

## GSK-3 $\beta$ Promotes Cell Migration and Inhibits Autophagy by Mediating the AMPK Pathway in Breast Cancer

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GSK-3 is a versatile protein kinase participating in many reactions. Currently, there is insufficient understanding of its influence on breast cancer (BC). In order to explore its influence on migration and invasion in BC, we investigated its expression in BC cell lines using qRT-PCR and Western blot (WB). Immunohistochemistry (IHC) was used to examine the potential of GSK-3 to predict clinical outcome in BC patients. GSK-3 knockdown was achieved using an shRNA plasmid vector in T47D cells. Our research explored the biological reactions and downstream pathways involved. We found excessive GSK-3 expression in BC tissues, which was correlated with worse clinicopathological parameters and clinical outcome. Progression of BC was suppressed by GSK-3 knockdown. Furthermore, suppression of GSK-3 function led to a noticeable decrease in ATP generation, and this was associated with stimulation of AMP-activated protein kinase (AMPK) in T47D cells. Activation of AMPK, a typical sign of autophagy stimulation, was triggered after suppression of GSK-3 function, in parallel with increased generation of LC3 II. Our findings therefore indicate that GSK-3 participates in regulation of migration as well as stimulation of autophagy via mediating activation of the AMPK pathway. This suggests that GSK-3 has potential as a predictor of clinical outcome and as a target for BC therapy.

**Key words: GSK-3 $\beta$ ; Migration; Autophagy; AMPK; Breast cancer (BC)**

### INTRODUCTION

Breast cancer (BC) is one of the most prevalent malignancies. Worldwide, it is the second largest contributor to mortality-linked malignancy in women<sup>1</sup>. It is particularly problematic as an early malignancy in Asia, where it displays a comparatively young median age upon diagnosis in comparison with the West<sup>2,3</sup>. Research into the generation of BC helps determine innovative therapeutic targets and is promising to improve methods of examination, diagnosis, and early treatment<sup>4-7</sup>.

Autophagy is triggered after stimulation of the ULK1/2 (Unc-51-like kinase 1/2) complex, which regulates autophagosome generation in mammals<sup>8</sup>. As the dominant energy sensor, the function of AMP-activated protein kinase (AMPK) is promoted by increased concentration of AMP and decreased ATP concentration, upon nutrient or metabolic stress, including ischemia<sup>9,10</sup>. As a heterotrimeric complex, AMPK consists of a catalytic as well as two regulatory / subunits, which are encoded by different genes<sup>11</sup>. Phosphorylation of AMPK on

threonine 172 (pT172) is crucial to complete stimulation<sup>12</sup>. As a persistently stimulated versatile kinase, glycogen synthase kinase-3 (GSK-3) participates in various reactions determining cellular fate<sup>13</sup>. Two isoforms ( and ) of GSK-3 exist in mammals. Previous work has indicated that suppression of GSK-3 function stimulates autophagic cellular reactions without serum, and this has also been seen in vivo in ischemic mice<sup>14,15</sup>. Nevertheless, understanding of the etiology of autophagy triggered via GSK-3 suppression is insufficient.

This study aimed to explore GSK-3 expression in clinical BC specimens and the relationship between expression of GSK-3 and prognosis in patients suffering from BC.

### MATERIALS AND METHODS

#### Cell Culture

Human BC cells (T47D) were bought from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells underwent cultivation in DMEM

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media (Gibco, Gaithersburg, MD, USA) including 10% newborn bovine serum deactivated by heat, 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA).

#### *Transduction and Clone Selection*

We bought shRNA targeting GSK-3 generated in pLKO.1-puro from Santa Cruz Biotechnology (Dallas, TX, USA). Steady knockdown (KD) clones were created. Immunoblotting of GSK-3 was applied to verification of the clones, which received steady transduction.

#### *Transwell Assay*

Approximately  $1 \times 10^5$  cells were suspended in 200 µl of media without serum, before being added to chambers on the top of a Transwell (8 µmol for 24-well plates). Full media (600 µl) were supplemented to the chambers below. After 24 h, cells underwent fixing with formalin and staining with 0.1% crystal violet.

#### *In Vitro Proliferation and Colony Generation Assay*

We used 96-well plates to plant the cells at a density of 500 cells per well. Cells underwent overnight cultivation and were then supplemented with cell counting kit-8 (CCK-8) (Promega, Madison, WI, USA). They were then incubated for 2 h at 37°C. Cell quantification was performed with a microplate spectrophotometer (BioTek, Winooski, VT, USA) at an absorbance of 450 nm. The reaction was allowed to continue for 5 days, and cellular growth curves were plotted. Procedures were carried out in triplicate. In order to investigate effects over longer time periods, 1,000 cells were planted in six-well plates with complete media and incubated at 37°C for 14 days. Cells underwent staining with 0.1% crystal violet.

#### *Immunohistochemistry (IHC) and Tissue Microarrays (TMAs)*

TMAs were provided by Shanghai Biochip Co. Ltd. (Shanghai, P.R. China). Histopathological diagnosis was made in conformity with World Health Organization (WHO) criteria. Overall survival (OS) referred to the period between operation and mortality. Data from deceased patients were examined on the day of mortality, while data from living patients were examined at the end of the follow-up period.

Paraffin sections were taken for IHC, which was conducted on slides with thickness of 4 µm. Malignancy was verified with the help of hematoxylin and eosin (H&E) staining. Slices underwent overnight incubation at 4°C with primary antibodies following antigen retrieval. They were then incubated for 30 min with secondary antibodies conjugated with HRP at 37°C. Diaminobenzidine was applied for visualization.

#### *Assessment of IHC Variables*

GSK-3 protein was found in the cytoplasm as well as the nuclei of cells and was stained as brown granules. GSK-3 expression was evaluated with a 4-point scale (0–4) based on quantity of positive cells. The scores for the proportion of positive cells were categorized as follows: <5% (0), 5%–25% (1), 25%–50% (2), 50%–75% (3), or >75% (4). Staining intensity was scored as follows: no staining (0), light brown (1), brown (2), and dark brown (3). A threshold score of 5 was chosen to delineate high from low expression. Patients with high or low expression were designated GSK-3<sup>high</sup> or GSK-3<sup>low</sup>, respectively.

#### *Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

We isolated total RNA using TRIzol reagent. An equivalent quantity of RNA underwent RT to obtain cDNA (Applied Biosystems, Foster City, CA, USA).  $\beta$ -Actin served as internal reference. An ABI Prism 7900HT system was used to quantify transcription. RT underwent assessment according to Ct values, which were normalized to  $\beta$ -actin using the comparative Ct method.

#### *Western Blot Assay*

Western blot (WB) was carried out as previously described<sup>16,17</sup>, with antibodies for GSK-3,  $\beta$ -actin (Santa Cruz Biotechnology), N-cadherin, E-cadherin, vimentin, ATG5, LC3 I/II, p-AMPK, or AMPK (Cell Signaling Technology, Danvers, MA, USA).

#### *ATP-Dependent Luciferase Assay, AMP/ATP Detection, and Glycolysis Assay Using High-Performance Liquid Chromatography (HPLC)*

Concentration of ATP in treated cells was measured using the ATPLite assay kit by PerkinElmer (Waltham, MA, USA). Cultivation media were acquired in preparation for detection of glycolysis function using a cell-based assay kit for quantifying L-lactate, the terminal product generated by cellular glycolysis. Prior to HPLC, cells were washed and resuspended in phosphate-buffered saline (PBS). Nucleotides (ATP and AMP) were isolated via fast lysing the cells with 0.05 M KOH solution, which was adjusted to pH 6 and was used to conduct reverse phase chromatography. The mobile phase (pH 6) included 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 0.008 M tetrabutylammonium hydrogen sulfate, and acetonitrile (containing 30% solvent B and 2% solvent A). Empower II software (Waters, Milford, MA, USA) was used for analyses and instrument control.

#### *In Vivo Generation of Malignancy and Metastasis*

A total of 10 female Balb/c nude mice aged from 4 to 6 weeks were bought from Shanghai SLAC Laboratory

Animal Co., Ltd. (Shanghai, P.R. China). Their body weights ranged from 15 to 20 g. These mice were randomly distributed into two groups (five mice in each group). One million T47D/shGSK-3 and T47D/vector cells in 100  $\mu$ l of PBS were administered by subcutaneous inoculation. Malignant nodules were examined weekly once their length exceeded 2 mm. Malignancy volume was determined using the following formula: volume=(width<sup>2</sup> length)/2. Procedures related to animals were approved by Jinan University.

#### Statistical Analysis

Results are presented in the form of mean $\pm$ standard deviation (SD). SPSS 19.0 for Macintosh (SPSS Inc., Chicago, IL, USA) was used for analyses. Significance was inferred with a value of two-tailed  $p < 0.05$ .

## RESULTS

### Expression of GSK-3 $\beta$ Was Correlated With Worse Clinical Outcome and Clinicopathological Parameters in BC Patients

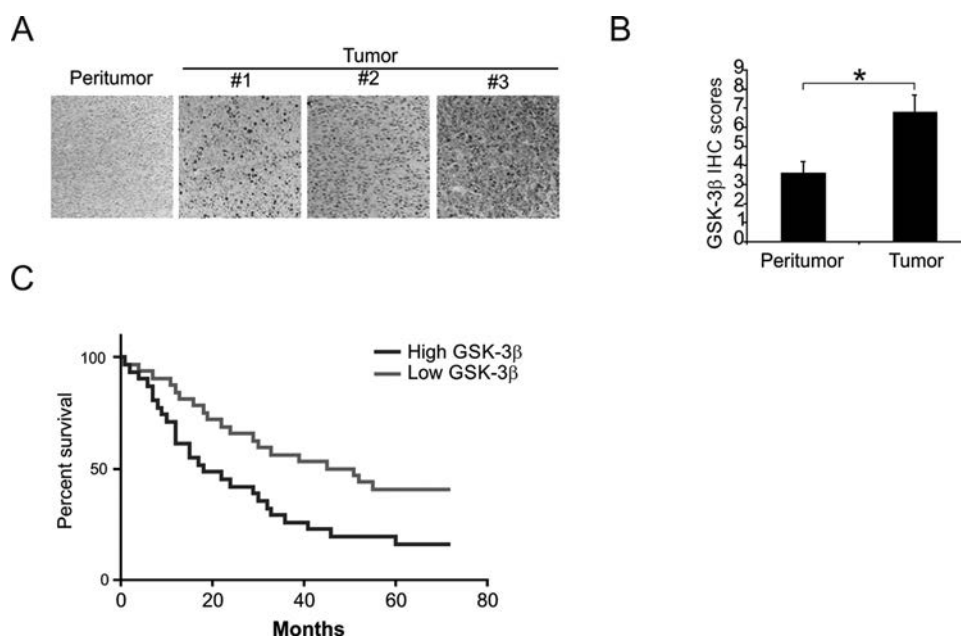
Detection of GSK-3 translation by IHC was carried out in 64 human BC tissues and paired non-malignant tissues. A typical IHC outcome is presented in Figure 1A. We found that GSK-3 protein displayed elevated IHC scores in BC specimens in comparison with surrounding normal tissues (Fig. 1B).

Kaplan–Meier analysis was used to examine the correlation between GSK-3 expression and prognosis. We discovered that increased GSK-3 expression was correlated with shortened OS. Furthermore, survival of GSK-3<sup>low</sup> patients was significantly higher than that of GSK-3<sup>high</sup> patients (Fig. 1C).

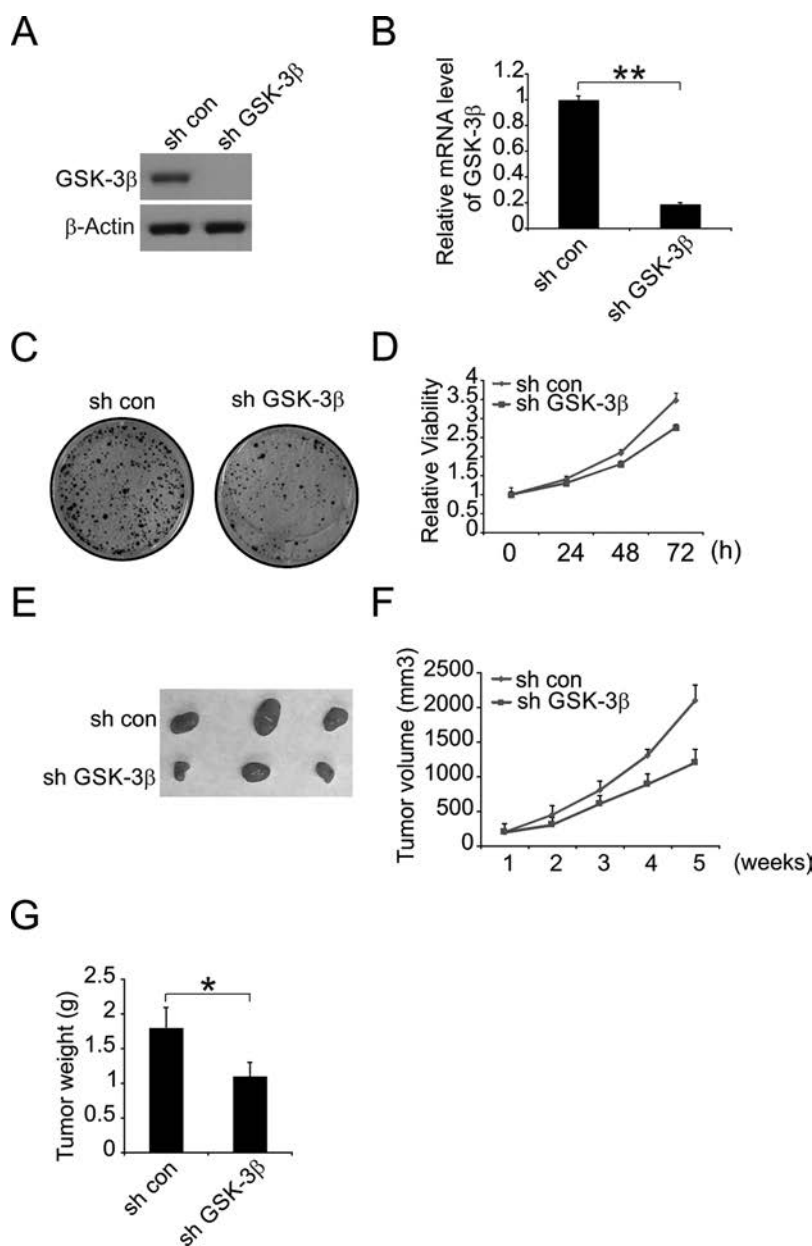
### GSK-3 $\beta$ KD Suppressed BC Progression

In order to investigate the effects of GSK-3 activity, we used shRNA to produce GSK-3-KD cells. GSK-3 shRNA supplement brought about a noticeable decline in translation as well as transcription of GSK-3 (Fig. 2A and B).

We initially investigated the influence of GSK-3 downregulation on cell proliferation in the T47D cell line. Colony generation assays showed greater quantities of T47D-NC clones in comparison with the T47D/shGSK-3 group. It was also discovered that the volume of clones generated in the T47D/shGSK-3 group was suppressed compared with that in the control group (Fig. 2C). Our proliferation assay showed inhibition of T47D proliferation following GSK-3 shRNA supplement (Fig. 2D). These results therefore indicate that GSK-3 enhances proliferation of BC cells. In vivo experiments with T47D cell xenografts in mice showed that GSK-3 KD noticeably hindered generation of malignancy (Fig. 2E and F). Similar to malignancy volume, malignancy weights of T47D-NC xenografts were



**Figure 1.** Expression of glycogen synthase kinase-3 (GSK-3) in breast cancer (BC) tissues. (A) Representative images of GSK-3 staining in peritumoral breast tissues and tumor tissues. (B) Immunohistochemistry (IHC) scores of GSK-3 expression levels in human breast cancer tissues and peritumoral breast tissues. (C) Kaplan–Meier analysis of overall survival for GSK-3 protein expression. \* $p < 0.05$ .

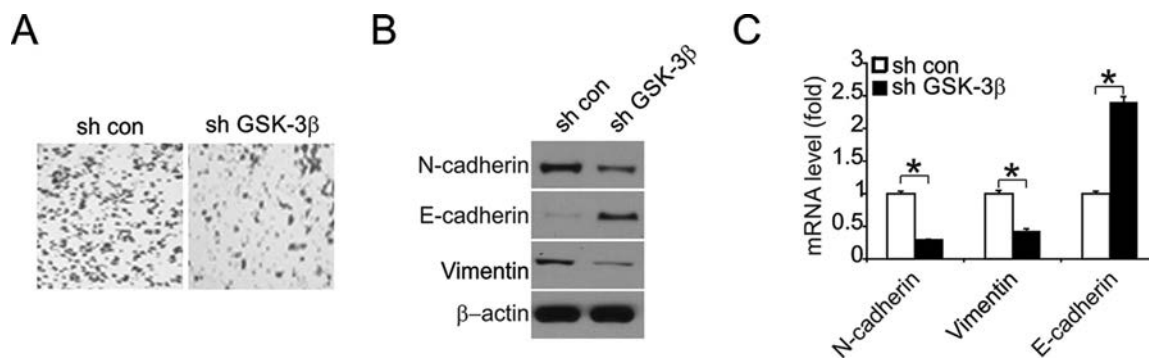


**Figure 2.** Effect of GSK-3 knockdown on T47D cell growth. (A, B) GSK-3 expression in T47D was modified by shRNA interference and verified with Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR). (C) Knockdown of GSK-3 in T47D attenuated colony formation. (D) Knockdown of GSK-3 in T47D attenuated cell proliferation, based on cell counting kit-8 (CCK-8) assay. (E) Tumor xenograft formed by implanted T47D cells with different expression levels of PKM2. (F, G) Tumor volume and weights of the xenograft. \* $p < 0.05$ , \*\* $p < 0.01$ .

noticeably increased in comparison with those of T47D/shGSK-3 cells (Fig. 2G).

Furthermore, *in vitro* migration assays showed that quantity of migrated cells was clearly higher for T47D-NC than for T47D/shGSK-3 (Fig. 3A). As it is widely accepted that epithelial–mesenchymal transition (EMT) is the transition from polarized epithelial cancer cells to motile and contractile mesenchymal cells during the progression and metastasis of

malignancy, we aimed to investigate the influence of GSK-3 on EMT development. Using WB and quantitative RT-PCR (qRT-PCR), we evaluated the expression of representative biomarkers linked with EMT in T47D cells. Following GSK-3 shRNA supplement, expression of E-cadherin was noticeably promoted. However, expression of the mesenchymal biomarkers N-cadherin and vimentin was remarkably suppressed (Fig. 3B and C).



**Figure 3.** GSK-3 knockdown inhibits the migration of T47D cells. (A) Silencing of GSK-3 expression led to a lower migration rate in T47D cells. (B, C) Expression levels of N-cadherin, E-cadherin, and vimentin in stably transfected T47D cells were analyzed by Western blot and qRT-PCR. \* $p < 0.05$ .

*GSK-3β Downregulation Suppressed Autophagy in BC*

Previous research has shown that GSK-3 inhibits autophagy and enhances radiosensitivity in non-small cell lung cancer<sup>18</sup>. Moreover, KD of GSK-3 increases basal autophagy in nutrient-laden human aortic endothelial cells<sup>19</sup>. We therefore examined whether GSK-3 KD affected autophagy of BC cells. WB was carried out to evaluate expression of the autophagy markers ATG5 and LC3 I/II in T47D cells with and without GSK-3 KD. GSK-3 KD promoted expression of ATG5 and LC3 I/II conversion (Fig. 4A). However, addition of 200 μmol of 3-MA (an autophagy inhibitor) for 24 h significantly inhibited GSK-3 -KD-stimulated excessive expression of ATG5 and LC3 I/II conversion (Fig. 4A–C).

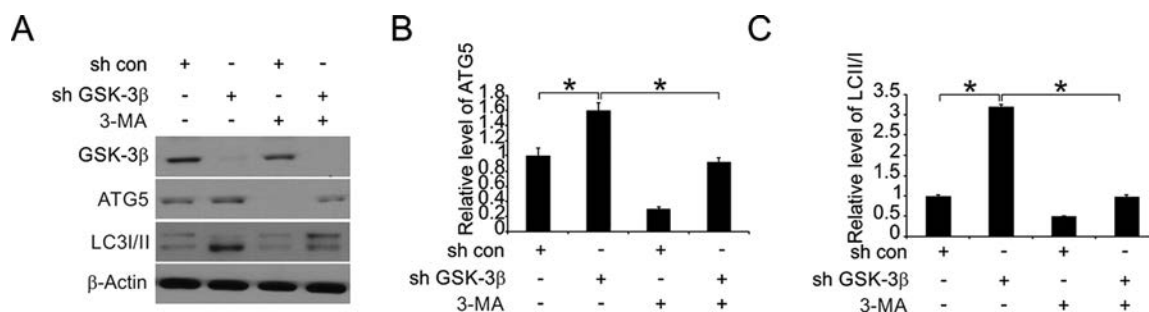
*GSK-3β Suppression Decreased ATP Generation*

Because energy crisis can participate in the stimulation of autophagy, we next checked whether GSK-3 suppression affected ATP concentration. T47D cells were starved of serum for 24 h and then supplemented with GSK-3 inhibitor. ATP concentration moderately decreased with time subsequent to serum deprivation, based on an

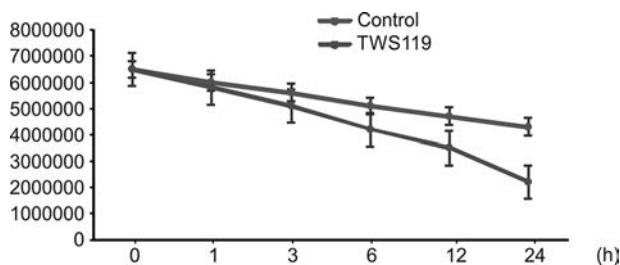
ATP-dependent luciferase assay (Fig. 5). Treatment with GSK-3 inhibitor TWS119 brought about a noticeable reduction of ATP concentration (Fig. 5). This indicated that GSK-3 suppression suppressed ATP generation after starvation of serum.

*GSK-3β Inhibition Promotes AMPK Activation*

As the dominant sensor of energy, AMPK is stimulated immediately after energy crisis. As GSK-3 suppression impaired ATP generation, we asked whether AMPK was activated parallel to autophagy stimulated by GSK-3 suppression subsequent to starvation of serum. We used TWS119 supplementation to investigate AMPK activation via assessment of its phosphorylation. Four hours after TWS119 supplementation, AMPK pT172 concentration was noticeably promoted in comparison with solvent supplement (Fig. 6A). Findings were similar when GSK-3 KD was achieved using specific siRNA (Fig. 6B). These findings suggested that generation of LC3 II was triggered via serum deprivation. Its processing was promoted subsequent to GSK-3 suppression, in parallel with the stimulation of AMPK.



**Figure 4.** GSK-3 regulates autophagy. (A) Western blotting analysis of GSK-3, ATG5, and LC3 in T47D cells with knockdown of GSK-3 in the presence of 200 μM 3-MA for 24 h. (B, C) Relative expression of ATG5 and LC3 II/I based on quantitative Western blot analysis results obtained using densitometric analysis. \* $p < 0.05$ .



**Figure 5.** GSK-3 inhibition reduces cellular ATP production. T47D cells were treated with solvent (DMSO) or TWS119 (5  $\mu$ M) for the indicated time periods. At the end of the experiments, cells were harvested, rinsed in cold phosphate-buffered saline (PBS), and then subjected to ATP-dependent luciferase assay using the ATPLite™ kit.

## DISCUSSION

GSK-3, a serine/threonine protein kinase, is a complex regulator of numerous cellular functions. GSK-3 is a unique kinase that is constitutively active in resting and nonstimulated cells<sup>20</sup>. GSK-3 has been implicated in a wide range of diseases including neurodegeneration, inflammation and fibrosis, noninsulin-dependent diabetes mellitus, and cancer<sup>21</sup>.

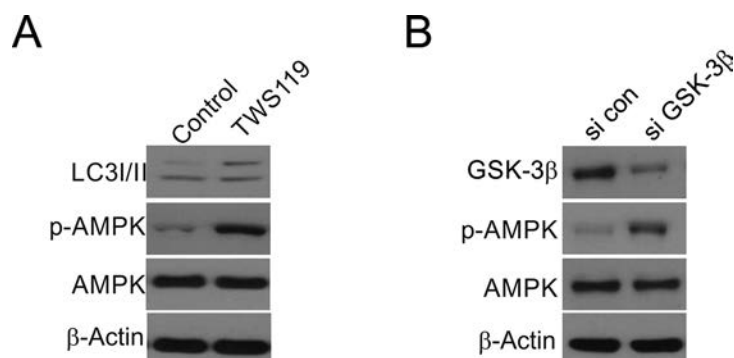
Elevated GSK-3 expression in BC tissues has been shown previously<sup>22</sup>. We investigated effects of GSK-3 KD using *in vitro* and *in vivo* assays. Our findings revealed that GSK-3 shRNA supplement suppressed proliferation and migration of BC cells *in vitro* and limited metastasis as well as growth of malignancy *in vivo*. Expression of GSK-3 in BC tissues was negatively correlated with OS of patients. We therefore conclude that GSK-3 can be an essential oncogene crucial to modulation of malignancy growth and metastasis in BC.

The influence of GSK-3 on metabolism of malignancy has been shown in previous research, but knowledge

of its metabolic activities remains unclear<sup>17,23,24</sup>. Emerging evidence reveals elevated expression of GSK-3 in multiple malignancies<sup>25–27</sup>. Nevertheless, understanding of the link between GSK-3 and BC is insufficient. Autophagy is initiated after activation of the ULK1/2 complex that controls the nucleation step of autophagosome formation in mammalian cells<sup>28</sup>. As the major energy sensor, the enzyme AMPK activity increases once cellular AMP levels elevate or ATP levels decrease under nutrient/metabolic stress such as ischemia<sup>29</sup>. AMPK is a heterotrimeric complex with one catalytic and two regulatory  $\alpha$  /  $\beta$  subunits encoded by distinct genes, and AMPK phosphorylation on threonine 172 (pT172) is required for full activation<sup>30</sup>. Several protein kinases are capable of phosphorylating this site, including the ubiquitously expressed liver kinase B-1 (LKB1) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase (CaMKK)<sup>31</sup>. Currently, it is conceivable that among several cellular signal pathways that regulate autophagy response under physiological or pathological conditions, the LKB1–AMPK pathway was demonstrated to directly modulate autophagy activity by suppressing the protein translation activator mammalian target of rapamycin (mTOR) pathway and by activating autophagy initiator ULK1<sup>32</sup>.

We showed that suppression of GSK-3 function brought about clear suppression of ATP generation and stimulation of AMPK. These results provide insight into our finding that GSK-3 suppression stimulates a strong autophagic reaction and subsequent necrotic apoptosis without serum. It has been regarded as a promising therapeutic target for neuronal degenerative diseases and type II diabetes, as well as malignancies linked with metabolic reprogramming and autophagy.

In summary, our results show that GSK-3 KD is able to suppress proliferation as well as migration of BC cells *in vivo* and *in vitro*. It can also enhance autophagy



**Figure 6.** GSK-3 inhibition leads to AMPK activation. (A) Cells were treated with 5  $\mu$ M TWS119 for 24 h before harvesting. Indicated protein level was analyzed by Western blotting. (B) T47D cells seeded in six-well plates were transfected with control siRNA or siRNA for GSK-3 for 24 h in serum-supplied media [10% fetal bovine serum (FBS)]. Indicated protein level was analyzed by Western blotting.

mediated by the AMPK pathway. Moreover, expression of GSK-3 could serve as an innovative predictor of clinical outcome in BC patients who underwent curative operation. In general, our research throws light upon the influence of GSK-3 on BC development, indicating that GSK-3 can be a promising target to treat BC.

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