Sox5 contributes to prostate cancer metastasis and is a master regulator of TGF-β-induced epithelial mesenchymal transition through controlling Twist1 expression

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Background: Metastatic castration-resistant prostate cancer (mCRPC) is one of the main contributors to the death of prostate cancer patients. To date, the detailed molecular mechanisms underlying mCRPC are unclear. Given the crucial role of epithelial–mesenchymal transition (EMT) in cancer metastasis, we aimed to analyse the expression and function of Transforming growth factor-beta (TGF-β) signal-associated protein named Sox5 in mCRPC.

Methods: The protein expression levels were analysed by western blot, immunohistochemistry and immunofluorescence. Luciferase reporter assays and chromatin immunoprecipitation were employed to validate the target of Sox5. The effect of Smad3/Sox5/Twist1 on PCa progression was investigated *in vitro* and *in vivo*.

Results: Here, we found that TGF-β-induced EMT was accompanied by increased Sox5 expression. Interestingly, knockdown of Sox5 expression attenuated EMT induced by TGF-β signalling. Furthermore, we demonstrated that Smad3 could bind to the promoter of Sox5 and regulate its expression. Mechanistically, Sox5 could bind to Twist1 promoter and active Twist1, which initiated EMT. Importantly, knockdown of Sox5 in prostate cancer cells resulted in less of the mesenchymal phenotype and cell migration ability. Furthermore, targeting Sox5 could inhibit prostate cancer progression in a xenograft mouse model. In clinic, patients with high Sox5 expression were more likely to suffer from metastases, and high Sox5 expression also has a lower progression-free survival and cancer specific-survival in clinic database.

Conclusions: Therefore, we propose a new mechanism in which Smad3/Sox5/Twist1 promotes EMT and contributes to PCa progression.

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It is estimated that 161 360 new cases of prostate cancer (PCa) were diagnosed and that 26 730 people died of PCa in 2017 (Siegel et al, 2017). Five-year survival rates can be 100% when PCa is localised; however, once it has spread, the chances of survival are only 28% (Hodson, 2015), and 34.4% of patients were presented with metastatic disease at diagnosis (Steinberger et al, 2016). Metastases are present in at least 80% of castration-resistant prostate cancer (CRPC) patients (Hussain et al, 2016). Cancer metastasis is a multistep process. First, metastases initiate as cancer cells and acquire invasive potential; cancer cells then grow expansively and invade the basement membrane in surrounding tissues, accompanied by angiogenesis; cancer cells eventually become circulating tumour cells (CTCs) and are transported around body; finally, CTC arrest and extravasations to secondary tissues or organs occur to form micro- or macro-metastases (Jiang et al, 2015). Epithelial–mesenchymal transition (EMT) has been shown to play a critical role in the acquisition of invasive potential, thereby promoting metastasis (Thiery and Lim, 2013).

EMT signifies the conversion of polarised immobile epithelial cells into spindle-shaped motile mesenchymal cells and enables cell migratory capacity and invasiveness (Kalluri and Weinberg, 2009; Tsai and Yang, 2013; Jadan et al, 2015). The transforming factor-β (TGF-β) signalling pathway is a potent inducer of EMT and plays a key role in cancer metastases (Massague, 2008). SMAD-mediated canonical signalling and SMAD-independent noncanonical signalling are then required for its downstream signalling activation. Smad2/3 phosphorylation and nuclear translocation are involved in canonical signalling, and the nuclear-localised SMAD complex initiates transcriptional activation or transcriptional repression of several genes (Pickup et al, 2013). Transforming growth factor-beta inhibition has shown certain clinical effects in lung cancer and hepatic cell carcinoma, suggesting that inhibition of TGF-β signalling is an emerging strategy for cancer therapy (Neuzillet et al, 2015). However, the mechanisms by which TGF-β promotes cancer metastasis are largely unknown.

SRY-related high-mobility-group box 5 (Sox5) is a member of the Sox family, consisting of more than 20 Sox genes falling into groups A to H, and belongs to the Sox D group (Lefebvre et al, 2007). Previous studies of Sox5 function focus mainly on chondrogenic differentiation (Lefebvre et al, 2001; Ikeda et al, 2005). Recently, research shows that Sox5 enhances progression of various tumours such as nasopharyngeal carcinoma, pituitary tumour, hepatocellular carcinoma, and breast cancer (Huang et al, 2008; Pei et al, 2014; Renjie W and Haiqian L, 2015; Wang et al, 2015). Its functions in PCa have not been explored yet. Here, we report that Sox5 is responsible for TGF-β-induced EMT and that Smad3–Sox5–Twist1 signalling acts as an axis to promote EMT and contribute to prostate cancer metastasis.

MATERIALS AND METHODS

Cell culture and transfection. CW22RV1, PC-3 and LNCaP cell lines were maintained in RPMI 1640 media containing penicillin (25 units ml^{-1}), streptomycin (25 g ml), and 10% foetal bovine serum (FBS). Cos-1 cell line was maintained in Dulbecco's modified Eagle's medium (Invitrogen, Shanghai, China) containing 10% FBS. Cells were incubated at 37 °C with 5% CO$_2$. For knocking down Sox5 (Gene Copoeia, Guangdong Province, PR China, Catalogue#HSH017632-mH1), X-tremeGENE HP DNA Transfection Reagent (Roche, Shanghai, China) was used. We seeded cells in six-well plates and grew them to 70–90% confluence for transfection. We prepared plasmid DNA-Transfection Reagent complexes (each well required 2000 ng DNA and 6 μl Transfection Reagent), incubated the complexes at room temperature for 25 min, and then added to cells. After 48 h transfection, cells were collected for experiments. Western blot assays were used to confirm the infection efficiency.

Immunohistochemistry. Tumour tissues were fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. The primary antibodies of the rabbit anti-Sox5 (Bioss Biosynth, Beijing, China, bs-17136R), the mouse anti-N-cadherin (Abcam, Shanghai, China, Catalogue# 124397), and the mouse anti-E-cadherin (BD biosciences, Beijing, China, Catalogue# 610181) were used for staining. The primary antibody was recognised by secondary antibody (ZSGB-BIO, PV-6000), and visualised by DAB kit (ZSGB-BIO, ZLI-9019). The IHC scores were calculated as reported with modification to normalise the score (Xie et al, 2015).

RNA isolation and quantitative analysis. Total cellular RNAs were extracted by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 ng) was used to synthesise first strand complementary DNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific, Shanghai, China, #K 1622) according to the manufacturer's protocol. The amount of certain cDNA was measured in real-time PCR assay using Fast Start Universal SYBR Green Master (Roche). Primers were: Sox5 Forward: 5'-AGGTTTGGACTCACTTGACAGG-3', Reverse: 5'-TCCATCTG CTTCCCCATACG-3'; Twist1 Forward: 5'-AGCTACGCCTTCT CCGTCTG-3', Reverse: 5'-CTCCTTCTCTGGAAACAATGACA-3'; and GAPDH Forward: 5'-TGGCTTCATAGG TGACTTCCA-3', Reverse: 5'-AAGGACCTGTCTAGGTTTGATGC-3'.

Western blot analysis. Harvested cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl/pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; 1 mM proteinase inhibitor; 1 mM Na$_3$VO$_4$; 1 mM NaF; 1 mM okadaic acid; and 1 mg ml^{-1} aprotinin, leupeptin, and pepstatin). Samples (30 μg protein) were separated on 8% SDS–PAGE gel and transferred to PVDF membranes at 4 °C (250 mA, 2 h). Membranes were blocked in 5% fat-free milk in TBST for 1 h at room temperature, and incubated with appropriate diluted primary antibodies GAPDH (Sungene Biotech, Tianjin, China, KM9002, 1 : 5000), Sox5 (Bioss Biosynth, bs-17136R, 1 : 1000), N-cadherin (Abcam, Catalogue# 124397, 1 : 1000), Twist1 (Abcam, ab50887, 1 : 50), Histone 3 (Abcam, ab8580, 1 : 1000), and E-cadherin (BD biosciences, Catalogue# 610181, 1 : 1000) were used for staining overnight at 4 °C, then washed 10 min three times, and incubated with HRP-conjugated anti-rabbit or anti-mouse antibody for 1 h at room temperature, washed 10 min three times. The blots were developed in ECL mixture and visualised by Imager.

Migration assay. Cells (105 for LNCaP and CWR22RV1, 5 × 104 for PC-3) after different treatments were re-suspended with serum-free media and seeded in the upper chambers of the transwells. Foetal bovine serum (10%) with or without 10 ng ml^{-1} TGF-β1 (R&D Systems, Shanghai, China) was put in the lower chambers. After 24 h (PC-3) or 48 h (LNCaP and CWR22RV1) incubation, the cells invaded to the lower part of the membrane were harvested, fixed with 75% ethanol, and stained with 0.1% crystal violet (Solarbio, Beijing, China, G1061) in PBS. The invaded cells were counted under microscope. The s.d. was calculated from three independent wells.

Nuclear protein extraction. We prepared cytoplasmic and nuclear extracts as described elsewhere (Wu, 2006). Before the procedure, following reagents were prepared and stored in stock concentrations. 0.5 M sodium fluoride (NaF; Sigma, Shanghai, China), stored at 4 °C; 100 mM phenylmethylsulphonyl fluoride (PMSF; Sigma) solution in isopropanol, stored at − 20 °C; 0.1 M dithiotreitol (DTT; Invitrogen), stored at − 20 °C; 1 mg ml^{-1} leupeptin (Sigma), stored at − 20 °C; 1.25 M β-glycerophosphate disodium salt (Sigma), stored at 4 °C; 1 M sodium vanadate (Sigma), stored at − 20 °C; 1 M potassium chloride (KCl; Aldrich, Milwaukee, WI, USA) stored at room temperature (RT); 1 M HEPES (Sigma), stored at 4 °C; 1 M magnesium chloride hexahydrate (MgCl 2, Sigma), stored at RT; 2 M sucrose (Sigma), stored at

RT; 10% Igepal CA-630 (NP-40; Sigma), stored at RT; 5 M sodium chloride (NaCl; Sigma), stored at RT; and 0.5 M EDTA (Invitrogen), stored at RT. Then the compound was prepared, buffer A: 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 0.5% NP-40, stored at 4 °C; buffer B: 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 2.5% glycerol, stored at 4 °C; and buffer D: 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 8% glycerol, stored at 4 °C. Medium from cultured cells were removed and washed with cold PBS, cells were harvested with a rubber scraper, centrifuged at 550 g for 5 min, and then the supernatant was discarded. To buffers A, B, and D, the following inhibitors were added: 0.5 mM PMSF, 1 mM Na_3VO_4, 0.5 mM DTT, 1 μg ml^{-1} leupeptin, 25 mM β-glycerophosphate, and 10 mM NaF. the pellet in two package cell volume of buffer A with inhibitors was resuspended and kept on ice for 10 min, vortexed briefly, and centrifuged at 2600 g for 30 s. The supernatant was collected and labelled as cytoplasm protein. The pellet in 2/3 package cell volume of buffer B with inhibitors was resuspended. The mixture was resonicated for 5 s and centrifuged at 10 400 g for 5 min. The supernatant with equal volume of buffer D with inhibitors was diluted and labelled nuclear protein.

Immunofluorescence. The cells were seeded on coverslips in six-well culture plates. After various treatments, the cells were fixed (15 min) with 4% paraformaldehyde, permeabilised (5 min) with PBS containing 0.1% Triton X-100, and fixed (10 min) with 4% paraformaldehyde. Then the cells were blocked with goat serum (BOSTER) for 1 h at 37 °C, antibodies Sox5, N-cadherin, or Twist1 were added, incubated at 4 °C overnight. After incubation with affinity-purified antibody Cy5 labelled goat anti-rabbit IgG (H + L) and affinity-purified antibody Dylight 488 labelled goat anti-mouse/rabbit IgG (H + L) (Kirkegaard&Perry Laboratories, Shanghai, China) at 37 °C for 1 h, the cells were washed three times with PBS and stained with DAPI. Images were acquired using confocal microscope with Olympus fluo view 4.0 version.

Chromatin immunoprecipitation (CHIP). LNCaP cells were treated with 10 ng ml^{-1} TGF-β1for 24 h to perform Smad3 (Abcam, ab28379) CHIP assay using EpiQuik Chromatin Immunoprecipitation Kit (Epigentek, Farmingdale, NY, USA) according to the protocol. CW22RV1 cells were used to perform Sox5 (Abcam, ab94396) CHIP assay. PCR was performed using primers specific for the Smad3 or Sox5 binding region in the Sox5 or Twist1 promoter. Sox5 promoter-Forward: 5′-AGTATGGGA-GACGTGTTAAATGAGT-3′.

Sox5 promoter-Reverse: 5′-ACTTCCAGCAGCGGAGTCTG-3′. Twist1 promoter-Forward: 5′-CTTAGGCGCTATCAAATTCCC-3′, Twist1 promoter-Reverse: 5′-AGCGACAGCAGCAATGG-CAAC-3′.
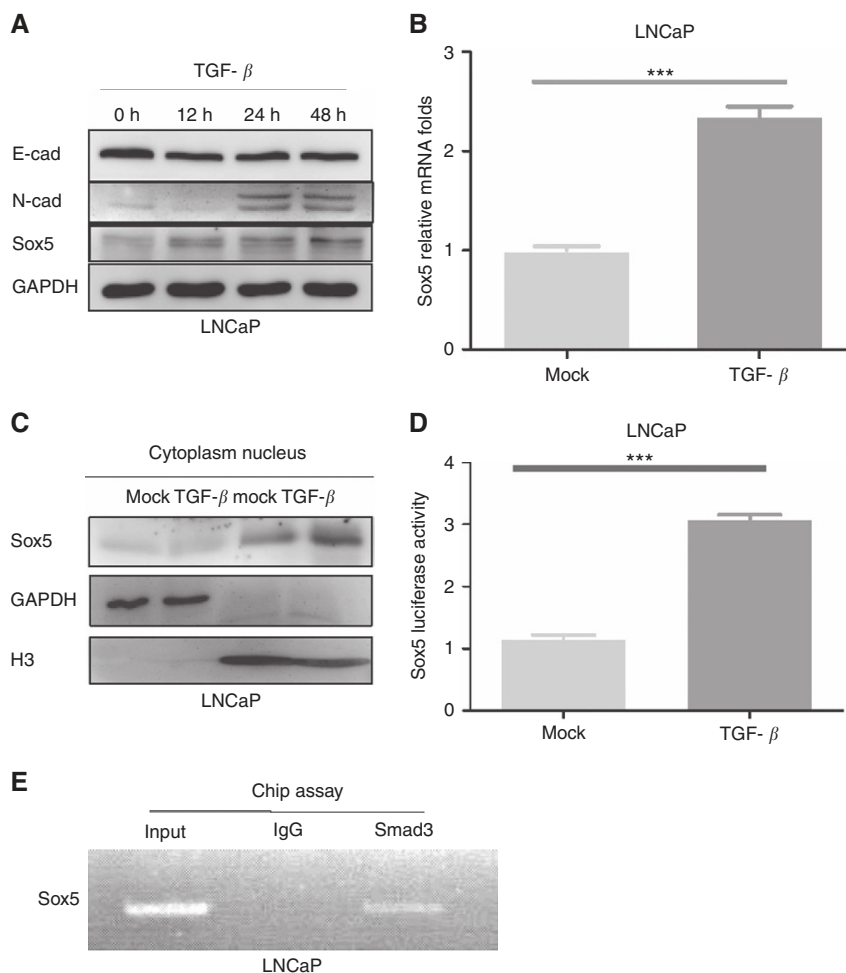

Figure 1. Sox5 is induced by TGF-β. (**A**) LNCaP cells were treated with 10 ng ml^{-1} TGF-β, whole cell lysates were extracted at the indicated time, E-cadherin, N-cadherin, and Sox5 were measured by immunoblotting, and GAPDH was used as a loading control. (**B**) LNCaP treated with vehicle or 10 ng ml^{-1} TGF-β for 24 h. Sox5 mRNA fold change was measured by Q-PCR. (**C**) LNCaP treated with vehicle or 10 ng ml^{-1} TGF-β for 24 h. Cytoplasm and nuclear protein were extracted and measured by immunoblotting. (**D**) Immunofluorescence showed Sox5 expression in LNCaP treated with vehicle or TGF-β. (**E**) LNCaP transfected with Sox5 luciferase plasmid and treated with vehicle or TGF-β. Luciferase values were measured. ***$P < 0.001$.
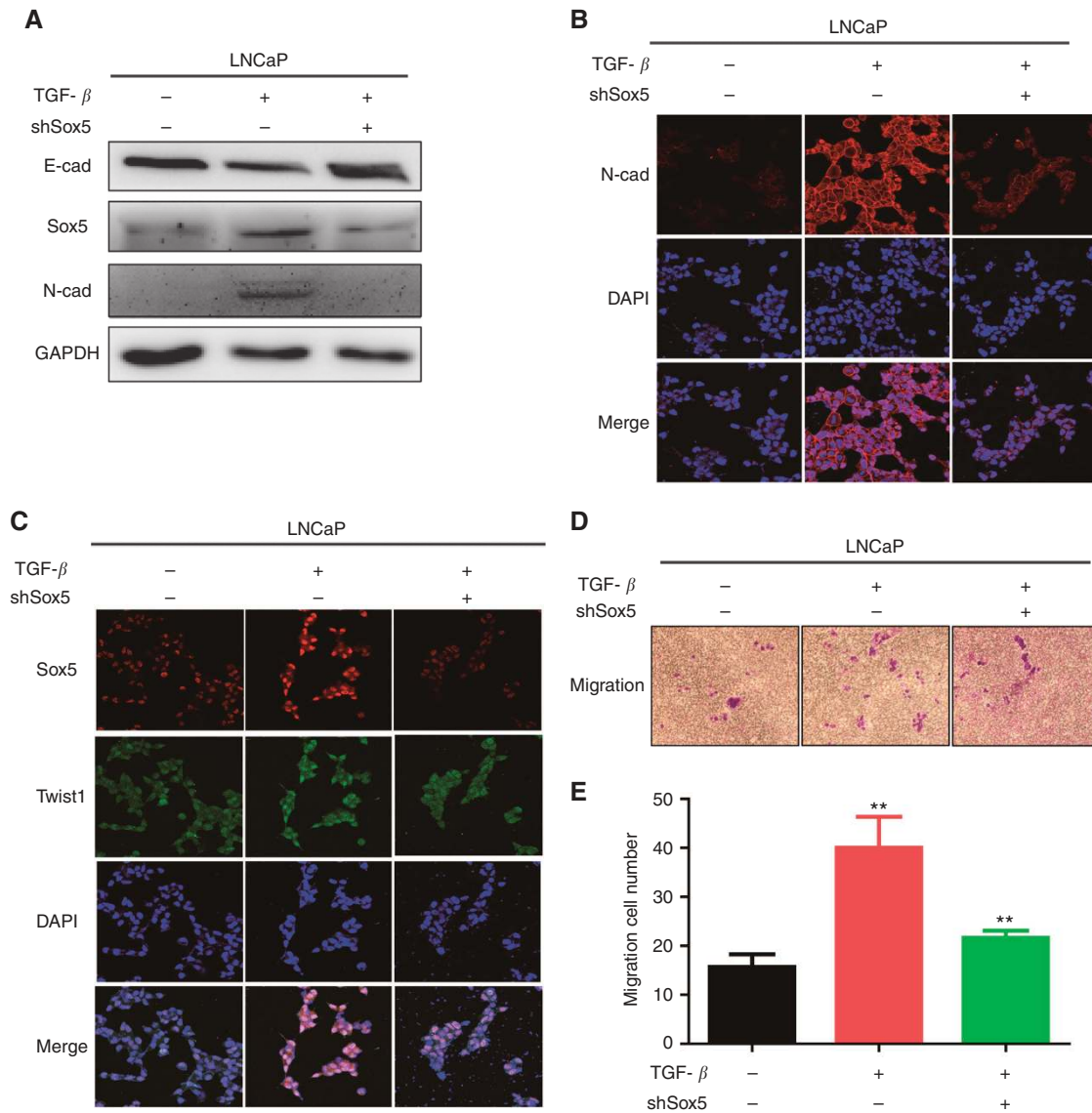
Figure 2. Sox5 is responsible for TGF-β-induced EMT. (A) LNCaP cells were transfected with vehicle or shSox5 plasmid, and then treated with vehicle or TGF-β. E-cadherin, N-cadherin and Sox5 expression were detected by immunoblotting. (B and C) The expression of N-cadherin and Twsit1 were detected by immunofluorescence. (D) The mobility of LNCaP cells with the indicated treatment was measured by migration assays (E). The migrated cells were counted. **$P < 0.01$.

Luciferase reporter assays. Primers with restriction enzyme sites *Hin*dIII/*Nhe*I were designed to amplify the promoter fragment of Sox5 or Twist1 from genomic DNA with a length of 459 or 626 bp. DNA fragments were cloned into pGL4.27 promoter luciferase vector (Promega, Beijing, China). LNCaP cells were transfected with the Sox5 luciferase reporter constructs; Cos-1 cells were transfected with the Twist1 luciferase reporter constructs with or without Sox5 over express plasmid (Gene Copoeia, Catalogue#EX-Z4528-Lv105-5). Supernatants were collected 24 h after stable transfection. Dual-Luciferase Reporter Assay System (Promega) was applied to measure luciferase value according to the manufacturer's instructions.

Xenograft mice model. Male 6- to 8-week-old nude mice were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). The CWR22RV1 cells transfected with vehicle or shSox5 plasmid were injected subcutaneous with matrigel. About 3 weeks later, the tumour was detached and cut into pieces around $1 \times 1 \times 1$ mm size. Tumours were implanted orthotopically in the anterior prostates of the mice, and also subcutaneously. About 2 weeks later, the tumour could be touched. We then monitored the tumour size every day, calculating the tumour volume using the formula $V = 1/2a \times b2$ (a

represents length, *b* represents width). After the mice were killed, the tissue samples were fixed, processed as paraffin tissue sections, and used for immunohistochemistry and H&E analysis.

Statistical analysis. Values were expressed as mean ± s.d. The Student's *t* and ANOVA tests were used to calculate *P*-values. *P*-values were two-sided, and considered statistically significant when $P < 0.05$.

RESULTS

Sox5 is induced by TGF-β/Smad3 signalling. Transforming growth factor-beta signalling has been associated with the invasion of cancer cells and metastasis, and it is a crucial regulator of EMT (Thakur *et al*, 2014; Buczek *et al*, 2016). We conjectured a relation between Sox5 and TGF-β, so we treated LNCaP cells with TGF-β and found that Sox5 expression increased gradually as well as N-cadherin (Figure 1A). The mRNA of Sox5 also increased with the addition of TGF-β (Figure 1B). As a transcription factor, Sox5 mainly resides in the nucleus and regulates gene expression by binding to
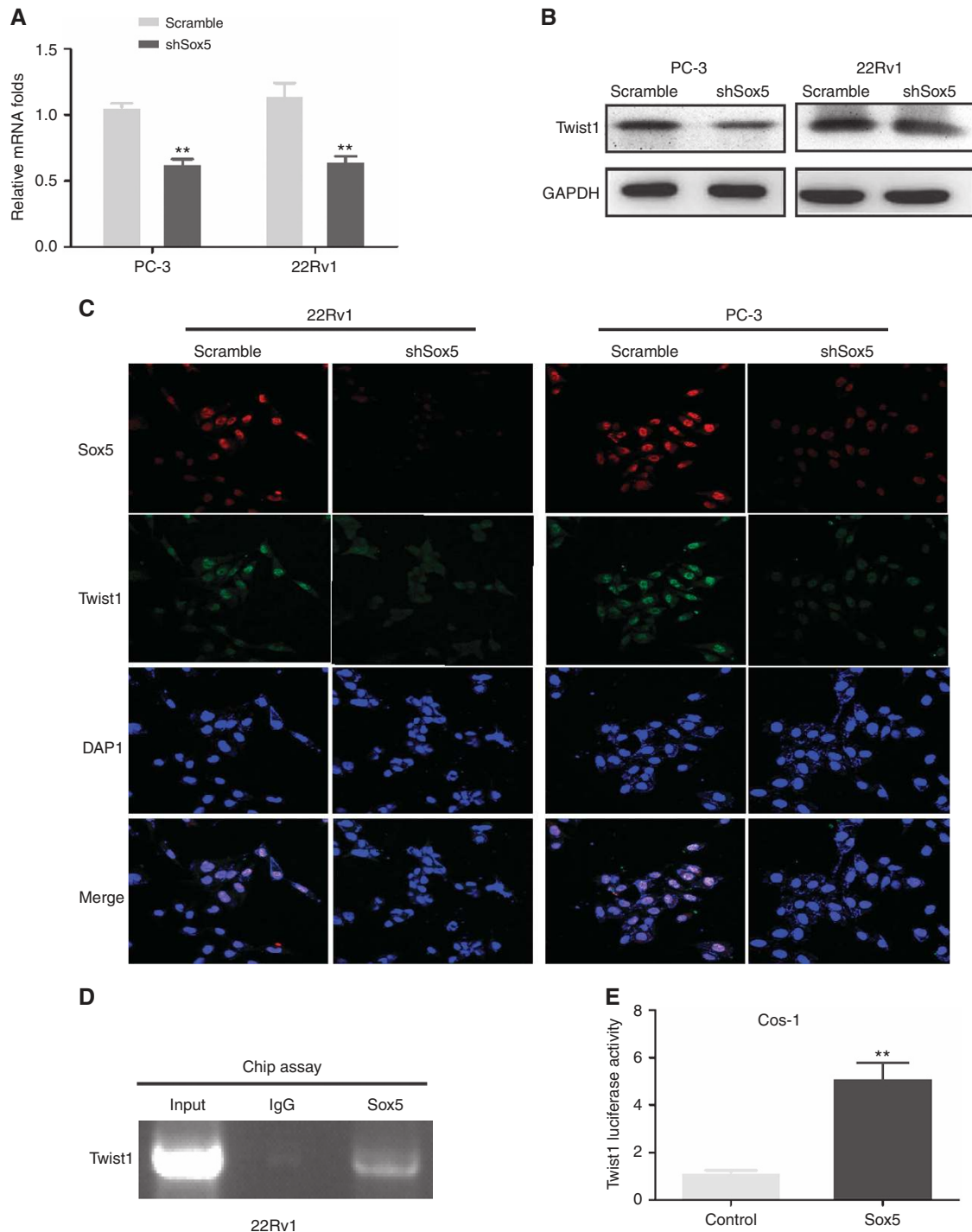
Figure 3. Sox5 regulates EMT transcription factor Twist1 expression. (A) PC-3 and 22Rv1 cells were transfected with control or shSox5 plasmids, Sox5 mRNA were detected by Q-PCR. (B) Twist1 expression in PC-3 and 22Rv1 cells transfected with control or shSox5 plasmids by immunoblotting. (C) Twist1 and Sox5 expression in PC-3 and 22Rv1 cells transfected with control or shSox5 plasmids by immunofluorescence. (D) CHIP-PCR was performed in 22Rv1 cells with IgG as a negative control, showing that Sox5 can bind to the Twist1 promoter. (E) Transfected Twist1 promoter plasmids with or without Sox5 plasmid in Cos-1 cells, then, luciferase values were detected. **$P < 0.01$.

enhancer or promoter regions (Mata-Rocha *et al*, 2014; Liu and Lefebvre, 2015). Nuclear protein extraction determined that Sox5 increased predominantly in the nucleus (Figure 1C). As TGF-β can induce EMT through Sma- and Mad-related family 2 and 3 (SMAD2 and SMAD3) phosphorylation (Thakur *et al*, 2014; Buczek *et al*, 2016), we used JASPAR once again to determine the mechanisms of how TGF-β induces Sox5 expression. We chose to test if Smad3 binds to the Sox5 promoter. Surprisingly, positive results retrieved

from JASPAR, as well as Smad3 CHIP in LNCaP after TGF-β treatment for 24 h, verified the prediction (Figure 1D). To verify Smad3 functional binding to the Sox5 promoter, we amplified a length of 459-bp Sox5 promoter fragments in the CW22RV1 genome, cloned them into the pGL4.27 promoter luciferase vector, and transfected the constructed plasmid into LNCaP. Transforming growth factor-beta treatment increased luciferase intensities (Figure 1E).
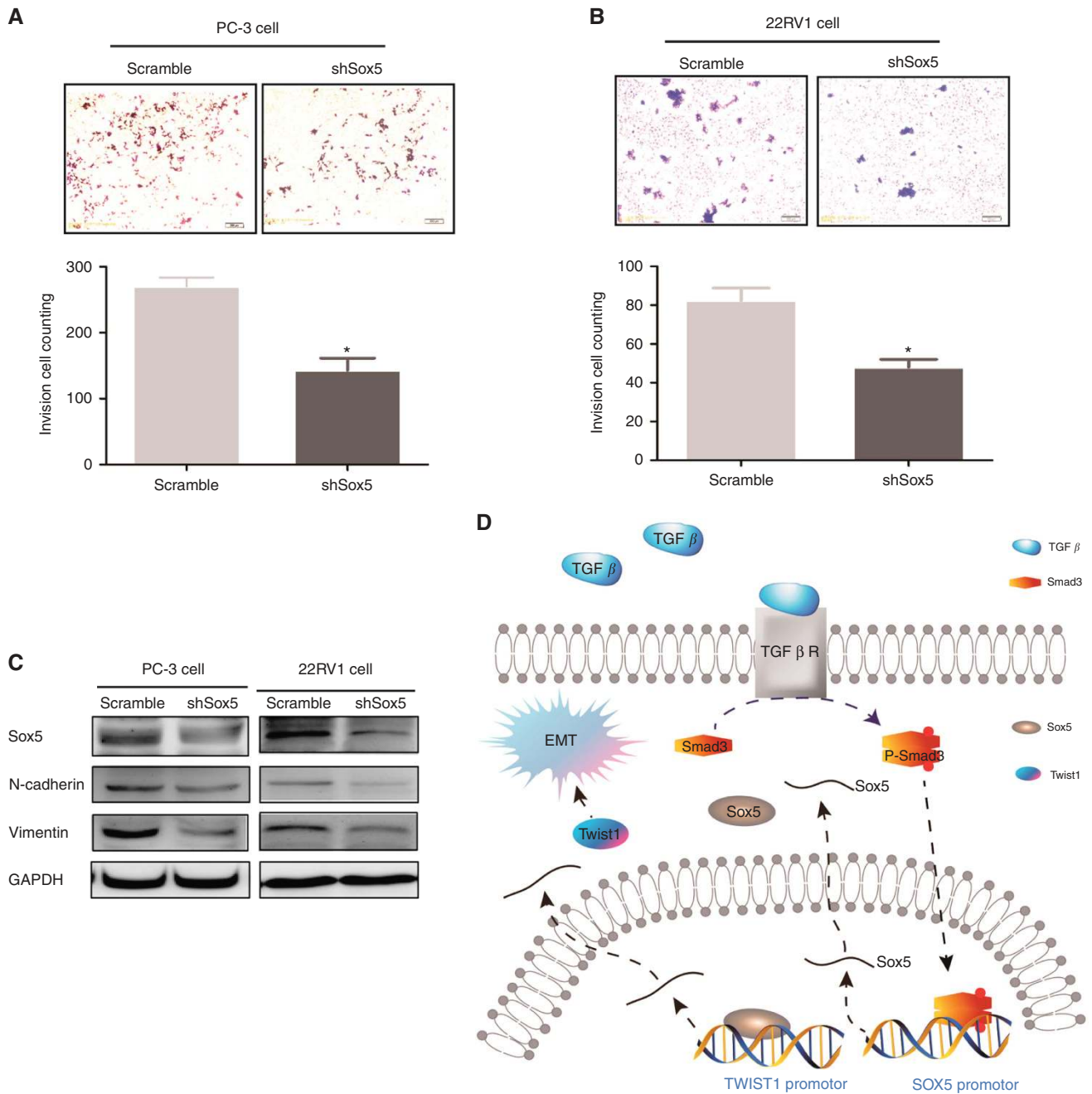
Figure 4. Targeting Sox5 will inhibit PCa cell migration and EMT *in vitro*. (**A**) Images of the invasion ability of control and shSox5 PC-3, and migrated cells were counted. (**B**) Images of the invasion ability of control and shSox5 22RV1, and migrated cells were counted. (**C**) Western blot shows that mesenchymal marker N-cadherin and vimentin expression was decreased in shSox5-PC-3 and shSox5-22RV1. (**D**) Schematic diagram of the Smad3–Sox5–Twist1 axis shows EMT regulating. $*P<0.05$.

Sox5 is responsible for TGF-β-induced EMT and metastasis. To further explore the role of Sox5 in TGF-β-induced EMT, we treated LNCaP with TGF-β and knocked down Sox5 simultaneously. When we treat LNCaP cell with TGF-β, Sox5 and N-cadherin expressions were increased simultaneously. However, E-cadherin expression was decreased. Then we knocked down Sox5 after TGF-β treatment, accompanied by N-cadherin reduction and E-cadherin increase (Figure 2A). The above results were further to be identified by immunofluorescence assay (Figure 2B). EMT regulator, Twist1, was also detected by immunofluorescence assay. The result was the same as N-cadherin that Sox5 could also reverse TGF-β-induced Twist1 expression (Figure 2C). We then performed a migration assay to observe cell mobility in various conditions. Transforming growth factor-beta increased LNCaP cell mobility, and knocking down Sox5

dampened the effect (Figure 2D). Cell counts indicated a significant difference between the shSox5 and vehicle groups (Figure 2E).

Sox5 regulates EMT transcription factor Twist1 expression. In order to find the downstream gene of Sox5 related to EMT regulation, we knockdown Sox5 expression in CWR22RV1 and PC-3 cells, which have high expression of Sox5 (Figure 3A). Previous data suggest that Sox5 regulates Twist1 expression in hepatocellular carcinoma and breast cancer (Pei *et al*, 2014; Wang *et al*, 2015). We speculated that Sox5 also regulates Twist1 expression in PCa. When we knocked down Sox5, Twist1 expression also downregulated in CWR22RV1 and PC-3 cells (Figure 3B). Immunofluorescence suggested a reliable Sox5 knockdown effect and confirmed the downregulation of Twist1 (Figure 3C). A prediction from JASPAR
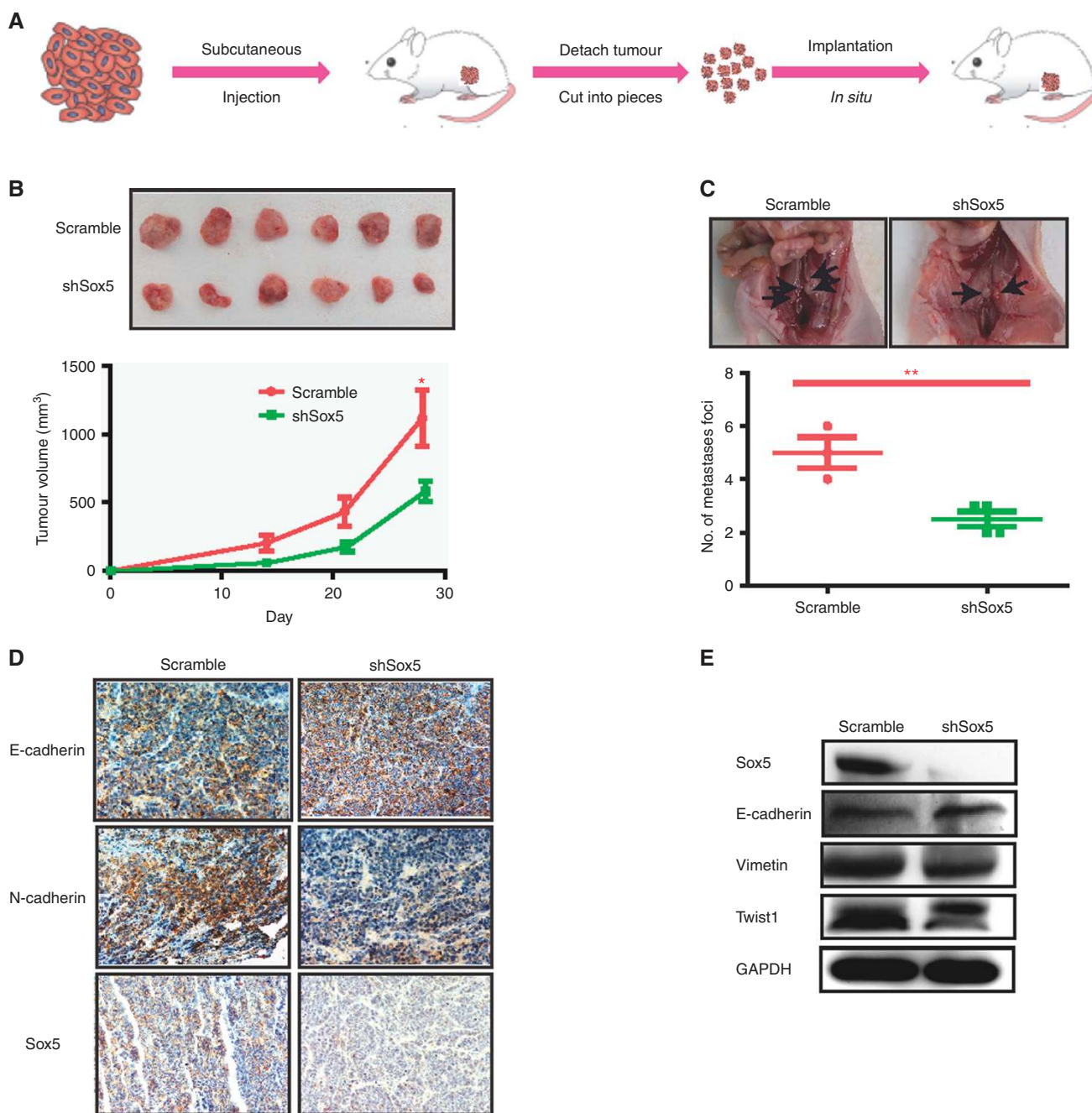
Figure 5. Target Sox5 inhibits prostate cancer progression in a xenograft mouse model. (**A**) Schematic diagram of the procedure. 22Rv1 cells were transfected with vehicle or shSox5 plasmid and injected subcutaneously. Approximately 3 weeks later, the tumours were detached and cut into pieces, then were implanted orthotopically and subcutaneously. (**B**) Subcutaneous tumour growth was monitored (down). The mice were killed and the tumours were separated from mice (top). (**C**) Lymph node metastases are indicated (black arrows, top). The numbers of metastases were calculated (down). (**D**) IHC was applied to evaluate Sox5, E-cadherin, and N-cadherin expression. (**E**) WB was applied to evaluate Sox5, E-cadherin, Vimentin, and Twist1 expression. *$P<0.05$, **$P<0.01$.

(http://jaspar.genereg.net/) indicated that Sox5 can bind to the Twist1 promoter region. We performed CHIP assay in CW22RV1 and confirmed that Sox5 can bind to the Twist1 promoter (Figure 3D). To verify that the binding is functional, we amplified a length of 626-bp Twist1 promoter fragments in the CW22RV1 genome and cloned them into a pGL4.27 promoter luciferase vector. Co-transfection of the constructed plasmid and the Sox5 over-expression plasmid into Cos-1 increased the luciferase intensities compared with the constructed plasmid and the vehicle plasmid (Figure 3E). Thus, we suggest that Sox5 regulates EMT transcription factor Twist1 expression, which may be responsible for Sox5-induced EMT.

Hence, the Smad3–Sox5–Twist1 axis plays a vital role in EMT, and the direct action of Smad3–Sox5 and Sox5–Twist1 interaction indicate that Sox5 is the mainstay of the axis.

The effects of Sox5 on prostate cancer cell mobility. We then performed migration assays to test cell mobility. The migration assay indicated that PC-3 and CWR22RV1 cells have decreased mobility after inhibiting Sox5 expression, and the number of cells migrating through the transwell was much less than in the control (Figure 4A and B). WB also suggested that N-cadherin and vimentin expression reduced in these two prostate cancer cells with lower Sox5 expression (Figure 4C).
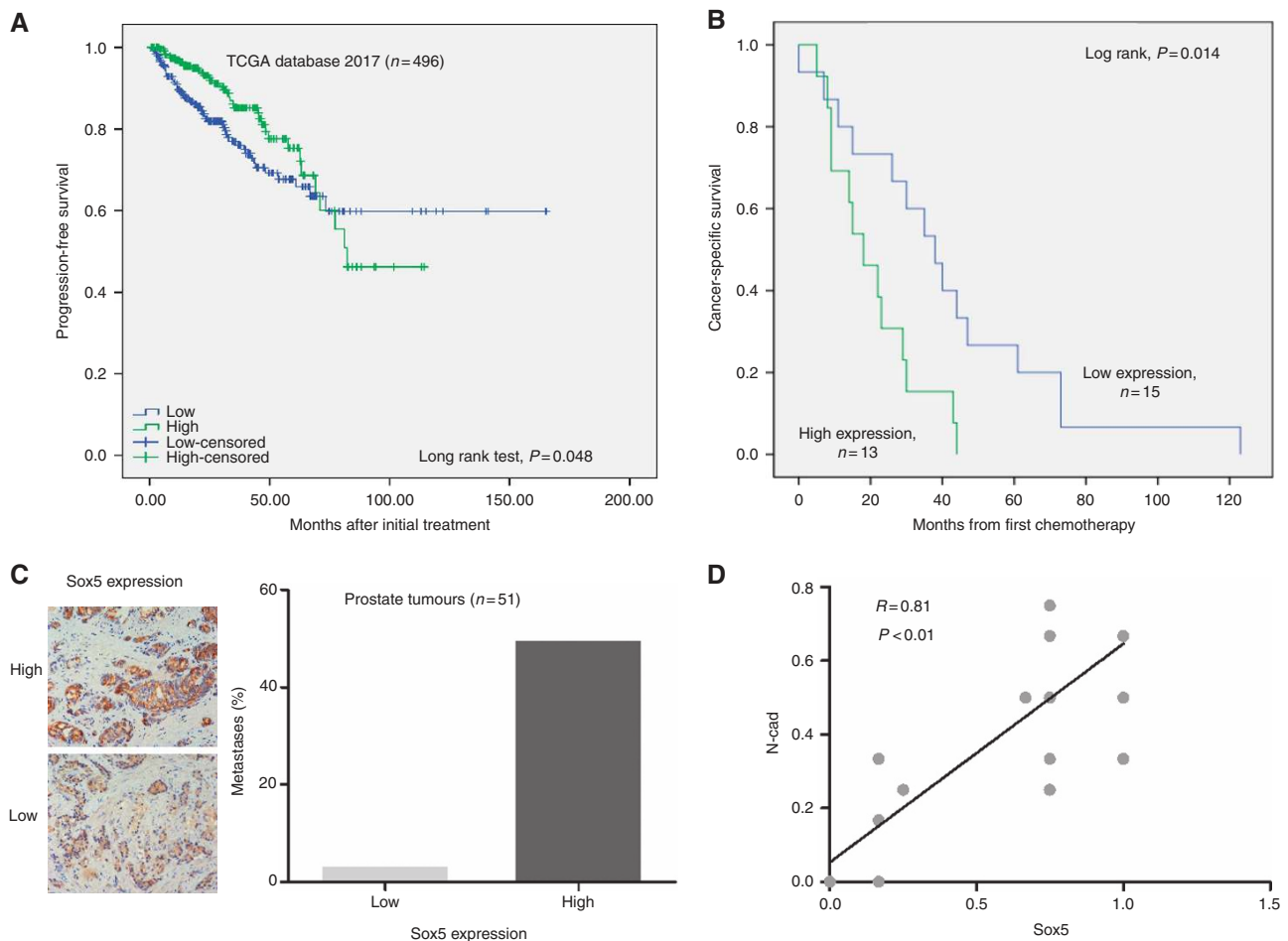
Figure 6. Sox5 is required for prostate cancer metastasis. (**A**) Progression-free survival of the TCGA prostate cancer patients with high or low levels of Sox5 expression. (**B**) Cancer-specific survival of the metastatic prostate cancer patients with high or low levels of Sox5 expression in Grasso et al. (2012). (**C**) Fifty-one samples were stratified into low and high Sox5 expression groups with a cut-off value of 0.5intensity (left). The metastases rates in low and high Sox5 expression groups were compared by Chi-square test of the four table (right). (**D**) Sox5 expression was positively correlated with N-cadherin expression with a correlation coefficient of 0.81.

The schematic diagram shows that the Smad3–Sox5–Twist1 axis invoices EMT regulating and contributes to PCa metastasis (Figure 4D).

Targeting Sox5 inhibits PCa progression in xenograft mouse model. *In vitro* data suggest that Sox5 promotes PCa progression via EMT, so we completed *in vivo* experiments to corroborate this. We prepared approximately 1×10^7 vehicle and shSox5 CW22RV1 cells to inject into subcutaneous tissues. Approximately 3 weeks later, the tumours were detached and cut into pieces and orthotopic and subcutaneous implantation were performed to observe tumour growth and metastases (Figure 5A). Subcutaneous tumours can be palpable approximately 2 weeks after implantation, and their growth was monitored every other day. Data showed that the tumours were much smaller in the Sox5 knockdown group (Figure 5B). To evaluate metastases, we dissected the mice and found enlargement of the abdominal aortic lymph node (Figure 5C). After counting the number of metastatic foci, we found that knockdown of Sox5 can repress tumour metastases (Figure 5C). IHC showed that tumours in the shSox5 group expressed low levels of Sox5 and N-cadherin, but high levels of E-cadherin (Figure 5D). WB in tumour samples identified that lower mesenchymal markers expression, such as Vimentin and Twist1, accompanied with higher epithelial marker expression, such as E-cadherin (Figure 5E).

Together, *in vitro* and *in vivo* data suggest that Sox5 promotes EMT and contributes to PCa metastases. Mechanistically, TGF-β in the microenvironment induces Sox5 expression via Smad3 phosphorylation. Twist1 is regulated by Sox5, resulting in EMT and metastasis in prostate cancer.

Sox5 is correlated with metastasis in clinical prostate tumours. The correlation of Sox5 expression with clinical progression was analysed for the TCGA cohort, and it was found that Sox5 elevation was linked to accelerated progression in prostate cancer (Figure 6A). In 28 prostate cancer patients with metastasis, low level of Sox5 had better cancer-specific survival (Figure 6B). To further investigate the clinical relevance of Sox5, we examined a cohort ($n = 51$) of prostate cancer clinical samples collected from the Second Hospital of Tianjin Medical University by IHC staining. The expression of Sox5 was stratified into low and high groups according to stain intensity and extent (Figure 6C, top). Combined analysis of the expression and clinical data showed that patients with high Sox5 expression had a propensity to suffer from metastases (Figure 6C, bottom). We also found that Sox5 expression was highly correlated with N-cadherin expression ($R = 0.81$, $P < 0.01$; Figure 6D).

Together, the results from clinical data and *in vitro* experiments suggested Sox5 contributes to prostate cancer metastases through EMT.

DISCUSSION

Prior studies show that Sox5 plays an important role in the progression of various cancers. In prostate cancer, proof from TCGA database showed Sox5 was correlated to prostate progression (Figure 6A). To further unclear the role of Sox5 in prostate cancer progression, we studied the relationship between the level of Sox5 and metastasis. As a result, we found that the patients with high level Sox5 was more likely to suffer from metastasis, which was consistent with previous research (Ma et al, 2009). In another cohort of 28 prostate cancer patients with metastasis, low level of Sox5 had a better cancer-specific survival (Figure 6B). According to the clinic data, we conclude that Sox5 will accelerate the death of late stage PCa patients. However, the function of Sox5 in PCa progression has not previously been explored. In our research, we found that Sox5 was highly correlated with N-cadherin expression. Sox5 and EMT were linked by transcription factor Twist1 in our data. We further demonstrated that Sox5 could regulate Twist1 expression by CHIP and luciferase assays. Twist1 is known to promote tumour metastasis through inducing EMT (Eckert et al, 2011). It is well known that TGF-β can induce Twist1 expression; one mechanism is by Smad-mediated expression of HMGA2 (Thuault et al, 2006). In the process of induction of chondrogenic differentiation of stromal stem cells by medium containing TGF-β3, dexamethasone, and bone morphogenetic protein (BMP)-6, Sox5 expression was increased five folds (Sekiya et al, 2002). Considering the common relationship between TGF-β-Twist1/EMT and Sox5-Twist1/EMT, we speculated that Sox5 can be induced by TGF-β. Evidence from our data confirmed that TGF-β could induce Sox5 expression via Smad3 phosphorylation. Targeting Sox5 could retard EMT induced by TGF-β signal. Thus, we propose a novel mechanism for Smad3–Sox5–Twist1 that involves TGF-β-induced EMT. The origins of TGF-β include carcinoma-associated fibroblasts, extra-cellular matrix, and tumour cells (Ao et al, 2007). TGF-β can act on tumour cells in an autocrine, paracrine, and sometimes endocrine manner (Ten Dijke and Arthur, 2007). There are many challenges to develop TGF-β inhibitors due to the tumour microenvironment and its manner of action, and it is difficult to balance proliferation and metastases as TGF-β has dual effects on cancer cell proliferation (Neuzillet et al, 2015). Our research prompted targeting Sox5, not only to inhibit EMT and metastases but also to repress PCa cell proliferation. It is worth of further study in future.

Overall, our results demonstrate that Sox5 is a critical component of TGF-β signalling. The uncovering of this TGF-β–Sox5–Twist1 axis will extend our comprehension of TGF-β network complexity and argue for Sox5 as a new option to target TGF-β signalling for cancer intervention.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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