SKA3 Promotes Cell Growth in Breast Cancer by Inhibiting PLK-1 Protein Degradation

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Abstract
Breast cancer (Bca) remains the most common form of malignancy affecting females in China, leading to significant reductions in the mental and physical health of those with this condition. While spindle and kinetochore associated complex subunit 3 (SKA3) is known to be linked with cervical cancer progression, whether it is similarly associated with Bca progression remains unknown. Using shRNA, we specifically knocked down the expression of SKA3 in Bca cell lines and then assessed the resultant changes in cell proliferation using CCK-8 and colony formation assays. In addition, we used western blotting to quantify the expression levels of relevant proteins in these cells, and we assessed the interaction between SKA3 and polo-like kinase-1 (PLK-1) via co-immunoprecipitation. In this study, we observed elevated SKA3 expression in Bca tissues and cell lines. When we knocked down SKA3 expression in Bca cells, we were able to determine that it functions in an oncogenic manner so as to promote the growth and proliferation of these cells in vitro. From a mechanistic perspective, we were able to show that in Bca cells SKA functions at least in part via interacting with PLK-1 and preventing its degradation. In summary, we found that SKA3 is able to regulate PLK-1 degradation in Bca cells, thus controlling their growth and proliferation. These results highlight SKA3 as a potentially viable target for anti-cancer drug development aimed at combatting Bca.

Keywords
SKA3, breast cancer, PLK-1, tumor growth

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Introduction
Breast cancer (Bca) remains the most prevalent form of cancer impacting females and the leading cancer-associated cause of death in China. Importantly, rates of Bca mortality have been rising in recent years, with death typically resulting from metastatic tumor progression that ultimately disrupts normal biological functionality. Even so, the specific molecular mechanisms governing Bca metastasis and associated patient mortality remain to be fully clarified.

Spindle and kinetochore associated complex subunit 3 (SKA3) is a component of the SKA complex which localizes to the outer layers of the kinetochore. This protein is essential both for ensuring that mitotic exit is appropriately regulated through its interactions with the NDC80 complex and for controlling meiotic spindle migration and the stability of the spindle during anaphase. A number of previous studies have highlighted roles for SKA in the onset and progression of cancer. For example, somatic mutations in SKA3 are often detected in Bca and are thought to influence the growth of impacted tumor cells. Multiple recent studies have further shown SKA3 to be directly related to both tumor aggression and patient outcomes in many cancer types. How SKA3 impacts Bca, however, remains to be fully clarified.

In this report, we assessed the mechanistic function of SKA3 in the context of Bca progression. We observed elevated SKA3 expression in both Bca cell lines and primary patient tumor samples, and we additionally determine that knocking down SKA3 in Bca cells was sufficient to interfere with their proliferative activity in vitro. We further found that from a mechanistic perspective, SKA3 was able to directly interact with

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PLK-1 and to thereby prevent its degradation, thus promoting Bca tumor growth and progression. Together, our results highlight SKA3 as a potentially viable therapeutic target in Bca.

Materials and Methods

Tissue Samples and Cell Culture

We obtained 12 total Bca primary tumor tissue samples and paired healthy paracancerous tissues (>5 cm distant from tumor tissue) were obtained from 30 female patients (age range, 33–79 years; mean age, 60 years) who underwent surgical resection at Shaoxing People’s Hospital (Dezhou, China) between January 2014 and July 2015 for qRT-PCR and WB assays. The Shaoxing People’s Hospital Institutional Review Board (Ethics number: SXPX-20130516) approved the present study, and all patients provided informed consent to participate. Following collection, samples were snap-frozen and stored at −80°C prior to RNA and protein extraction.

The BT549, T47D, MCF-7, SKBR3, and MDA-MB-231 human Bca cell lines were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). In addition, the normal control MCF-10A human breast cell line was obtained from the American Type Culture Collection (ATCC) (VA, USA). All cells were cultured using DMEM (Gibco, NY, USA) containing 10% FBS and penicillin/streptomycin (Invitrogen, CA, USA) in a 37°C humidified incubator with a 5% CO2 atmosphere.

qRT-PCR

Trizol was used to isolate RNA from all tissue and cell samples based on provided directions, after which a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA) was used to measure RNA concentrations and the A260/A280 absorbance ratios for each sample. qRT-PCR assays were then conducted as in previous studies,12,13 with the primers used in this study being detailed in Table S1.

Transfection and Lentiviral Transduction

Control, SKA3-specific shRNA SKA3-specific shRNA (shSKA3-1: 5’-GGTGAAGATCCGGTTTGAACAA-3’; shSKA3-2: 5’-GACCAAACATGTCCCAGTGCAA-3‘) and PLK-1-specific shRNA constructs were synthesized by Gene Pharma Co (Shanghai, China). In addition, HA-SKA3, HA-PLK-1 and FLAG-SKA3 expression constructs were purchased from Genechem (Shanghai, China). Cells were transiently transfected with these plasmids, and high titer lentiviral preparations were prepared using Bca cells. All lentiviral transduction reactions were performed based on provided instructions.

Cell Proliferation and Colony Formation Assays

A Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) approach was used to quantify the proliferation of Bca cells in the present study. Briefly, cells were plated at $5 \times 10^3$ cells/well in 96-well plates, and CCK-8 assay solution (10 μl) was added to wells after an appropriate incubation period. Absorbance at 450 nm was then analyzed via microplate reader (Thermo Fisher Scientific, Shanghai, China). For anchorage-independent soft agar colony formation assays, cells were plated at $5 \times 10^3$ cells per well in 6-well plates and were cultured for an appropriate period of time, with colonies being counted once foci were clearly evident.

Co-Immunoprecipitation (co-IP) and Western Blotting

A modified lysis buffer (NaF, 50 mM; NaCl, 100 mM; Tris-HCl pH 7.5, 50 mM; Na3VO4, 1 mM; Na4P2O7, 30 mM; 0.5% NP-40 and PMSF, 0.5 mM; Sigma-Aldrich) containing a protease inhibitor cocktail (EDTA-free; Roche) was used to lyse Bca cells, with these lysates then being processed for co-IP and western blotting as in previous studies.14 Antibodies used for these 2 experimental approaches are listed in Table S2.

Statistical Analysis

SPSS v20.0 (SPSS Inc., IL, USA) was used for all statistical testing. Data are means ± standard deviations (SDs) and represent a minimum of 3 independent experiments. The Student’s t-test was used to compare the SKA3 expression between Bca and normal tissue samples (Figure 1A). One-way analysis of variance followed by Turkey’s post hoc test was used to compare 3 or more groups (Figure 1C and E, 2A and D, 3B and C). Repeated Measures Anova was used to compare values between groups for Figure 2B and C, 3E and D. F. P < 0.05 was the significance threshold. (ns P > 0.05; **P < 0.01; ***P < 0.05).

Results

SKA3 Is Upregulated in Bca

In order to begin to assess the expression dynamics of SKA3 in Bca, we first queried the GEPIA database (http://gepia.cancer-pku.cn/index.html), which revealed a significant increase in SKA3 expression in Bca tissues relative to normal tissue samples (Fold-change >2, P < 0.05; Figure 1A). The Oncomine database similarly indicated that SKA3 mRNA levels are higher in Bca tissues, thus confirming this result (Figure 1B). We then next directly measured the mRNA expression of SKA3 in primary human Bca tissues and paired adjacent normal tissue (ANT) samples. This analysis further confirmed that SKA3 expression is elevated in Bca samples relative to ANT samples (Figure 1C). This was also evident when SKA3 expression was assessed at the protein level via Western blotting (Figure 1D). Similarly, when we assessed the expression of SKA3 at the mRNA and protein levels in 5 Bca cell lines (BT549, T47D, MCF-7, SKBR3, and MDA-MB-231), we found it to be expressed at significantly higher levels in these cells relative to a control normal human breast cell line (MCF-
10A) (Figure 1E and F). These results thus highlighted that SKA3 upregulation is closely linked to the development of breast cancer.

**SKA3 Promotes Bca Cell Growth In Vitro**

In order to assess the functional importance of SKA3 in Bca cell lines, we next generated SKA3-specific shRNA constructs which were then used to knock down SKA3 expression in the MCF-7 and MDA-MB-231 Bca cell lines, as they had high levels of endogenous SKA3 expression (Figure 2A). We found that knockdown of SKA3 led to a significant reduction of MCF-7 and MDA-MB-231 cell proliferation in vitro (Figure 2B and C). Similarly, a colony formation confirmed that SKA3 knockdown suppressed colony formation in both of these cell lines (Figure 2D). We also found that SKA3 knockdown was associated with significant reductions in the expression of the pro-proliferative proteins Ki67 and PCNA (Figure 2E). These findings thus indicate that SKA3 plays a key role in regulating Bca cell proliferation.
SKA3 Enhances Bca Cell Growth At Least in Part Via Interacting With PLK-1

In order to further understand the functional role played by SKA3 in Bca cells, we next generated a STRING functional protein-protein interaction network in order to identify potential SKA3-interacting proteins (https://string-db.org/). This network revealed that the key serine/threonine kinase polo-like kinase-1 (PLK-1) was predicted to be associated with SKA3 (Figure 3A). We therefore hypothesized that SKA3 may interact with or regulate PLK-1 so as to control the growth of Bca cells. When SKA3 was knocked down in Bca cells, we found that PLK-1 protein levels rose, whereas corresponding mRNA levels were unchanged (Figure 3B), indicating that SKA3 regulated PLK-1 expression independent on mRNA levels. In contrast, knocking down PLK-1 had no impact on SKA3 protein or mRNA expression (Figure 3C), suggesting that SKA3 functions upstream of PLK-1. We next performed rescue experiments, which revealed that PLK-1 overexpression was sufficient to rescue cells from SKA3 knockdown-associated reductions in proliferation and viability as measured via CCK-8 assay (Figure 3D-F). Together, these findings thus indicated that SKA3 was able to regulate the proliferation of Bca cells at least in part via interacting with PLK-1.

SKA3 Enhances the Stability of the PLK-1 Protein

Given the above results, we next wanted to assess whether SKA3 was able to directly interact with PLK-1. To that end, we next co-expressed FLAG-tagged SKA3 and HA-PLK1 or HA-empty vector control constructs in Bca cells, with western blotting and co-IP assays then being used to confirm a direct interaction between SKA3 and PLK-1, meanwhile we co-expressed HA-PLK1 and FLAG- SKA3 or FLAG–empty vector control constructs in Bca cells, with western blotting and co-IP assays then being used to confirm a direct interaction between SKA3 and PLK-1 (Figure 4A). In addition, we found that when cells were treated with the proteasome inhibitor MG132, this was sufficient to rescue the SKA3 knockdown-induced reduction in PLK-1 protein stability, thus suggesting that SKA3 is able to prevent proteasome-mediated degradation of PLK-1 in Bca cells (Figure 4B). We then assessed whether SKA3 was able to alter PLK-1 protein stability by treating cells with cycloheximide (CHX) in order to disrupt de novo protein synthesis. This experiment revealed that SKA3 knockdown was associated with reduced PLK-1 stability in these Bca cell lines (Figure 4C). Together these findings thus suggested that SKA3 is able to promote the proliferation of Bca cells at least in part via binding to PLK-1 and enhancing its stability (Figure 4D).
While there have been many recent medical advances that have significantly improved the prognosis of Bca patients, patients with advanced disease still frequently suffer a poor prognosis owing to high rates of metastasis and recurrence. The molecular basis for such metastasis, however, remains incompletely understood, and more reliable biomarkers of Bca recurrence are needed in order to reliably predict which patients are at the highest risk so as to guide patient treatment and disease management. In addition, such biomarkers have the potential to serve as therapeutic targets in order to effectively treat this deadly disease. In the present study, we found that SKA3 expression is significantly increased in Bca, and that it plays a key role in facilitating Bca cell proliferation, making it crucial for the progression of this form of cancer. From a mechanistic perspective, we found that SKA3 was able to interact with PLK-1 and thereby stabilize it, with such stabilization being essential for the efficient proliferation and survival of Bca cells. These results thus strongly suggest that SKA3 may represent a viable therapeutic target for efforts aimed at suppressing Bca progression. In this study, we initially assessed the expression of SKA3 in a range of human Bca samples and cell lines. An initial analysis of the GEPIA and Oncomine databases revealed that clinical patient Bca samples typically exhibit SKA3 upregulation, and we confirmed this finding in our own independent set of Bca patient tissue samples. This same SKA3 upregulation was also evident in Bca cell lines relative to normal control cells, and when SKA3 was knocked down in these Bca cell lines we found this to be associated with a significant reduction in their in vitro proliferation. These results thus suggested that SKA3 is a key pro-proliferative factor in Bca that may be targeted in order to disrupt the growth of these tumor cells.

**Discussion**

![Figure 3](Image)

**Figure 3.** SKA3 enhances Bca cell growth at least in part via interacting with PLK-1. A The STRING database predicted PLK-1 to be closely associated with SKA3. B Levels of PLK-1 were assessed via Western blotting and qRT-PCR in SKA3-knockdown Bca cell lines. Data are means ± SEM; One-way analysis of variance followed by Turkey’s post hoc test. C Levels of SKA3 were assessed via Western blotting and qRT-PCR in PLK-1-knockdown Bca cell lines. Data are means ± SEM; One-way analysis of variance followed by Turkey’s post hoc test. D Confirmation of successful PLK-1 overexpression (HA-PLK-1) in Bca cell lines. Data are means ± SEM; One-way analysis of variance followed by Turkey’s post hoc test. E and F A CCK-8 assay revealed that SKA3 knockdown-associated reductions in Bca cell viability were reversed upon PLK-1 overexpression. Data are means ± SEM, repeated measures ANOVA. *shNT vs.shSKA3 -1 or shSKA3 -1 vs. shSKA3-1+HA-PLK-1.

In this study, we initially assessed the expression of SKA3 in a range of human Bca samples and cell lines. An initial analysis of the GEPIA and Oncomine databases revealed that clinical patient Bca samples typically exhibit SKA3 upregulation, and we confirmed this finding in our own independent set of Bca patient tissue samples. This same SKA3 upregulation was also evident in Bca cell lines relative to normal control cells, and when SKA3 was knocked down in these Bca cell lines we found this to be associated with a significant reduction in their in vitro proliferation. These results thus suggested that SKA3 is a key pro-proliferative factor in Bca that may be targeted in order to disrupt the growth of these tumor cells.
We next sought to identify the mechanisms whereby SKA3 is able to regulate the proliferation of Bca cells. An initial STRING interaction network suggested that SKA3 was closely associated with PLK-1, which is a key serine/threonine kinase\(^\text{23-27}\) that has been shown to play a role in the context of mitotic spindle function.\(^\text{28}\) The phosphorylation of heat shock transcription factor 1 (HSF1) by PLK-1 is a key step in the mitotic process.\(^\text{29}\) Previous reports suggest that PLK-1 downregulation can strongly disrupt the proliferation of oral squamous cell carcinoma cells.\(^\text{29}\) PLK-1 knockdown similarly has been shown to interfere with tumor cell migration and invasion in other contexts,\(^\text{30}\) and as such PLK-1 has been proposed as a possible therapeutic target in a range of cancers. In the present study, we determined that SKA3 was able to directly bind to PLK-1 and to thereby stabilize it, protecting it from proteasomal degradation. Through rescue experiments, we were able to confirm that SKA3 knockdown-associated reductions in Bca cell proliferation could be rescued by PLK-1 overexpression. This thus suggests that SKA3 is able to enhance Bca cell proliferation primarily via regulating PLK-1 stability. This stabilization may result from either of the following mechanisms: 1) The interaction between SKA3 and PLK-1 results in the degradation of PLK-1 via the lysosome-ubiquitination pathway, or 2) SKA3 promotes PLK-1 dimer formation, thereby leading to stabilization of this protein. There are still some deficiencies in our research, which is worth further study in the future, such as 1): absence of in vivo studies to confirm the effect of SKA3 on breast cancer cell proliferation; 2) lacking of Rescue experiment such as SKA3 overexpression on cell proliferation and colony formation. In future studies, we will seek to more fully clarify the molecular mechanisms whereby SKA3 is able to stabilize PLK-1 in order to promote Bca cell proliferation.

Figure 4. SKA3 enhances the stability of the PLK-1 protein. A Bca cells were transfected with FLAG-SKA3 and HA-PLK-1 or HA-empty vector constructs, as indicated, and cell lysates were then subjected to anti-FLAG or anti–HA antibody-mediated immunoprecipitation and Western blotting. B SKA3 knockdown-associated PLK-1 degradation was disrupted when cells were treated with the MG132 proteasome inhibitor. C SKA3 knockdown is associated with reduced PLK-1 protein stability in CHX-treated cells. D A proposed mechanism whereby the SKA3-PLK-1 axis regulated Bca cell growth.
In summary, our results suggest that SKA3 can promote the growth and proliferation of Bca cells at least in part via binding to PLK-1 and enhancing its stability. This SKA3-PLK-1 axis may therefore be a viable therapeutic target for the treatment of Bca.

Declaration of Conflicting Interests
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References
1. Singh A, Greninger P, Rhodes D, et al. A gene expression signature associated with “K-Ras addiction” reveals regulators of EMT and tumor cell survival. Cancer Cell. 2009;15(6):489-500. doi: 10.1016/j.ccc.2009.03.022
2. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136(5):E359-E386. doi: 10.1002/ijc.29210
3. DeSantis CE, Ma J, Gaudet MM, et al. Breast cancer statistics, 2019. CA Cancer J Clin. 2019;69(6):438-451. doi:10.3322/caac.21583
4. Schnitt SJ. The diagnosis and management of pre-invasive breast disease: flat epithelial atypia—classification, pathologic features and clinical significance. Breast Cancer Res. 2003;5(5):263-268. doi:10.1186/bcr625
5. Jeyaprakash AA, Santamaria A, Jayachandran U, et al. Structural and functional organization of the Ska complex, a key component of the kinetochore-microtubule interface. Mol Cell. 2012;46(3):274-286. doi:10.1016/j.molcel.2012.03.005
6. Sivakumar S, Daum JR, Tipton AR, Rankin S, Gorbsky GJ. The spindle and kinetochore-associated (Ska) complex enhances binding of the anaphase-promoting complex/cyclosome (APC/C) to chromosomes and promotes mitotic exit. Mol Biol Cell. 2014;25(5):594-605. doi:10.1091/mbc.E13-07-0421
7. Daum JR, Wren JD, Daniel JJ, et al. Ska3 is required for spindle checkpoint silencing and the maintenance of chromosome cohesion in mitosis. Curr Biol. 2009;19(17):1467-1472. doi:10.1016/j.cub.2009.07.017
8. Zhang QH, Qi ST, Wang ZB, et al. Localization and function of the Ska complex during mouse oocyte meiotic maturation. Cell Cycle. 2012;11(5):909-916. doi:10.4161/cc.11.5.19384
9. Jiao X, Hooper SD, Djureinovic T, et al. Gene rearrangements in hormone receptor negative breast cancers revealed by mate pair sequencing. BMC Genomics. 2013;14(1):165. doi:10.1186/1471-2164-14-165
10. Hu R, Wang MQ, Niu WB, et al. SKA3 promotes cell proliferation and migration in cervical cancer by activating the PI3K/Akt signaling pathway. Cancer Cell Int. 2018;18(1):1-6. doi:10.1186/s12935-018-0670-4
11. Lee M, Williams KA, Hu Y, et al. GNL3 and SKA3 are novel prostate cancer metastasis susceptibility genes. Clin Exp Metastasis. 2015;32(8):769-782. doi:10.1007/s10585-015-9745-y
12. Zheng H, Bi FR, Yang Y, et al. Downregulation of miR-196-5p induced by hypoxia drives tumorigenesis and metastasis in hepatocellular carcinoma. Horm Cancer. 2019;10(4-6):177-189. doi:10.1007/s12672-019-00370-5
13. Zheng H, Yang Y, Wang MC, et al. Low CDX1 expression predicts a poor prognosis for hepatocellular carcinoma patients after hepatectomy. Surg Oncol. 2016;25(3):171-177. doi:10.1016/j.suronc.2016.05.026
14. Zheng H, Yang Y, Hong YG, et al. Tropomodulin 3 modulates EGFR-PI3K-AKT signaling to drive hepatocellular carcinoma metastasis. Mol Carcinog. 2019;58(10):1897-1907. doi:10.1002/mc.23083
15. De Blasio C, Zonfrilli A, Franchitto M, et al. PLK1 targets NOTCH1 during DNA damage and mitotic progression. J Biol Chem. 2019;294(47):17941-17950. doi:10.1074/jbc.RA119.009881
16. Liu Z, Sun Q, Wang X. PLK1, a potential target for cancer therapy. Transl Oncol. 2017;10(1):22-32. doi:10.1016/j.tranon.2016.10.003
17. Gutteridge RE, Ndiaye MA, Liu X, Ahmad N. Plk1 inhibitors in cancer therapy: from laboratory to clinics. Mol Cancer Ther. 2016;15(7):1427-1435. doi:10.1158/1535-7163.MCT-15-0897
18. Combès G, Alharbi I, Braga LG, Elowe S. Playing polo during mitosis: PLK1 takes the lead. Oncogene. 2017;36(34):4819-4827. doi:10.1038/onc.2017.113
19. Anastasiadi Z, Lianos GD, Ignatiadou E, Harissis HV, Mitsis M. Breast cancer in young women: an overview. Updates Surg. 2017;69(3):313-317. doi:10.1007/s13304-017-0424-1
20. Bonilla JM, Tabanera MT, Mendoza LR. Breast cancer in the 21st century: from early detection to new therapies. Surg Oncol Clin N Am. 2020;29(1):51-62. doi:10.1016/j.soc.2019.08.004
21. Greenwalt I, Zaza N, Das S, Li BD. Precision medicine and targeted therapies in breast cancer. Surg Oncol. 2017;26(1):4-15. doi:10.3109/09540261.2016.852971
22. Ahonen LJ, Kallio MJ, Daum JR, et al. Polo-like kinase 1 creates the tension-sensing 3F3/2 phospho-switch that stabilizes kinetochore-microtubule attachments. J Cell Biol. 2012;199(2):285-301. doi:10.1083/jcb.201203091
26. Kumar S, Sharma G, Chakraborty C, Sharma AR, Kim J. Regulatory functional territory of PLK-1 and their substrates beyond mitosis. *Oncotarget*. 2017;8(23):37942. doi:10.18632/oncotarget.16290
27. Kumar S, Kim J. PLK-1 Targeted inhibitors and their potential against tumorigenesis. *Biomed Res Int*. 2015;2015:705745. doi:10.1155/2015/705745
28. Pidoux AL, LeDizet M, Cande WZ. Fission yeast pkll is a kinesin-related protein involved in mitotic spindle function. *Mol Biol Cell*. 1996;7(10):1639-1655. doi:10.1091/mbc.7.10.1639
29. Kim SA, Kwon SM, Yoon JH, Ahn SG. The antitumor effect of PLK1 and HSF1 double knockdown on human oral carcinoma cells. *Int J Oncol*. 2010;36(4):867-872. doi:10.3892/ijo_00000564
30. Nakamura Y, Fujimoto M, Fukushima S, et al. Heat shock factor 1 is required for migration and invasion of human melanoma in vitro and in vivo. *Cancer Lett*. 2014;354(2):329-335. doi:10.1016/j.canlet.2014.08.029