Sox2 targets cyclinE, p27 and survivin to regulate androgen-independent human prostate cancer cell proliferation and apoptosis

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Received 21 November 2011; revision accepted 12 January 2012

Abstract

Objectives: Sox2 is a major transcription factor and the transforming growth factor- α (TGF- α)/EGFR autocrine loop is a hallmark of prostate cancer progression. In this study, we have evaluated the effects and potential mechanisms of Sox2 on cell proliferation and apoptosis, and investigated effects of TGF- α on expression of Sox2 on androgen-independent human prostate cancer cells.

Materials and methods: Expression of Sox2 has been determined by RT-PCR, western blot analysis and immunocytochemistry, using RNAi and over-expression strategy to study functions of Sox2 in DU145 and PC-3 cells. Changes in level of proliferation, cell cycle and apoptosis profiles were measured by MTT, colony-forming, bromodeoxyuridine incorporation assays, cell cycle and annexin V analysis.

Results: Sox2 was expressed in six human prostate cancer cell lines, and its inhibition reduced cell proliferation and induced apoptosis in DU145 cells. We have shown that knock-down of Sox2 inhibited G₁ to S phase transition concomitantly with down-regulation of cyclin E and up-regulation of p27 proteins. Conversely, over-expression of Sox2 led to the opposite effect in PC-3 cells but its inhibition induced apoptosis by down-regulation of sur-

vin in DU145 cells. We also found that TGF- α up-regulated Sox2 and survivin protein expression *via* the EGFR/PI3K/AKT pathway.

Conclusions: Sox2 expression is necessary for cell proliferation and evasion of apoptosis in prostate cancer cells and TGF- α could regulate Sox2 and survivin expression by activating the EGFR/PI3K/AKT pathway.

Introduction

Prostate cancer (PCa) is one of most malignant types amongst older men (1). Considering ageing populations, numbers of men diagnosed with PCa have increased over the last decades. Tumour growth and maintenance of prostate cancer cells are androgen-dependent in early stages of the disease, however at later stages, tumour progress changes from androgen dependence to independence. Early-stage prostate cancer can be cured effectively by radical prostatectomy, but prognosis of high-risk prostate cancer is not good. To a large extent, lack of effective therapies for advanced PCa is related to poor understanding of the molecular mechanisms underlying progression of the disease. Thus, identification of new predictive biomarkers, which could serve as effective targets for therapy and chemopreventative interventions, will improve clinical management of PCa (2).

The *Sox* [Sex-determining region Y (SRY)-related high-mobility group (HMG) box] gene family encodes a series of transcription factors, which share the HMG domain of the SRY protein (3). *Sox2* belongs to the B1 group of the *Sox* gene subfamily and the full-sequence of the cDNA is 2418 bp (4). It is a 317 residue critical transcription factor in embryonic and cancer stem cells (5) and plays an important role in cell self-renewal, differentiation, proliferation and apoptosis (6–14). It is over-expressed in most human tumour types, including

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in neural system tumours (12), respiratory system tumours (13,15,16), reproductive system tumours (17,18) and in digestive system tumours (19,20). It has also shown that *Sox2* silencing causes cessation of proliferation and loss of tumourigenicity, and over-expression of *Sox2* is associated with increased cancer aggressiveness and reduced patient survival, in breast cancer. Immunohistochemistry assays have showed that quantity of *Sox2* protein expression is significantly increased in prostate cancer tissues compared to normal prostate or benign prostate hyperplasias (14). *Sox2* is involved in proliferation of prostate cancer cells, but the mechanisms involved remain largely unknown, both in prostate and in other cancers.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is capable of regulating cell proliferation and apoptosis (21). It has been shown to be over-expressed in at least 60 cancer cell lines and in most human tumour types, including lung, breast, stomach, liver, bladder, ovarian cancers and endometrial carcinoma (22,23). It has been reported that loss-function of survivin causes spontaneous apoptosis or sensitizes cancer cells to apoptotic stimuli, whereas gain-function of survivin confers cytoprotection against a variety of apoptotic stimuli (24). In prostate cancer, over-expression of survivin has also been associated with increasing cancer aggressiveness and decreasing patient survival (25), whereas *survivin* gene silencing sensitizes prostate cancer cells to selenium population growth inhibition (26).

In the present study, we investigated whether *Sox2* would contribute to prostate cancer cell proliferation and/or to apoptosis and its mechanisms. We exploited RNAi technology to knockdown expression of *Sox2* or up-regulated it in androgen-independent prostate cancer DU145 or PC-3 cells, and studied effects of TGF- α on *Sox2* and survivin expression. Our results confirmed that TGF- α can induce *Sox2* and survivin expression by activating the EGFR/PI3K/AKT pathway.

Materials and methods

Materials

All reagents were obtained from Sigma (St Louis, MO, USA) unless otherwise stated. Primary antibody against *Sox2* was obtained from Abcam and antibodies against survivin, Bcl-2, AKT and p-AKT were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies to β -actin, cyclin E, p27, p21, and goat anti-rabbit and anti-mouse secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TGF- α was from Peprotech, Rocky Hill, NJ, USA and LY294002 was purchased from Sigma. All

primers used in this research were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

Cell culture

Human prostate cancer cell lines LNCap, DU145 and PC-3 were purchased from American Type Culture Collection, and PC-3M, PC-3M-1E8 and PC-3M-2B4 were obtained from the Peking University Health Science Center (Beijing, China). Cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1 $\times$ penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), at 37 °C in humidified atmosphere (5% CO₂/95% air).

In vitro transfection

Vectors of pGFP-V-RS-*Sox2*-shRNA (OriGene Technologies, Inc., Rockville, MD, USA) or pcDNA3.0-vector (Invitrogen) with *Sox2* cDNA (pcDNA3.0-*Sox2*) were used. DU145 cells were transfected with PGFP-V-RS-*Sox2*-shRNA and PC-3 cells were transfected with pcDNA3.0-*Sox2*, using Lipofectamine 2000 (Invitrogen). 2 μ g/ml puromycin (for DU145 cells) or 850 μ g/ml G418 (for PC-3 cells) were applied to select puromycin or G418 resistant cells.

RNA extraction and RT-PCR

RNA was extracted by using TRIzol reagent (Gibco, Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized with Superscript II reverse transcriptase and oligo dT from 2 μ g of total RNA. Specific RT-PCR primers for this assay were used as follows: *β -actin*, sense: 5'-TAC CTCATGAAGATCCTCACC-3'; anti-sense: 5'-TTTC-GT GGATGCCACAGGAC-3'; *Sox2*, sense: 5'-CCCTGTGG TTACCTCTTCC-3'; anti-sense: 5'-CCTCCCATTTCCC TCGTTTT-3'. Annealing temperature was 55 °C. Amplification cycles were 30 for *Sox2* and 23 for *β -actin*. Expression of *β -actin* was monitored in all RT-PCR experiments, as internal control.

Western blot analysis

After being treated with the appropriate compounds, cells were harvested and washed in ice-cold phosphate buffer. Protein concentration of extracts was determined using BCA reagent (Beyotime Institute of Biotechnology, Nanjing, China). Equal amounts of protein (40 μ g/lane) were loaded, separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. After being blocked with 5% non-fat milk, membranes were incubated with the appropriate primary antibody [*Sox2*

(1:1000), survivin (1:1000), cyclin E (1:500), p27 (1:500), p21 (1:500), β -actin (1:000), AKT (1:500), p-AKT (1:500) and Bcl-2 (1:1000)] at 4 °C, overnight. After being incubated with the respective second antibody (1:2000), immune complexes were detected using ECL western blotting reagents (Applygen Technologies Inc., Beijing, China). Expression levels of β -actin were monitored as internal control, and band intensities were normalized to that of β -actin.

Cell proliferation and analysis of DNA synthesis

MTT assay was used as the qualitative index of cell viability. Cells were plated in 96-well microtitre plates at density of 3×10^3 cells/well, and after 24, 48, 72 and 96 h incubation, 10 μ l of MTT (5 mg/ml; Amresco, Solon, OH, USA) solution was added to each well; then samples were incubated for 4 h. Subsequently, cells were lysed using dimethylsulphoxide (100 μ l/well; Sigma). When the formazan crystals were completely dissolved, optical absorbance was measured at 450 nm.

Colorimetric immunoassay kits [Cell proliferation ELISA, bromodeoxyuridine (BrdUrd; colorimetric), Roche Diagnostics GmbH, Mannheim, Germany] were used for quantification of DNA synthesis. This assay is based on measurement of BrdUrd incorporation during DNA synthesis. Briefly, cells were seeded in 96-well plates; 24, 48, 72 and 96 h after, they were labelled with BrdUrd for 3 h at 37 °C, washed and anti-BrdUrd antibody was applied for 90 min at 25 °C. After three washes, substrate solution was added, followed by incubation for 30 min. Blocking solution (1 M H_2SO_4) was then added, and absorbance of samples was measured at 490 nm, with reference wavelength of 690 nm, using an ELx800 multiwell plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Analysis of cell cycles and apoptosis

Cell cycle analysis was performed using flow cytometry. Twenty-four hours after being harvested, cells were fixed in 70% ethanol and stored at 4 °C overnight, then were incubated in RNase (25 μ g/ml) at 37 °C for 30 min, followed by staining of their DNA with propidium iodide (50 μ g/ml), for 30 min in the dark. Each sample was then analysed using fluorescence-activated cell sorting (FACS) (BD, San Jose, CA, USA).

Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kits (Beyotime) and flow cytometry were used for analysis of apoptosis. Twenty-four hours after being harvested, 1×10^6 cells were incubated in annexin V-FITC, at room temperature for 10 min in the dark,

followed by staining of their DNA with propidium iodide in the dark. Then, each sample was analysed by fluorescence-activated cell sorting (FACS) (BD).

Analysis of apoptosis

Cells were plated into 6 cm diameter dishes and allowed to proliferate for 24 h. They were then trypsinized, washed in cold PBS, resuspended in binding buffer, and annexin V-FITC and propidium iodide were added according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

After staining, flow cytometry distinguished between apoptotic cells and viable cells. Percentages of early apoptotic cells (annexin V-positive and propidium iodide-negative) were calculated from the flow-cytometric data.

Assay of EGFR/PI3K/AKT signal transduction pathway

Populations of DU145 and PC-3 cells were expanded as described in the Cell culture section and were serum-starved for 16 h, washed twice in RPMI1640 medium, and incubated in TGF- α (50 ng/ml) (Peprotech) for up to 120 min (for AKT phosphorylation assay), or for up to 48 h (for Sox2 and survivin protein assays). To evaluate effects of PI3K/AKT inhibitor LY294002 (Sigma, St. Louis, MO, USA) on the epidermal growth factor receptor (EGFR)/PI3K/AKT pathway, we pre-treated cells with 30 μ M LY294002 for 2 h, then with 50 ng/ml TGF- α for 30 min (for AKT phosphorylation assay) or 24 h (for Sox2 and survivin protein assays). Cells were then harvested and proteins extracted for western blot analysis.

Statistical analysis

Each experiment was repeated in triplicate and results are expressed as mean $\pm$ SEM. Statistical analyses were performed using Statistics Package for Social Science (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL, USA) and $P < 0.05$ was considered significant.

Results

Expression of Sox2 in prostate cancer cell lines

We used RT-PCR and western blot analysis to study expression of Sox2 in six human prostate cancer cell lines. Results showed that mRNA and protein expression of Sox2 were higher in the androgen-independent prostate cancer lines DU145, PC-3, PC-3M, PC-3M-1E8 and PC-3M-2B4 than in the androgen-dependent line, LNCap (Fig. 1a,b). Immunocytochemical detection showed

that Sox2 was localized in cytoplasm and nucleoli of DU145, PC-3 and PC-3M cells (Fig. 1c).

Down-regulation of Sox2 inhibited cell proliferation

To evaluate the biological role of Sox2 on proliferation of androgen-independent human prostate cancer cells, stable Sox2 shRNA transfected clones were constructed. Three plasmids of Sox2 RNAi (Sox2-shRNA1, Sox2-shRNA2 and Sox2-shRNA3) and 1 negative control plasmid (CTLshRNA) were used and tested for their ability to knock down Sox2 expression in DU145 cells. As shown in Fig. 2a,b, DU145-Sox2-shRNA1 and DU145-Sox2-shRNA2 cells had lower levels of Sox2 mRNA and protein (RT-PCR and western blotting), whereas DU145-CTLshRNA, transfected with negative control shRNA (Fig. 2a,b), did not have reduced levels of Sox2 mRNA nor protein expression. Efficiency of Sox2-shRNA1 and Sox2-shRNA2 was better than Sox2-shRNA3, so Sox2-shRNA1 and Sox2-shRNA2 were chosen for the next assays. MTT assay showed that cell proliferation was lower in DU145-Sox2-shRNA2 cells, compared to DU145-CTLshRNA (Fig. 2c). To assess the effect of Sox2 on DNA synthesis in DU-145 cells, BrdUrd assay had been carried out; this showed that down-regulated expression of Sox2 reduced DNA synthesis in DU145 cells (Fig. 2d). Colony-forming unit assays demonstrated that down-regulation of Sox2 inhibited clone formation ability, including clone number and volume, in DU145-Sox2-shRNA2 cells, compared to

DU145-CTLshRNA (Fig. 2e). Such results indicate that Sox2 regulated cell proliferation, which, of course, is associated with reduction in DNA replication.

Down-regulation of Sox2 induction of cell-cycle

To further verify the possibility that Sox2 contributed to the prostate cancer cells' cell-cycle distribution, their DNA content was measured using flow cytometry. We found that DU145-Sox2-shRNA2 caused cells to accumulate in G₁ phase and the proportion of cells in S phase was lower compared to DU145-CTLshRNA cells (Fig. 3a), consistent with results of the BrdUrd assay (Fig. 2d). We used western blot analysis to examine levels of 3 cell cycle regulators, cyclin E, p27 and p21. Results demonstrated that knock-down of Sox2 in DU145 cells decreased expression of cyclin E and increased expression of p27, but expression of p21 remained unaltered.

Down-regulation of Sox2 induction of apoptosis via inhibition of survivin expression

To reveal the biological role of Sox2 on apoptosis in androgen-independent human prostate cancer cells, annexin V-FITC staining was used. DU145-Sox2-shRNA2 increased proportions of annexin V-positive cells compared to DU145-CTLshRNA (Fig. 4a,b). To identify molecules responsible for the apoptotic effect caused by knock-down of Sox2 in DU145 cells, expression of

Figure 1. Expression and localization of Sox2 in human prostate cancer cell lines. (a) RT-PCR analysis shows the mRNA expression of Sox2 in human prostate cancer cells, β -actin was used as an internal control in RT-PCR. (b) Western blotting shows the protein expression of Sox2 in human prostate cancer cells, β -actin was used a loading control in western blot. (c) Immunocytochemical detection shows the localization of Sox2 in DU145, PC-3 and PC-3M cells

Figure 2. Downregulation of Sox2 inhibits proliferation in prostate cancer DU145 cells. Selective silencing of Sox2 expression in DU145 cells by RNAi. Sox2 mRNA and protein expression was tested by (a) RT-PCR and (b) Western blot analysis, respectively. Transfections were performed with plasmids Sox2-shRNA1, Sox2-shRNA2, and Sox2-shRNA3 and 1 control plasmid (Sox2-shRNACTL). The transfections with plasmids Sox2-shRNA_{neg} was used as negative controls. β -actin was used as an internal control in RT-PCR and western blot. (c) MTT assay for Sox2 knockdown by shRNAs in DU145 cells. Cells were collected after 24, 48, 72 and 96 h, and the absorbance was measured at 490 nm. (d) BrdUrd assay for Sox2 knockdown by shRNAs in DU145 cells. Cells were incubated with BrdUrd after 24, 48, 72 and 96 h to quantify DNA synthesis. The absorbance was measured at 450 nm. (e) colony-forming unit assays for DU145-CTLshRNA and DU145-Sox2-shRNA2 cells. 100 DU145-CTLshRNA and DU145-Sox2-shRNA2 cells were cultured for 9 days, crystal violet staining of clones at day 9. Three separate experiments were carried out, all of which exhibited similar trends. Band intensity was calculated using Quantity one. Statistical significance was considered $**P < 0.01$, $*P < 0.05$, compared with control group. Data are means $\pm$ SEM of three independent experiments.

Figure 3. Down-regulation of Sox2 induces cell-cycle arrest in prostate cancer DU145 cells. (a) Cell cycle analysis for the effect of Sox2 on cell cycle. DU145-Sox2-shRNA2 and DU145-CTLshRNA cells were fixed, stained with propidium iodide and the DNA content measured by flow cytometry. (b) Western blot analysis for cyclin E, p21 and p27 in Sox2 knockdown cells. Band intensity was calculated using Quantity One. Statistical significance was considered at $**P < 0.01$, $*P < 0.05$, compared with control group. Data are means $\pm$ SEM of three independent experiments.

survivin and Bcl-2 were detected by western blotting. We found that DU145-Sox2-shRNA1, DU145-Sox2-shRNA2 cells contained lower amounts of survivin pro-

teins, compared to DU145-CTLshRNA cells, while expression of Bcl-2 remained unaltered (Fig. 4c). These data indicate that Sox2 regulated an anti-apoptotic

Figure 4. Down-regulation of Sox2 induces apoptosis in prostate cancer DU145 cells through inhibiting survivin expression. (a) Annexin V-FITC assays for the effect of Sox2 on apoptosis. DU145-Sox2-shRNA2 and DU145-CTLshRNA cells were stained with propidium iodide and Annexin V-FITC, and then measured by flow cytometry. (b) Western blot analysis for survivin, Bcl-2 in DU145-Sox2-shRNA2 and DU145-CTLshRNA cells. Statistical significance was considered at $^{***}P < 0.01$, compared with control group. Data are means $\pm$ SEM of three independent experiments.

program in DU145 cells and inhibition of Sox2 lead to rapid induction of apoptosis by inhibiting survivin.

Overexpression of Sox2 enhancement of cell proliferation and promotion of G₁ to S phase transition in androgen-independent human prostate cancer cells

To further verify the biological role of Sox2 in proliferation of human prostate cancer cells, stable over-expression of Sox2 transfection clones were made. We chose PC-3 cells for this part of the overall study, as this cell line had been detected to have relatively low endogenous Sox2 protein expression. As shown in Fig. 5a, PC-3-Sox2 was demonstrated to have higher levels of Sox2 protein compared to the PC-3-Mock. MTT assay showed that cell proliferation was higher in PC-3-Sox2 cells compared to PC-3-Mock cells (Fig. 5b). Colony-forming unit assays showed that over-expression of Sox2 induced clone formation ability, including by clone number and by volume (Fig. 5c). We found that over-expression of Sox2 decreased proportion of cells in G₁ (from 51.74% to 41.45%) and increased that in S phase (from 30.38% to 51.75%), indicating that high expression of Sox2 could promote G₁ to S phase transition of the cell cycle (Fig. 5d).

We used western blot analysis to examine levels of three cell cycle regulators, cyclin E, p27 and p21. Results showed that over-expression of Sox2 in PC-3 cells elevated expression of cyclin E and reduced

expression of p27, but expression of p21 remained unaltered (Fig. 5e).

TGF- α induced Sox2 expression via the EGFR/PI3K/AKT signalling pathway

Activity of the TGF- α /EGFR autocrine loop is a hallmark of PCa progression (27). Our laboratory team has demonstrated that TGF- α induces EGFR mRNA and protein expression and that the EGFR/PI3K/AKT pathway plays a key role in proliferation and apoptosis of prostate cancer cells (work in progress). To assess the function of the EGFR/PI3K/AKT pathway in regulating Sox2 expression, we treated DU145 and PC-3 cells with a EGFR/PI3K/AKT pathway inhibitor; on the basis of our previous studies, we treated cells with 50 ng/ml TGF- α . With the use of anti-p-AKT antibody, we conducted time-course analysis of AKT during treatment with the inhibitor LY294002. LY294002 completely blocked phosphorylation of AKT activated by TGF- α (Fig. 6a,b).

Next investigated regulation of Sox2 and survivin protein expression by TGF- α . TGF- α induced Sox2 and survivin expression over time (Fig. 6c,d); maximal levels were reached 24 h after cells were exposed to TGF- α . When we pre-treated DU145 and PC-3 cells with LY294002 for 2 h before TGF- α was added, Sox2 and survivin protein expression markedly declined when LY294002 was applied (Fig. 6b). Taken together, these

Figure 5. Over-expression of Sox2 enhances cell proliferation and promote G₁ to S phase transition in prostate cancer PC-3 cells. Sox2 protein expression was tested by (a) Western blot analysis in PC-3-Mock and PC-3-Sox2 cells. β-actin was used as an internal control. (b) MTT assay for Sox2 over-expressed PC-3 cells. Cells were collected after 24, 48, 72 and 96 h, and the absorbance was measured at 490 nm. (c) Colony-forming unit assays for PC-3-Mock and PC-3-Sox2 cells. 100 PC-3-Mock and PC-3-Sox2 cells were cultured for 9 days, crystal violet staining of clones at day 9. (d) Cell cycle analysis for the effect of Sox2 on cell cycle. PC-3-Mock and PC-3-Sox2 cells were fixed, stained with propidium iodide and the DNA content measured by flow cytometry. (e) Western blot analysis for cyclin E, p21 and p27 in Sox2 over-expressed cells. Band intensity was calculated using Quantity One. Statistical significance was considered at * $P < 0.05$, ** $P < 0.01$, compared with control group. Data are means $\pm$ SEM of three independent experiments.

data indicate that TGF- α up regulated Sox2 and survivin protein expression *via* the EGFR/PI3K/AKT pathway.

Discussion

Sox2, an important transcription factor, has been shown to be over-expressed in most human cancer types so far studied, including prostate cancer. It plays a key role in cell self-renewal, differentiation, proliferation and apoptosis in stem cells and in cancer cells. In the current study, we showed that Sox2 mRNA and protein were expressed in most of types of prostate cancer cells used. Moreover, levels of both Sox2 mRNA and protein were significantly higher in androgen-independent human prostate cancer cells than in androgen-dependent human

prostate cancer cells. These findings suggest that Sox2 may play a key role in progression of prostate cancer.

Evidence has shown that over-expression of Sox2 is oncogenic and significantly induces cell population growth in lung and breast cancers (28,29). Sox2 silencing in glioblastoma tumour-initiating cells causes inhibition of proliferation and loss of tumorigenicity (30). Jeter *et al.* have found that Sox2 was not expressed at the mRNA level in LNCap, DU145 and PC-3 cells (20) and on the contrary, Bae *et al.* found that Sox2 was highly expressed at mRNA and protein level in DU145 and PC-3 cells. They used RNAi to specifically repress expression of Sox2 in DU145, which caused inhibition of tumorigenicity, but the mechanism was not shown to be clear (14). We have demonstrated, here, that Sox2

Figure 6. TGF- α /EGFR/PI3K/AKT signal pathway induce Sox2 expression in prostate cancer DU145 and PC-3 cells. LY294002 inhibition of Sox2 and survivin up-regulation induced by TGF- α in (a) DU145 and (b) PC-3 cells. Cells were treated with or without 50 μ M LY294002 for 2 h and exogenous soluble TGF- α were treated for 24 h and were harvested. Total cell lysates from cells were subjected to immunoblot with specific antibodies as indicated. Time course of Sox2 and survivin protein expression was regulated by 50 ng/ml TGF- α in (c) DU145 and (d) PC-3 cells. Exogenous soluble TGF- α was added to the medium for up to 48 h and Sox2, survivin protein expression level were assessed. The same membrane was also stripped later and reprobed with antibody to s-actin. Quantitative analysis of band density is shown in the right panel normalized to s-actin. Each bar represents the means $\pm$ SEM of three independent experiments. ** P < 0.01 versus normal, ## P < 0.01 compared with TGF- α treatment group.

was expressed at mRNA and protein levels in prostate cancer cells generally; western blot and RT-PCR assays showed that Sox2 expression levels were higher in androgen-independent prostate cancer cell lines DU145, PC-3, PC-3M, PC-3M-1E8 and PC-3M-2B4, compared to LNCap, androgen-dependent prostate cancer cell line. mRNA and protein levels of Sox2 were considerably higher in highly metastatic human prostate cancer cell line DU145, PC-3M-1E8, compared to PC-3M-2B4, the poorly metastatic cell line. Low and moderate levels of Sox2 were detected in PC-3 and PC-3M respectively (Fig. 1b,c). We will, in the future, seek to elucidate whether Sox2 contributes to prostate cancer cell invasion, as well as to determine mechanisms involved in the process. We also observed lower cell proliferation and DNA synthesis when Sox2 expression was inhibited. We observed that knockdown of Sox2 expression induced G₁ cell cycle arrest, while over-expression of Sox2 promoted G₁ to S phase promotion. Thus, Sox2 may regulate the G₁/S phase checkpoint in prostate cancer. Sherr *et al.* have concluded that CDK2 complexed with cyclin E is essential for DNA replication and G₁/S

transition (31) as the cell cycle may be altered by various treatments, through different pathways. Then, we examined expression of three proteins that played key roles in G₁/S transition. Our evidence has shown cyclin E protein expression decreased markedly after Sox2 knockdown and increased after Sox2 over-expression, whereas p27, both an inhibitor and a substrate of cyclin E-CDK2 complex (32), increased markedly after Sox2 knockdown and decreased after Sox2 over-expression. p21, tumour suppressor protein by binding to cyclin-CDK complexes (33), remained unaltered. Our results imply that Sox2 could target p27 and cyclin E proteins to promote cell proliferation and cell cycle progression.

In our study, we have demonstrated that knock down of Sox2 can induce apoptosis in prostate cancer DU145 cells, by suppressing expression of survivin.

EGFR and its downstream signalling pathways are key regulators of tumour cell proliferation in a variety of tumours (30). EGFR is over-expressed in about 30% of PCa (34) and EGFR can be activated by one of seven possible ligands, including TGF- α (35,36). Previous studies have demonstrated that when the disease

progresses to more advanced stages, TGF- α is the pre-dominant ligand for EGFR (37,38). The TGF- α /EGFR autocrine loop is a hallmark of PCa invasion and progression (27,39) and Hu *et al.* have shown that EGFR can up-regulate Sox2 expression in neural precursor cells (40).

Thus, on the basis of these studies and our results, we demonstrate that TGF- α activated PI3K/AKT and up-regulated Sox2 and survivin protein expression in DU145 and PC-3 cells. When LY294002 was applied to block phosphorylation of AKT, expression of Sox2 and survivin proteins decreased markedly, but did not disappear completely. From these results, we conclude that the EGFR/PI3K/AKT pathway is a signal pathway upstream of Sox2 and that TGF- α can up-regulate Sox2 expression by activating it in prostate cancer cells.

Together, our findings indicate that Sox2 is generally expressed in prostate cancer cells, especially in androgen-independent ones. Knockdown of Sox2 expression by RNAi reduced androgen-independent prostate cancer cell proliferation and induced apoptosis by up-regulating p27, down-regulating cyclin E protein expression and inhibiting survivin. Ectopic over-expression of Sox2 resulted in increased cell proliferation, and promoted G₁ to S phase transition, as well as up-regulated cyclin E and down-regulated p27 proteins. TGF- α could up-regulate Sox2 and survivin protein expression via the EGFR/PI3K/AKT pathway. This suggests that Sox2 is involved in the function of TGF- α in PCa progression and thus that Sox2 can be regarded as a new candidate target for prostate cancer treatment.

Acknowledgements

This study has been supported by grants from the Natural Youth Science Foundation of China (No. 81101942) and the Science Foundation of Health Department, Heilongjiang province (No. 2010-201).

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