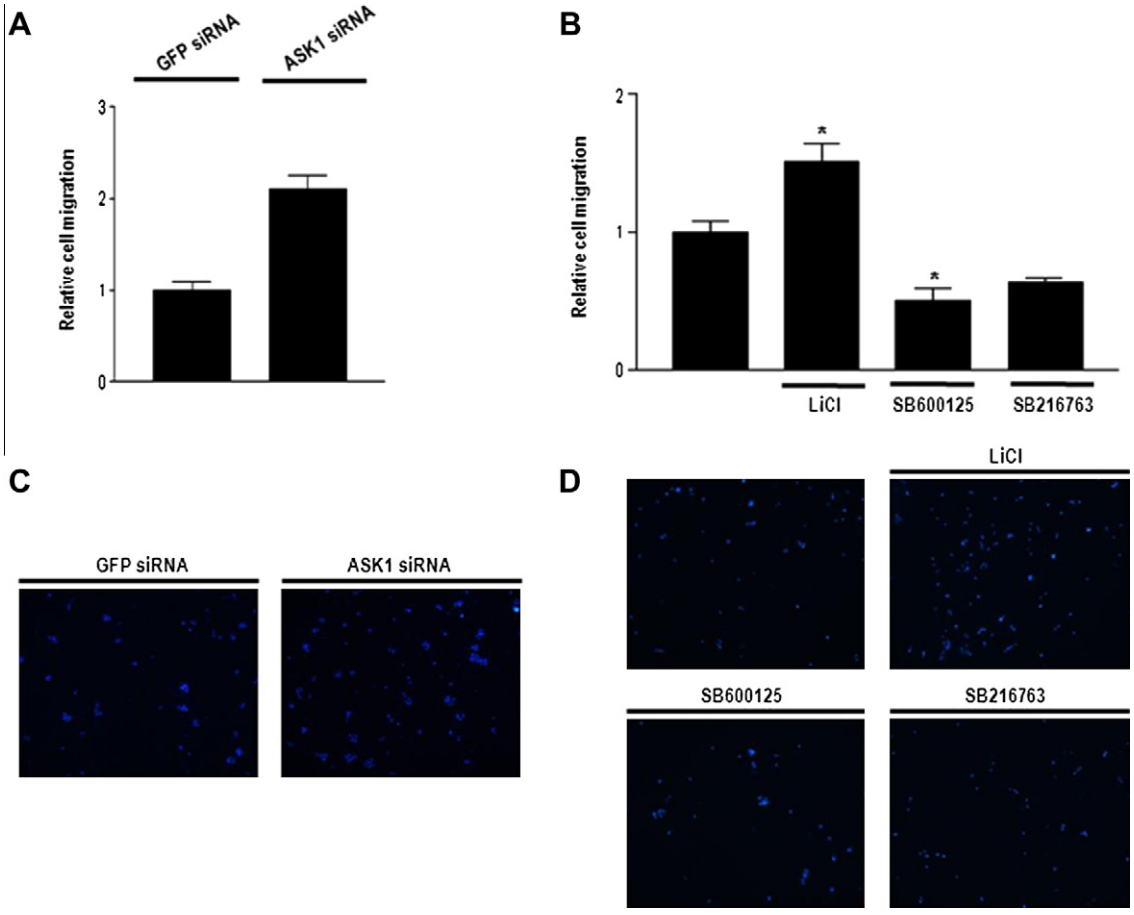

Fig. 2. MCF7 ASK1 siRNA cells migrate rapidly, compared to MCF7 GFP control cells, as the result of GSK3 inhibition but not JNK or P38 inhibition. (A) MCF7 GFP control cells and ASK1 siRNA cells (1×10^4 cells) were added to the upper chambers of 24-well transwell plates in serum-reduced media (DMEM with 0.5% FBS). MCF7 GFP control cells and ASK1 siRNA cells were cultured with DMEM media containing 5% FBS in the lower well for 24 h. The migration was measured as described in Section 2. Data are reported in terms of migrated cells. The *error bars* represent the means ± S.D. of two experiments. (B) MCF7 cells were added to the upper chamber of 24-well transwell plates in serum-reduced media (DMEM with 0.5% FBS). MCF7 cells were cultured in the absence or presence of 10 mM GSK3 inhibitor (LiCl) or 25 μM JNK inhibitor (SB600125) or 10 μM P38 inhibitor (SB203580) in the lower well for 24 h and motile cells were countered. (C and D) Photograph of the cells that migrated through the transwell inserts. The *error bars* represent the means ± S.D. of three experiments. $^{*}P < 0.05$ compared with untreated control.

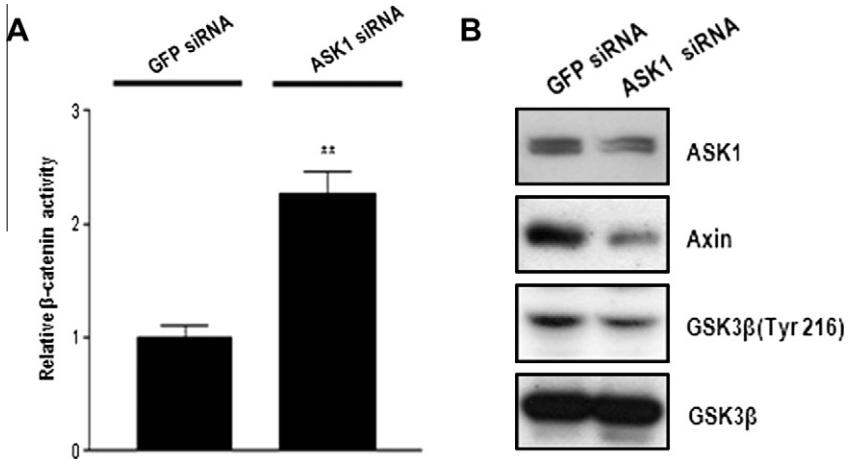

Fig. 3. ASK1 inhibits β-catenin activity via the regulation of GSK-3 activity. (A) MCF7 GFP control cells and ASK1 siRNA cells were transiently cotransfected with a pTopflash and a control pβ-galactosidase report construct. After 36 h of incubation, these transfectants were subsequently incubated for 12 h in the absence or presence of 10 mM LiCl. The data are expressed in terms of relative luciferase activity (normalized to β-galactosidase activity). The *error bars* are expressed as the means ± S.D. of three experiments. $^{**}P < 0.001$. (B) Whole-cell extracts were prepared from MCF7 GFP control cells and ASK1 siRNA cells, and were analyzed via immunoblot analysis using anti-ASK1, anti-Axin, anti-GSK-3β and anti-phospho-GSK3 (Tyr279/Tyr216) antibodies.

stability, as a crucial regulator of basal GSK-3β kinase activity. However, the phosphorylation of Tyr 279 on GSK-3α was not altered significantly, regardless of the presence of ASK1 (Supplementary Fig. 3). This result demonstrates that the increased activity of β-catenin in the absence of ASK1 is attributable to reduced GSK-3β activity rather than GSK-3α activity.

3.4. ASK1 participates in the Wnt signaling pathway via the regulation of GSK-3β

Next, we assessed the profile of Axin–GSK-3β–β-catenin destruction complex components under Wnt signaling conditions. Whereas LiCl treatment (Wnt signaling mimic condition) induced a diminished level of Axin via GSK-3β inhibition and the abnormally launched β-catenin production caused by the reduced Axin, the scaffolding protein of Axin–GSK-3β–β-catenin destruction complex in the presence of ASK1 [10,13], these phenomena were not noted under ASK1 knockdown conditions (Fig. 4). Additionally, LiCl treatment also reduced the levels of ASK1, as well as Axin. According to these results, we hypothesized that ASK1 performs a pivotal function in the formation of the Axin–GSK-3β–β-catenin destruction complex via the regulation not only of GSK-3β activity, but also levels of Axin, and leads to the regulation of β-catenin, the effector protein of the Wnt signaling pathway.

3.5. ASK1 regulates the formation of Axin–GSK-3β–β-catenin destruction complex

To evaluate our hypothesis, we evaluated this destruction complex via a co-immunoprecipitation assay. The destruction complex was disrupted in the MCF7 ASK1 siRNA cells, whereas the Axin–GSK-3β–β-catenin destruction complex was basally formed in the MCF7 GFP control cells (Fig. 5). As shown in Figs. 4 and 5, we inferred that ASK1 is involved in the Wnt signaling pathway by Axin dynamics via the regulation of GSK-3β activity. In other words, the Axin–GSK-3β–β-catenin destruction complex was not formed, because of the inhibited GSK-3β activity and the ASK1-mediated reduction of GSK-3β-induced Axin stability. This means that ASK1 regulates the basal GSK-3β kinase activity critical for the formation of the Axin–GSK-3β–β-catenin destruction complex.

4. Discussion

GSK-3β is known as a basally active kinase, and it stabilizes Axin via subsequent phosphorylation. Next, the stabilized Axin, a scaffolding protein, forms a complex with GSK-3β and β-catenin, and this complex formation is necessary for the maintenance of basal GSK-3β activity. However, the precise mechanism for the activation of GSK-3β in a basal state is not defined. In this study, we have demonstrated that the basal activity of GSK-3β, the Axin stability, the formation of the destruction complex and the activity of β-catenin, are regulated by ASK1 in resting cells. Therefore, we suggest that ASK1 is closely correlated with the regulation of GSK-3β basal activity.

Among various cellular processes regulated by GSK-3β, we focused on the regulation of cell motility. Previous studies have documented that β-catenin, a downstream transcription factor of GSK-3β, promotes migration [14,15]. Recently, the regulation of EMT, one of the processes inherent to cancer progression, was reported to occur via the inhibition of GSK-3 [16,17]. By all accounts, GSK-3 functions as a repressor of cell motility at the basal level and protects against cancer progression. According to our results, these GSK-3 functions might be controlled by ASK1 in the basal state. Furthermore, we determined that ASK1 is related closely with breast cancer metastasis. Further researches to define the mechanism underlying the regulation of GSK-3 by ASK1 and to illuminate the precise relevance of ASK1 in cancer progression are currently underway. A more precise understanding of the interrelationship between ASK1 and the Axin–GSK-3β–β-catenin destruction complex components will provide us with insights critical to the development of effective cancer drugs.

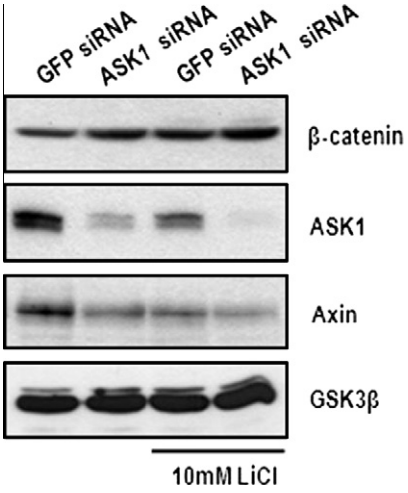

Fig. 4. ASK1 activates GSK-3β and regulates Wnt signaling pathway. MCF7 GFP control cells and ASK1 siRNA cells were incubated for 4 h in the absence or presence of 10 mM LiCl. The cell lysates were also subjected directly to immunoblot analysis with anti-β-catenin, anti-ASK1, anti-Axin and anti-GSK-3β antibodies.

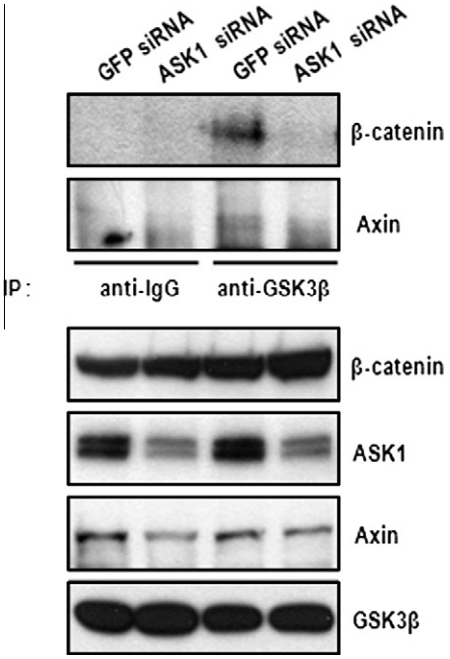

Fig. 5. ASK1 regulates the formation of the Axin–GSK-3β–β-catenin destruction complex. The formation of Axin–GSK-3β–β-catenin complex was reduced in MCF7 ASK1 siRNA cells. Cell lysates were subjected to immunoprecipitation with anti-GSK-3β antibody, and the resultant precipitates were subjected to immunoblot analysis with the indicated antibodies. Cell lysates were also directly assessed via immunoblot analysis with anti-β-catenin, anti-ASK1, anti-Axin and anti-GSK-3β antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.08.029.

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