SOX2 enhances cell survival and induces resistance to apoptosis under serum starvation conditions through the AKT/GSK-3β signaling pathway in esophageal squamous cell carcinoma

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Abstract. The human *SOX2* gene was recently identified as a novel major oncogene, recurrently amplified and overexpressed in esophageal squamous cell carcinoma (ESCC). However, the role and molecular mechanism of SOX2 in the carcinogenesis of ESCC remain to be elucidated. The present study investigated the effect of SOX2 on ESCC cell survival and resistance to apoptosis under serum starvation conditions. An adenoviral vector-mediated expression system and RNA interference were used to study the effect of SOX2. The present results revealed that SOX2 promoted ESCC cell survival and enhanced resistance to apoptosis under serum starvation conditions, but not in culture conditions with serum. Mechanistically, SOX2 increased the expression levels of phosphorylated AKT and glycogen synthase kinase-3β (GSK-3β), a downstream factor of AKT, under serum starvation conditions, leading to the promotion of ESCC cell survival. Additionally, SOX2 activated AKT through the PTEN/PI3K/phosphoinositide-dependent protein kinase 1 and mammalian target of rapamycin complex 2 signaling pathways. Therefore, SOX2 may facilitate the survival of ESCC cells under poor nutrient conditions by activating the AKT/GSK-3β signaling pathway.

Introduction

Worldwide, esophageal cancer is the fifth and ninth leading cause of cancer mortality in males and females, respectively (1). Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype in Asia, Africa, and South America (2).

SOX2, a member of the SOX family of transcription factors (3), is critical for the maintenance of pluripotency and self-renewal of embryonic stem cells (ESCs) (4). In the esophagus, SOX2 plays an important role in differentiation and morphogenesis (5).

Our previous study (6), together with the study of others (7), showed that the human *SOX2* gene, located at chromosome 3q26.3, is frequently amplified in ESCC, and that the expression of the *SOX2* mRNA and the encoded protein is elevated in most primary ESCCs. *SOX2* recently was identified as a novel major oncogene, recurrently amplified and activated in squamous cell carcinomas of multiple organs and tissues, including the esophagus (6,7), lung (8), and skin (9). Furthermore, we showed that SOX2 promotes ESCC cell proliferation *in vitro* and *in vivo* via the AKT/mammalian target of rapamycin complex 1 (mTORC1) signaling pathway (10). However, the role of SOX2 in the carcinogenesis of ESCC is not fully understood. Moreover, the mechanism by which SOX2 activates AKT remains unknown.

AKT, a serine/threonine kinase, regulates many biological processes, such as cell survival, proliferation, and growth (11,12). AKT is activated by extracellular signals through a process mediated by phosphatidylinositol 3-kinase (PI3K) activation. PI3K produces phosphatidylinositol (3,4,5)-trisphosphate, which recruits two protein kinases, AKT and phosphoinositide-dependent protein kinase 1 (PDK1), to the plasma membrane. PDK1 phosphorylates AKT at T308, increasing AKT kinase activity. In turn, AKT phosphorylates mitogen-activated protein kinase (MAPK) associated protein 1 (also known as SAPK interacting protein 1; SIN1), a key component of mTORC2, enhancing mTORC2 kinase activity and leading to phosphorylation of AKT at S473 by mTORC2, thereby catalyzing full activation of AKT (13). Conversely, AKT activity is negatively regulated by phosphatase and tensin homologue (PTEN).

Despite having an abundance of blood vessels, tumors usually are hypoxic and nutrient-deprived because of dysfunction of their vessels. Since AKT is involved in enhancing cell survival, we

hypothesized that SOX2, through AKT activation, may promote survival of ESCC cells under poor nutrient conditions.

In the present study, we sought to investigate the effect of SOX2 on ESCC cell survival and resistance to apoptosis under serum starvation conditions. Furthermore, we examined the underlying molecular mechanisms mediating SOX2's effects, focusing on the role of AKT.

Materials and methods

Reagents and antibodies. Antibodies against SOX2 (#3579), total AKT (#9272), phospho-AKT (p-AKT) (T308) (#13038), p-AKT (S473) (#4060), total ERK (extracellular signal-regulated kinase) (#4695), p-ERK (#4370), p-GSK-3β (glycogen synthase kinase-3β) (#5558), total GSK-3β (#9832), and PARP (poly (ADP-ribose) polymerase) (#9542) were purchased from Cell Signaling Technology. The antibody against β-actin (#A1978) was purchased from Sigma-Aldrich Japan; Merck KGaA. Doxorubicin was obtained from Toronto Research Chemicals. MK2206 (S1078), AR-A014418 (S7435), GSK2334470 (S7087), and AZD8055 (S15555) were obtained from Selleck Chemicals. LY294002 (154447-36-6) was obtained from Cayman Chemical Company.

Cell culture. Four ESCC cell lines (KYSE30, KYSE140, TE4, and TE6) were obtained from the American Type Culture Collection (ATCC) or the JCRB Cell Bank. All cell lines were cultured at 37˚C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, except for KYSE140 cells, which was maintained in Ham's F-12 medium supplemented with 5% fetal calf serum.

Adenovirus vector. Recombinant adenovirus vectors expressing SOX2 or control green fluorescent protein (GFP), which we here refer to as Ad-SOX2 and Ad-control, respectively, were obtained from ViGene. Cells were infected with Ad-SOX2 or Ad-control at a multiplicity of infection (MOI) of 50.

Immunoblotting. Immunoblotting was performed as described previously (14). Dilutions were as follows: 1:1,000 for antibodies against SOX2, AKT, p-AKT (T308), p-AKT (S473), ERK, p-ERK, PARP, p-GSK3β, and PARP; and 1:5,000 for the antibody against β-actin. For immunodetection, anti-rabbit IgG (#7074) or anti-mouse IgG (#7076) (Cell Signaling Technology, Inc.) was used as the secondary antibody at a dilution of 1:5,000 or 1:10,000, respectively. Antibody binding was detected using the ECL system (Amersham Biosciences). Densitometric analysis was performed using ImageJ software (version 1.53; National Institutes of Health).

RNA interference. Two small interfering RNAs (siRNAs) targeting *SOX2* (#1 and #2; ID s13294 and s13296, respectively; Ambion), as well as a control (non-silencing) siRNA, were delivered into cells using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Cell viability assay. Cell viability was determined using the WST-8 assay (Cell Counting Kit-8; Dojindo) according to the manufacturer's instructions.

Apoptosis assay. Apoptosis was evaluated based the level of cleaved PARP, which was detected by immunoblotting.

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics 24.0 (SPSS, Inc.). Comparisons were made using a two-tailed unpaired Student's t-test. P-values <0.05 were considered to indicate statistical significance.

Results

SOX2 overexpression activates AKT. To express SOX2 in ESCC cells using the adenoviral vector, two cell lines (KYSE30 and TE4) that are known to express SOX2 only at low levels (6) were infected with Ad-SOX2 or Ad-control. Cells were cultured under standard conditions and harvested at 48 h after infection. Immunoblot analyses revealed that SOX2 protein was successfully expressed in both of the lines infected with Ad-SOX2, and that levels of p-AKT (S473), but not those of total AKT, were higher in cells infected with Ad-SOX2 than in those infected with Ad-control (Fig. 1A). However, p-ERK levels were not increased in KYSE30 cells infected with Ad-SOX2 (Fig. 1A) compared to the same line infected with Ad-control.

To examine the time courses of accumulation of SOX2 and p-AKT, TE4 cells were infected with Ad-SOX2 and harvested 6, 12, 24, 48 or 72 h later. SOX2 accumulation was detected starting at 6 h; SOX2 protein levels gradually increased thereafter, reaching a maximum at 48 h after infection. Levels of p-AKT (T308 and S473) increased at 24 h and reached a maximum at 48 h. The time course analyses showed that the increase in SOX2 accumulation preceded the increase in p-AKT levels (Fig. 1B). These results suggested that overexpression of SOX2 increased the activation of AKT.

To examine whether, conversely, AKT activation increased SOX2 accumulation in ESCC cells, TE6 cells, which express a high endogenous level of SOX2 (6), were treated with MK2206, an inhibitor of AKT, for 48 h. Exposure to MK2206 resulted in decreased p-AKT (S473) levels in a dose-dependent manner. However, the decrease in p-AKT (S473) levels did not influence the level of SOX2 (Fig. 1C). The same results were obtained when TE6 cells were treated with 2 μM of MK2206 for different periods of time (0, 24, 48 and 72 h) and harvested for immunoblot analysis (Fig. 1D).

SOX2 activates AKT under serum starvation conditions. The activation of AKT by SOX2 was examined under serum starvation conditions. KYSE30 cells were infected with Ad-SOX2 or Ad-control. After a 24-h incubation, cells were cultured in serum-free medium for 24 h. The cells then were left untreated or treated with serum (10%) for 15 min. Immunoblot analysis showed that, for cells maintained under serum starvation conditions, p-AKT (S473) levels were higher in cells infected with Ad-SOX2 than in those infected with Ad-control (Fig. 2A). However, increases in p-AKT (S473) levels did not appear to differ when comparing serum-stimulated Ad-SOX2-infected cells to serum-stimulated Ad-control infected cells (Fig. 2A). To confirm this finding, KYSE140 cells, which have high endogenous expression of SOX2 (6), were transfected with either of two siRNAs (*SOX2* siRNA #1 and #2) targeting SOX2 expression, or with a control siRNA. After a 48-h
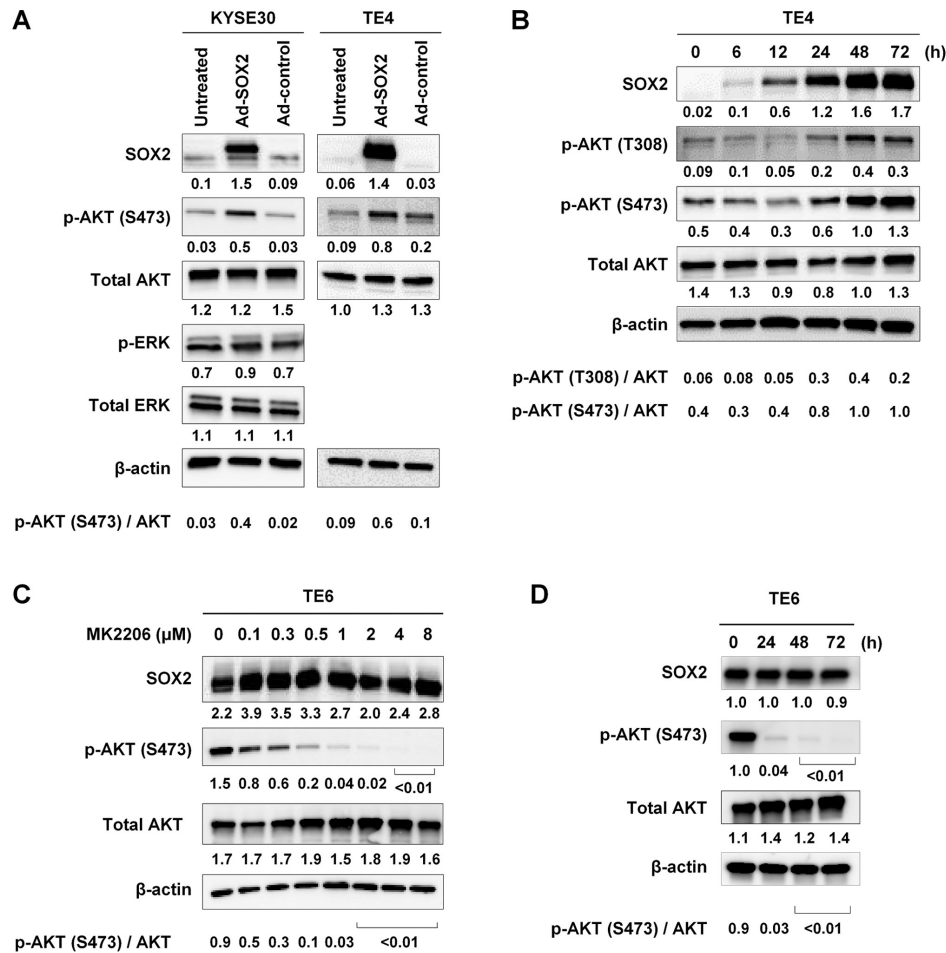
Figure 1. Activation of AKT by adenoviral vector-mediated overexpression of SOX2. (A) KYSE30 and TE4 cells, which are known to express SOX2 at low levels, were infected with Ad-SOX2 or Ad-control. Cells were harvested 48 h after infection and subjected to immunoblot analysis of SOX2, p-AKT (S473), total AKT, p-ERK and total ERK expression. (B) Time course of changes in the levels of SOX2, p-AKT (T308), p-AKT (S473) and total AKT. TE4 cells were infected with Ad-SOX2 and harvested at the indicated time points after infection, then subjected to immunoblot analysis. (C) Effect of MK2206. TE6 cells, which express a high endogenous level of SOX2, were treated for 48 h with MK2206, an AKT inhibitor, at the indicated concentrations for 48 h, then subjected to immunoblot analysis of SOX2, p-AKT (S473) and total AKT levels. (D) TE6 cells were treated with 2 μM MK2206 for different time periods and harvested at the indicated time points, then subjected to immunoblot analysis of SOX2, p-AKT (S473) and total AKT expression. For all experiments, β-actin was detected as a loading control. The numbers presented below the gels represent the expression levels of each protein relative to those of β-actin. Values were normalized so that β-actin expression in each well had a value of 1. The ratio of p-AKT (S473 or T308) to total AKT is shown. Ad, adenovirus vector; p-, phosphorylated.

incubation, cells were cultured in serum-free medium for 12 h. Cells then were incubated for 15 min in growth medium without or with serum (10%). SOX2 knockdown by siRNAs #1 and #2 was confirmed by immunoblotting (Fig. 2B). Levels of p-AKT (S473) were decreased following knockdown of SOX2 under serum starvation conditions, but not following knockdown under serum-stimulated conditions (Fig. 2B). Taken together, these results suggested that SOX2 increases AKT activation under serum starvation conditions. However, this effect of SOX2 appears to be masked by serum stimulation.

SOX2 promotes cell survival under serum starvation conditions. To examine the effect of SOX2 on cell viability under serum starvation conditions, TE4 cells were infected with Ad-SOX2 or Ad-control, and then cultured in growth medium in the presence or absence of serum. A cell growth assay showed that cell viability in serum-free medium was higher in cells infected with Ad-SOX2 than in those infected with Ad-control. Notably, this pattern was not seen in medium

containing serum (Fig. 3A). However, the difference between Ad-SOX2 and Ad-control in serum-free medium was no longer seen when cells were treated with MK2206 (Fig. 3B), suggesting the involvement of AKT in the effect of SOX2 on promotion of cell survival under serum starvation conditions.

SOX2 promotes resistance to apoptosis through activation of the AKT/GSK-3β pathway. We next investigated the effect of SOX2 on resistance to apoptosis under serum starvation conditions. TE4 cells were infected with Ad-SOX2 or Ad-control, then treated with doxorubicin, an anticancer drug, in serum-free medium. A cell viability assay showed that cells infected with Ad-SOX2 were more resistant to doxorubicin than those infected with Ad-control (Fig. 3C), exhibiting 50% inhibitory concentrations (IC50s) of 5.8 μM and 2.8 μM, respectively. However, when TE4 cells were infected with Ad-SOX2 or Ad-control and then treated with doxorubicin combined with MK2206, the difference in doxorubicin resistance was no longer seen (Fig. 3D). This result suggested the
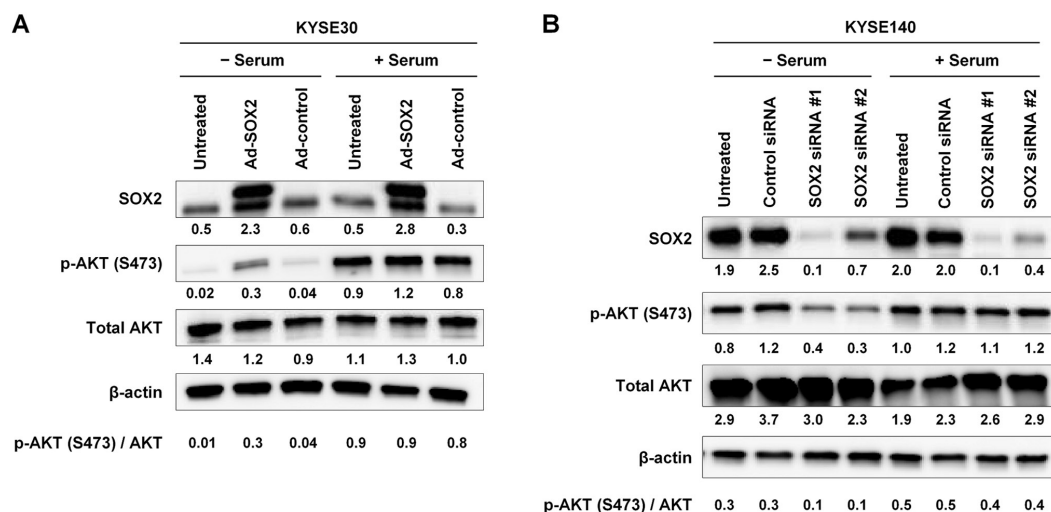
Figure 2. Activation of AKT by SOX2 under serum starvation conditions. (A) KYSE30 cells were infected with Ad-SOX2 or Ad-control. After a 24-h incubation, cells were cultured in serum-free medium for 24 h. Subsequently, cells were incubated in growth medium without or with serum (10%) for 15 min (- serum or + serum, respectively), harvested and subjected to immunoblot analysis of SOX2, p-AKT (S473) and total AKT expression. (B) KYSE140 cells were transfected with one of two siRNAs targeting SOX2 (SOX2 siRNA #1 and #2) or with a control siRNA. After a 48-h incubation, cells were cultured in serum-free medium for 12 h. Subsequently, cells were incubated in growth medium without or with serum (10%) for 15 min (- serum or + serum, respectively), harvested and subjected to immunoblot analysis of SOX2, p-AKT (S473) and total AKT expression. For all experiments, β-actin was detected as a loading control. The numbers presented below the gels represent the expression levels of each protein relative to those of β-actin. Values were normalized so that β-actin expression in each well had a value of 1. The ratio of p-AKT (S473) to total AKT is shown. Ad, adenovirus vector; p-, phosphorylated; siRNA, small interfering RNA.
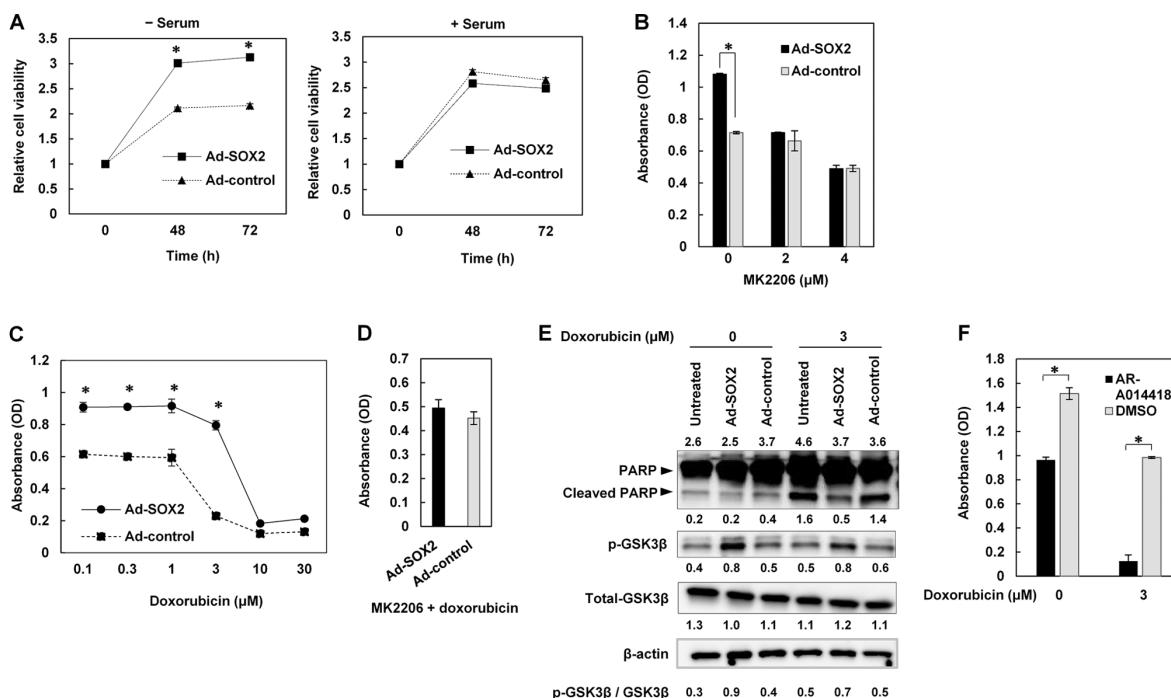

Figure 3. Promotion of cell survival under serum starvation conditions and resistance to apoptosis through activation of the AKT/GSK-3β signaling pathway by SOX2. (A) TE4 cells were infected with Ad-SOX2 or Ad-control, and then cultured in serum-free medium (- serum) or medium with serum (+ serum). Relative cell viabilities were measured at the indicated time points. (B) TE4 cells were infected with Ad-SOX2 or Ad-control, then treated 24 h later with the indicated concentrations of MK2206 in serum-free medium. Cell viabilities were measured 48 h after MK2206 treatment. (C) TE4 cells were infected with Ad-SOX2 or Ad-control, then treated 24 h later with the indicated concentrations of doxorubicin in serum-free medium. Cell viabilities were measured 48 h after doxorubicin treatment. (D) TE4 cells were infected with Ad-SOX2 or Ad-control, then treated 24 h later with 3 μM doxorubicin and 2 μM MK2206 in serum-free medium. After a 48-h incubation, cells were subjected to cell viability assays. (E) TE4 cells were infected with Ad-SOX2 or Ad-control, then treated 24 h later with or without 3 μM doxorubicin in serum-free medium. Cells were harvested 48 h after doxorubicin treatment and subjected to immunoblot analysis of PARP, cleaved PARP, p-GSK-3β and total GSK-3β expression. β-actin was detected as a loading control. The numbers presented above and below the gel for PARP and cleaved PARP represent the expression levels of PARP and cleaved PARP relative to those of β-actin, respectively. The numbers presented below the gels for p-GSK-3β and total GSK-3β represent the expression levels of p-GSK-3β and total GSK-3β relative to those of β-actin, respectively. Values were normalized so that β-actin expression in each well had a value of 1. The ratio of p-GSK-3β to total GSK-3β is shown. (F) TE4 cells were infected with Ad-SOX2, and treated 24 h later with AR-A014418 or DMSO. The cells were treated 24 h later with or without 3 μM doxorubicin in serum-free medium. Cell viability was measured 72 h after doxorubicin treatment. All data are presented as the mean ± SD (n=3). *P<0.01 analyzed by two-tailed unpaired Student's t-test. OD, optical density; Ad, adenovirus vector; p-, phosphorylated; GSK-3β, glycogen synthase kinase-3β; PARP, poly (ADP-ribose) polymerase.
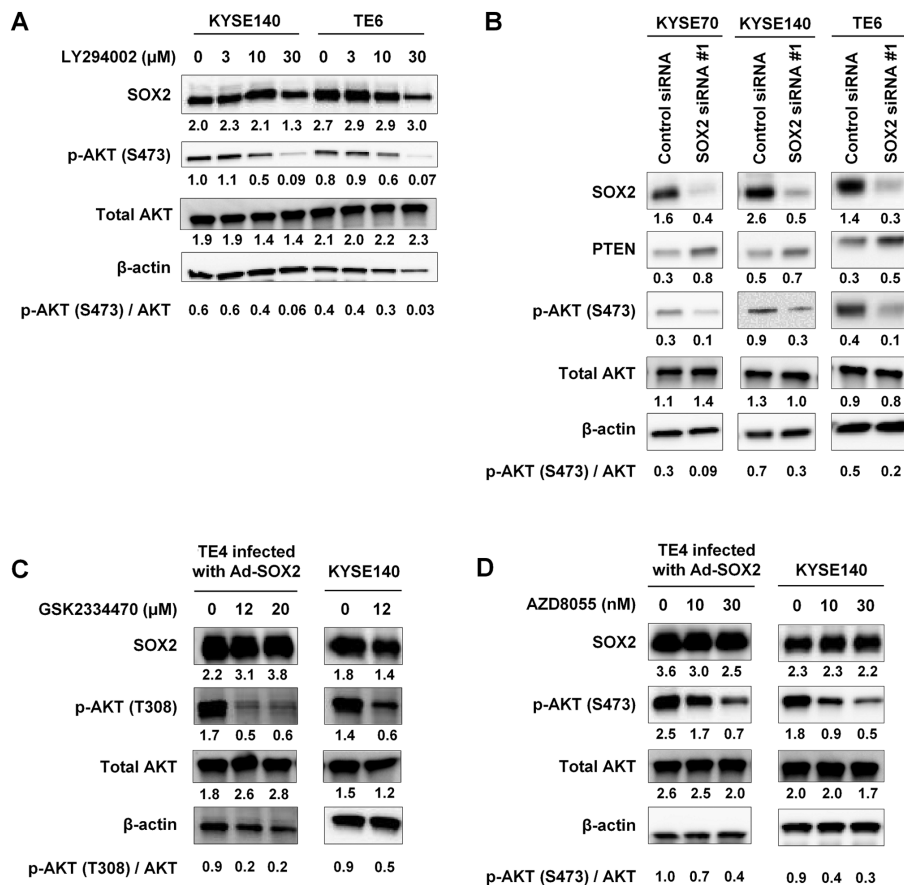
Figure 4. PTEN/PI3K/PDK1- and mTORC2-mediated phosphorylation of AKT in SOX2-overexpressing cells. (A) KYSE140 and TE6 cells were treated with the indicated concentrations of LY294002, a PI3K inhibitor, in serum-free medium for 48 h, harvested and subjected to immunoblot analysis of SOX2, p-AKT (S473) and total AKT expression. (B) KYSE70, KYSE140 and TE6 cells were transfected with either SOX2 siRNA#1 or with a control siRNA. After a 48-h incubation in serum-free medium, cells were harvested and subjected to immunoblot analysis of SOX2, PTEN, p-AKT (S473) and total AKT expression. TE4 cells infected with Ad-SOX2 and KYSE140 cells were treated for 1 h in serum-free medium containing the indicated concentrations of (C) GSK2334470, a PDK1 inhibitor, or (D) AZD8055, an mTORC2 inhibitor. Cells were harvested and subjected to immunoblot analysis of SOX2, p-AKT (T308 or S473) and total AKT expression. For all experiments, β-actin was detected as a loading control. The numbers presented below the gels represent the expression levels of each protein relative to those of β-actin. Values were normalized so that β-actin expression in each well had a value of 1. The ratio of p-AKT (S473 or T308) to total AKT is shown. PI3K, phosphatidylinositol 3-kinase; Ad, adenovirus vector; p-, phosphorylated; siRNA, small interfering RNA; PDK1, phosphoinositide-dependent protein kinase 1; mTORC2, mammalian target of rapamycin complex 2.

involvement of AKT in the effect of SOX2 on resistance to doxorubicin.

Immunoblotting of cleaved PARP, a marker of apoptosis, showed that TE4 cells infected with Ad-SOX2 were attenuated for doxorubicin-induced apoptosis compared to Ad-control-infected cells (Fig. 3E). We screened among factors known to lie downstream of AKT to identify those involved in resistance to apoptosis in cells overexpressing SOX2. We found that the p-GSK-3β levels were increased in TE4 cells infected with Ad-SOX2 compared to those infected with Ad-control (Fig. 3E). Similar results were seen when TE4 cells were treated with doxorubicin after Ad-SOX2 or Ad-control infection (Fig. 3E). To test the role of GSK-3β, TE4 cells infected with Ad-SOX2 were treated with AR-A014418, an inhibitor of GSK-3β, or dimethyl sulfoxide (DMSO), and then were untreated or treated with doxorubicin in serum-free medium. Although a single treatment with AR-A014418 decreased cell viability, combination treatment with AR-A014418 and doxorubicin synergistically reduced cell viability (Fig. 3F). Taken together, these findings suggested that SOX2 promotes resistance to apoptosis through activation of the AKT/GSK-3β pathway.

The PTEN/PI3K/PDK1 and mTORC2 pathways mediate phosphorylation of AKT in SOX2-overexpressing cells. In a further set of experiments, we examined the role of the PTEN/PI3K/PDK1 and mTORC2 pathways in SOX2-mediated activation of AKT by testing the effects of inhibitors of components of these pathways or by knock-down of SOX2 expression. KYSE140 and TE6 cells were treated with LY294002, an inhibitor of PI3K, in serum-free medium. The levels of p-AKT (S473) were decreased in a dose-dependent manner in the presence of LY294002, as determined by immunoblot analysis (Fig. 4A).

KYSE70, KYSE140, and TE6 cells were transfected with SOX2 siRNA #1 or control siRNA and then cultured in serum-free medium. Immunoblot analysis showed that SOX2 knockdown resulted in increased expression of PTEN and decreased expression of p-AKT (S473) (Fig. 4B).

TE4 cells infected with Ad-SOX2 and KYSE140 cells were treated with GSK2334470, a PDK1 inhibitor, in serum-free medium. Immunoblot analyses indicated that exposure of these cells to GSK2334470 diminished SOX2-mediated phosphorylation of AKT's T308 residue (Fig. 4C).

TE4 cells infected with Ad-SOX2 and KYSE140 cells growing in serum-free medium were treated with AZD8055, which inhibits the phosphorylation of the mTORC2 substrate AKT (15). AZD8055 exposure provided dose-dependent decreases in the levels of p-AKT (S473) in these cells (Fig. 4D). Considered together, these findings suggested that SOX2 may activate AKT through the PTEN/PI3K/PDK1 and mTORC2 pathways under conditions of serum starvation.

Discussion

In the present study, we used an adenoviral vector-mediated expression system to demonstrate that SOX2 overexpression results in the phosphorylation and activation of AKT, but not ERK, in ESCC cells. Although the AKT and MAP kinase pathways, both of which are downstream of Ras, are major pathways involved in the growth and proliferation of cancer cells, our results suggest that SOX2 activates only the AKT pathway in ESCC.

Previous studies have shown that the SOX2 protein level is determined by AKT activity in embryonic stem cells (ESCs) (16), non-small-cell lung cancer (17), and breast cancer (18). However, our time course assay after Ad-SOX2 infection showed that increased accumulation of SOX2 precedes the activation of AKT. Moreover, our experiments using the AKT inhibitor MK2206 suggested that AKT activation does not increase SOX2 accumulation in ESCC cells. Although the reason for the discrepancy between those previous results and ours is unclear, it may reflect the use of different types of cells and experimental systems.

Our experiments showed that SOX2 overexpression increased AKT activation under serum starvation conditions, but this effect was masked by serum stimulation. Furthermore, SOX2's promotion of cell survival was mediated through AKT activation under serum starvation conditions, but not in medium containing serum. These findings indicated that SOX2 facilitates ESCC cell survival under poor nutrient conditions. Therefore, overexpression of SOX2 may be beneficial for the survival of cancer cells suffering from insufficient nutrient supply, as often faced by solid tumors, and may be involved in the development and progression of ESCC.

Resistance to apoptosis contributes not only to the survival of cancer cells, but also to resistance to chemotherapy. Our experiments indicated that overexpression of SOX2 led to resistance to doxorubicin-induced apoptosis through AKT activation. Moreover, our results suggested that GSK-3β, a factor that lies downstream of AKT, may be involved in resistance to apoptosis in cells that overexpress SOX2.

GSK-3β was first identified as a negative regulator of glycogenesis and subsequently was found to regulate various signaling pathways, including the Wnt/β-catenin pathway (19). GSK-3β phosphorylates multiple substrates, including β-catenin, cyclin D1, MYC, BAX (Bcl-2-associated X protein), and nuclear factor-kappa B (NF-κB), and induces the degradation of the substrates or inhibition of their enzymatic activities. GSK-3β is directly phosphorylated by AKT and inhibitory phosphorylation of GSK-3β has numerous cellular functions such as promoting glycogen metabolism, cell cycle progression, and cell survival. In the context of cancer treatment,

GSK-3β inhibition has been studied as a possible therapeutic strategy (20). Our results showed the combinatorial effect of doxorubicin and AR-A014418, an inhibitor of GSK-3β, in reducing cell viability in ESCC cells that overexpress SOX2. Therefore, AKT and GSK-3β may be appropriate and important targets for the development of novel therapies for SOX2-overexpressing ESCC.

Finally, we explored the mechanism whereby SOX2 phosphorylates AKT under serum starvation conditions. Our findings suggested that both the PTEN/PI3K/PDK1 and mTORC2 pathways may be involved in the activation of AKT by SOX2.

Certain limitations should be considered in the interpretation of our findings. First, although SOX2 activates the AKT pathway, the direct target of SOX2 remains unclear. Second, downstream factors of AKT other than GSK-3β may be involved in apoptosis resistance in ESCC cells that overexpress SOX2. Third, results of the present study need to be confirmed by further *in vivo* studies.

In conclusion, our results showed that SOX2 promotes cell survival and enhances resistance to apoptosis under serum starvation conditions in ESCC cells; these effects are mediated by activation of the AKT/GSK-3β signaling pathway. SOX2 may phosphorylate AKT though the PTEN/PI3K/PDK1 and mTORC2 pathways under serum starvation conditions. We postulate that molecular-targeted therapy against the SOX2/AKT/GSK-3β pathway may be effective against ESCC cells that overexpress SOX2.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KT, KoY and YG contributed to the study conception, data collection and data interpretation. KT contributed to statistical analysis and manuscript preparation. KoY contributed to manuscript preparation and finalization. NI, TS, TK, OD, HT, YS, AU, TN, KaY, MM, HK, YN and YI contributed to acquisition of data, or analysis and interpretation of data. KT and KoY confirmed the authenticity of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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