

Zeb1 promotes androgen independence of prostate cancer via induction of stem cell-like properties

Ping Li¹, Jia Wang², Mingliang Chu¹, Kaiqing Zhang², Ru Yang¹ and Wei-Qiang Gao^{1,2}

¹State Key Laboratory of Oncogenes and Related Genes, Stem Cell Research Center, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200030, China; ²Med-X Research Institute, Shanghai Jiao Tong University, Shanghai 200030, China
Corresponding authors: Wei-Qiang Gao. Email: gao.weiqiang@sjtu.edu.cn and Ru Yang. Email: yangru@yahoo.com

Abstract

Current androgen deprivation therapy often leads to androgen independence. However, mechanism of the therapeutic failure is still not well understood. Here, we demonstrate elevated expression of Zeb1 in androgen-independent prostate cancer cells and prostate tumors of castrated PTEN conditional knockout mice. While Zeb1 shRNA resulted in a sensitization of androgen-independent prostate cancer cells, forced Zeb1 expression caused androgen-dependent prostate cancer cells to be more resistant to androgen deprivation. Moreover, such effects appeared to be mediated by induction of pluripotent genes or stem cell-like properties. Collectively, these findings suggest that inhibition of Zeb1 might be a potential therapeutic strategy for treatment of androgen-independent prostate cancer.

Keywords: Zeb1, epithelial–mesenchymal transition, androgen-independent prostate cancer, cancer stem cells

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Introduction

Although androgen deprivation therapy remains the mainstay therapeutic strategy for prostate cancer, there still exists a serious clinical problem, that is, initially androgen-dependent prostate cancer (ADPC) would eventually progress to androgen-independent prostate cancer (AIPC). Once it reaches the androgen independence stage, the disease will have a very poor prognosis, with a median survival of only approximately 12 months.¹ However, to date, the molecular mechanism underlying the androgen independence remains not well understood.

Our previous study has reported an interesting phenomenon that castration induces epithelial–mesenchymal transition (EMT) via a possible AR-Zeb1 bidirectional, negative feedback loop.² The EMT transcription factor Zinc-finger E-box-binding homeobox factor 1 (Zeb1), also known as TCF8 or deltaEF1, is proposed to play a crucial role in many types of human carcinomas such as breast cancer³ and pancreatic cancer.⁴ Very importantly, a recent study by Weinberg group indicates that Zeb1 is sufficient to switch the cells from a non-cancer stem cell to a cancer stem cell state and is required for the maintenance of the stemness of breast cancer stem cells.³ However, whether and how down-regulation or up-regulation of Zeb1 influences androgen independence of prostate cancer have not been directly determined.

In the present study, we set forth to investigate the biological functions of Zeb1 in the development of androgen

independence of prostate cancer. Our findings provide direct evidence indicating that Zeb1 promotes prostate cancer androgen independence and suggest that Zeb1 can be a potential therapeutic target for treatment of AIPC.

Materials and methods

Cell lines

Human prostate cancer cell lines, including LNCaP, C4-2B, PC3 AR(+), PC3, CW22-RV1, and DU145 (ATCC), were grown in RPMI-1640 or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco), 100 U/ml penicillin, and 0.1 mg/mL streptomycin (Gibco) at 37°C in humidified 5% CO₂ atmosphere.

Quantitative real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and 2 µg total RNA was reverse transcribed for cDNA synthesis using the high-capacity reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) amplification was performed on the Applied Biosystems Step One Plus[®] Real-Time PCR Systems (Singapore). A panel of primer pairs was used following the SYBR[®] Green method (Toyobo, Japan) (Supplementary Table 1). Specific gene expression was normalized to β-actin levels using the ΔΔCt method, with each sample being carried out in triplicates.

Immunoprecipitation and Western blotting

For immunoprecipitation (IP), cells were washed with ice-cold PBS and lysed in a lysis buffer supplemented with proteinase inhibitors (Cocktail Kit, Prod#78410, Rockford, USA) and phosphatase inhibitors (Roche). Cell lysates were incubated with a specific antibody or equal quality IgG for IP overnight at 4°C. Protein G Agarose anti-Rabbit IP Beads (Roche) were then added and incubated for another 4 h at 4°C. The immunoprecipitates were washed six times with the IP buffer and boiled for 10 min in 1 × SDS loading buffer. Immunoprecipitated proteins were detected by following Western blot.

For Western blot assays, total cell proteins were extracted from all the cell lines using a modified radioimmunoprecipitation buffer (RIPA, Prod#89901, Rockford, USA) supplemented with proteinase inhibitors. The protein concentration was examined by the BCA Protein Assay (Bio-Rad, Hercules, CA). All primary antibodies were used at a 1:1000 dilution and incubated at 4°C overnight. Antibody against Zeb1 was purchased from Cell Signaling Technology Inc. (#3396). Antibody against E-cadherin was purchased from BD Transduction Laboratories™ (#610181). Antibody against Vimentin (#2707-1) and Sox2 (#2683-1) were purchased from Epitomics, an Abcam Company. After washing three times with TBST, the membrane was incubated with secondary antibodies (1:1000, Cell Signaling Technology) against horseradish peroxidase-conjugated rabbit or mouse immunoglobulin G and the signals were measured by enhanced chemiluminescence Western blotting system (Millipore). Antibody against β-actin (Cell Signaling Technology Inc. #4970) was used as an internal loading control.

Immunofluorescent staining

All the cell lines were grown on Chamber slides in an appropriate medium as described above for 24 h. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature. After washing three times with PBS for 5 min each, the cells were blocked with a blocking buffer for 1 h at room temperature. After blocking, the cells were incubated with primary antibodies at 4°C overnight. Then, the cells were washed three times with PBS followed by incubation with a relevant fluorochrome-conjugated secondary antibody for 2 h at room temperature in dark. After three washes with PBS, the slides were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., #H-1200, Burlingame, CA).

Plasmids, transfection, and lentivirus production

Zeb1 shRNA were cloned into lentiviral vector pUCTP. The cDNA of Zeb1 was cloned into lentiviral expression vector pLV.Des2d.P/puro. The sequences of Zeb1 shRNA were listed in Supplementary Table 2.

Lentiviruses were produced by co-transfection of 293 T cells with a lentiviral vector, and packaging vectors pCMV-DR8 and pMD2.2 using the calcium phosphate transfection method. At 48 h post-transfection, lentiviral supernatant

was collected, filtered and mixed with polybrene (8 μg/mL), then added directly into target cell lines for infection. Starting 48 h post-infection, infected cells were then selected with 5 μg/mL puromycin for about 14 days to generate the stable transfectants.

Cell proliferation assay and drug sensitivity assay

Cell Counting Kit 8 (CCK-8) (Dojindo, Kumamoto, Japan) was used to analyze the rate of cell proliferation. Cells were seeded into 96-well plates with 5000 cells per well and cultured for 24 h, 48 h, 72 h, and 96 h. For drug sensitivity assay, cells were treated with different doses of bicalutamide. Then, 10 μL of CCK-8 solution was added to each well and incubated for 2 h. The optical density (OD) value was detected at a wave-length of 450 nm on the microplate reader (BioTek). Cell survival rate was calculated follow the formula below: $(OD \text{ (treated)} - OD \text{ (blank)}) / (OD \text{ (control)} - OD \text{ (blank)}) \times 100\%$.

Colony assay and sphere assay

For colony assay, 1000 cells per well were plated in 6-well culture plate and cultured for about two weeks. Then, colony cells were fixed with 4% paraformaldehyde and using 0.1% crystal violet for stain. For sphere assay, 500 DU145 cells and 1000 LNCaP cells per well were plated in six-well ultra low cluster plate (Corning, NY, USA) and cultured in DMEM:F12 with EGF (20 ng/mL), bFGF (20 ng/mL) and B27 (2%) for about one week. Further analysis was performed by counting the colony and sphere numbers under a Nikon microscope.

Cell migration assays

Transwell assay. The top chambers were seeded with 3×10^4 cells in serum-free media and 20% FBS was used as the chemoattractant in the bottom chamber (Corning, #3422). Then, migrated cells were fixed with 4% paraformaldehyde and using 0.1% crystal violet for stain. For further quantification of the migrated cells, five random regions of the filter were counted under a Nikon microscope. Three filters were used and the experiments were carried out in triplicates.

Wound healing assay. Scratch wounds were made in confluent cell monolayers using a pipette tip. Monolayers were washed twice with PBS and the cells were cultured in serum-free media. Cell migration was recorded in five different microscopic fields.

In vivo growth assay and mouse strains

Five-week-old male BALB/C nude mice (SLAC, Shanghai) were injected s.c. in the flank with 5×10^6 LNCaP cells or 2×10^6 DU145 cells mixed with an equal volume of matrigel (BD Biosciences, Bedford, MA, USA). Tumor growth was evaluated by measuring the length and width with a caliper and the volume of each tumor was calculated by the following formula: $\text{volume} = \pi/6 \times \text{tumor length} \times \text{tumor width}^2$.

PTEN conditional knockout mice were generated as previously described.⁵ To investigate the association of Zeb1

with androgen independence, some PTEN deletion mice and male athymic nude mice which harbored prostate tumor cells were castrated and some received sham surgery as controls. All mice were housed and bred in the SPF animal facility of Ren Ji Hospital in a pathogen-free environment with controlled temperature and humidity.

Statistical data analysis

Statistical evaluation was conducted using the Student's *t*-test and presented as the mean \pm SEM of at least three independent experiments. Two-sided probability value less than 0.05 was considered to be statistically significant. Prism GraphPad5 was used to perform all the plots and data analysis.

Results

Zeb1 is up-regulated in AIPC cells compared to ADPC cells

To examine whether Zeb1 expression is up-regulated during development of androgen independence, we utilized three pairs of AIPC and ADPC cell lines including C4-2B vs. LNCaP, PC3 vs. PC3-AR+, and DU145 vs. CW22-RV1^{6,7} and compared their expression levels of Zeb1. As shown in Figures 1(a) to (d), androgen-independent cells showed a higher level of Zeb1 and Vimentin, but a lower level of E-cadherin at both mRNA and protein levels, as revealed by qRT-PCR and immunoblotting analyses. Immunofluorescence staining also showed the elevated expression of Zeb1 in the AIPC cells as compared to those ADPC cells (Figure 1(e)).

To extend the observations from these cell lines to clinical scenarios, we analyzed microarray data from the Gene Expression Omnibus (GEO) database and found that Zeb1 was indeed significantly increased in the patients suffering from AIPC compared with those with ADPC (Figure 2(a), $P < 0.05$) (GDS1390). To provide additional supporting data for the positive correlation between Zeb1 expression and AIPC, we used the PTEN conditional knockout mouse prostate cancer model.⁸ As shown in Figure 2(b), prostate tumors from the *Pten*^{-/-} mice after castration displayed enhanced Zeb1 expression compared to those mice with a sham surgery. Similar enhanced level of Vimentin was also observed in the *Pten*^{-/-} prostate tumors after castration (Figure 2(c)). Interestingly, double immunostaining for Vimentin and E-cadherin showed that some of the tumor cells co-expressed both Vimentin and E-cadherin (Figure 2(c)), supporting the notion that these cells are in the process of undergoing EMT. Collectively, these data strongly suggest that there is a positive correlation between Zeb1 expression and AIPC.

Zeb1 expression is elevated in prostate cancer cell lines that show higher migration ability and in clinical prostate tumor specimens with angio-lymphatic invasion

An important clinical symptom of AIPC is the distal metastasis. As expected, AIPC PC3 cell had a higher migration ability than ADPC PC3 AR (+) cell (Figure 3(a) to (b), $P < 0.01$). In addition, data analysis from the Oncomine

database revealed significantly increased Zeb1 expression in the patients with angio-lymphatic invasion compared with those without angio-lymphatic invasion (Figure 3(c), $P < 0.05$).^{9,10} To further investigate whether Zeb1 plays a role in the metastasis of AIPC, we analyzed the GEO database that contains benign, malignant, and metastatic prostate cancer specimens. Notably, compared to the benign prostates, Zeb1 level was significantly higher not only in prostate cancer tissues but also in metastatic prostate tumor specimens (Figure 3(d)) ($P < 0.01$, GDS 3289). Taken together, these results demonstrate that Zeb1 expression is elevated in AIPC cells that have a higher migration capability and its expression level is positively correlated with the tumors with angio-lymphatic invasion.

Forced expression of Zeb1 enhances androgen independence and migration ability of prostate cancer cells

To directly validate the significance of Zeb1 expression in the development of AIPC, we transfected a lentiviral vector containing ectopic Zeb1 expression into LNCaP cells which showed relatively low Zeb1 expression at both mRNA and protein levels (Figure 1(a) and (d)). Both RT-PCR and immunostaining confirmed overexpression of Zeb1 in LNCaP cells at both mRNA and protein levels (Supplementary Figures 1(a) and (b)). As shown in Figure 4(a), after treatment with different doses of bicalutamide, an androgen agonist, cell survival rate was much higher in LNCaP-Zeb1 cells than in the vector control cells, indicating that overexpression of Zeb1 significantly enhanced androgen independence in the LNCaP cells that were originally androgen-dependent. To further investigate the effect of Zeb1 on the development of AIPC *in vivo*, we performed xenograft experiments in adult male athymic nude mice with LNCaP-Ctl and LNCaP-Zeb1 cells in Matrigel. When the tumors reached approximately 600 mm³ in size, the mice were castrated. As shown in Figure 4(b), the LNCaP-Zeb1 cells had a significantly higher growth advantage following castration of the host than those LNCaP-Ctl cells. These data indicate that overexpression of Zeb1 causes these cells to become more resistant to androgen deprivation treatment.

In addition, overexpression of Zeb1 in LNCaP cells also led to an elongated and scattered mesenchymal morphology *in vitro* (Supplementary Figure 2(a)), one of the properties of cells undergoing EMT. We also found that overexpression of Zeb1 in LNCaP cells caused a significant decrease of E-cadherin expression compared with the vector control (Supplementary Figure 2(b)). Because expression of the epithelial marker E-cadherin is negatively associated with the ability of cell migration, we studied the migration capacity between Zeb1 transfected cells and vector control cells using a transwell migration assay. As shown in Figure 4(c) to (d), overexpression of Zeb1 resulted in a higher migration rate in LNCaP cells ($P < 0.05$).

Knockdown of Zeb1 inhibits androgen independence and migration ability of prostate cancer cells

To determine whether knockdown of Zeb1 could influence androgen independence and migration ability of prostate

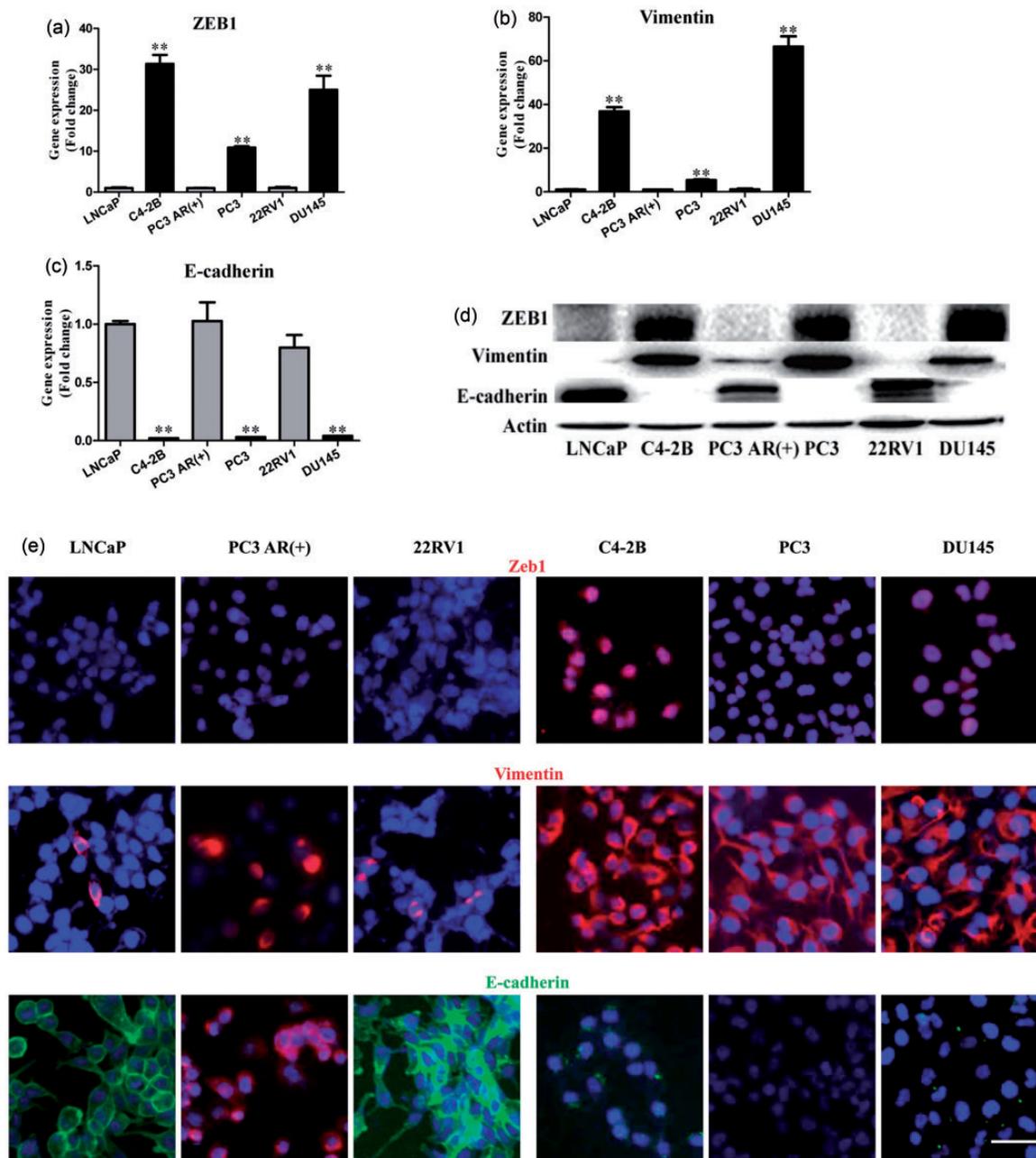


Figure 1 The level of Zeb1 is higher in AIPC cell lines than ADPC cell lines. (a) to (d) qRT-PCR and Western blot analysis of Zeb1, Vimentin, and E-cadherin expression in ADPC cell lines (LNCaP, PC3 AR(+), and 22RV1) and AIPC cell lines (C4-2B, PC3, and DU145). Actin was used as a negative control. (e) Further validation of Zeb1, Vimentin, and E-cadherin expression in all the cells mentioned above using immunofluorescent staining. Bars, 50 μ m; **, $P < 0.01$.

cancer cells, we transfected a lentiviral shRNA targeting Zeb1 into DU145 and PC3 cell lines which displayed relatively high Zeb1 expression at both mRNA and protein levels (Figure 1 (a) and (d)). RT-PCR analysis confirmed an approximate 70% reduction in mRNA level (Supplementary Figure 1(c) and (d)). Down-regulation of Zeb1 caused DU145 cells to be more sensitive to bicalutamide (Figure 5(a), $P < 0.05$). In addition, we also found that down-regulation of Zeb1 in PC3 cells caused a reversed EMT process, called as mesenchymal-to-epithelial transition (MET), with morphological changes from an elongated to a more round, tightly packed shape (Supplementary

Figure 2(c)). Furthermore, as expected, increased E-cadherin expression was observed in these cells after transfection with sh-Zeb1 (Supplementary Figure 2(d), $P < 0.01$). We then investigated whether down-regulation of Zeb1 led to an inhibition of prostate cancer cell migration capacity. Using a transwell assay and a wound healing assay, we found that the migration rate of DU145 sh-Zeb1 cells was significantly lower than DU145 vector control cells (Figure 5(b) to (d), $P < 0.01$), indicating that Zeb1 knock-down suppresses the ability of cell migration. Similar results were also observed in PC3 cells (Supplementary Figure 3(a) to (c)).

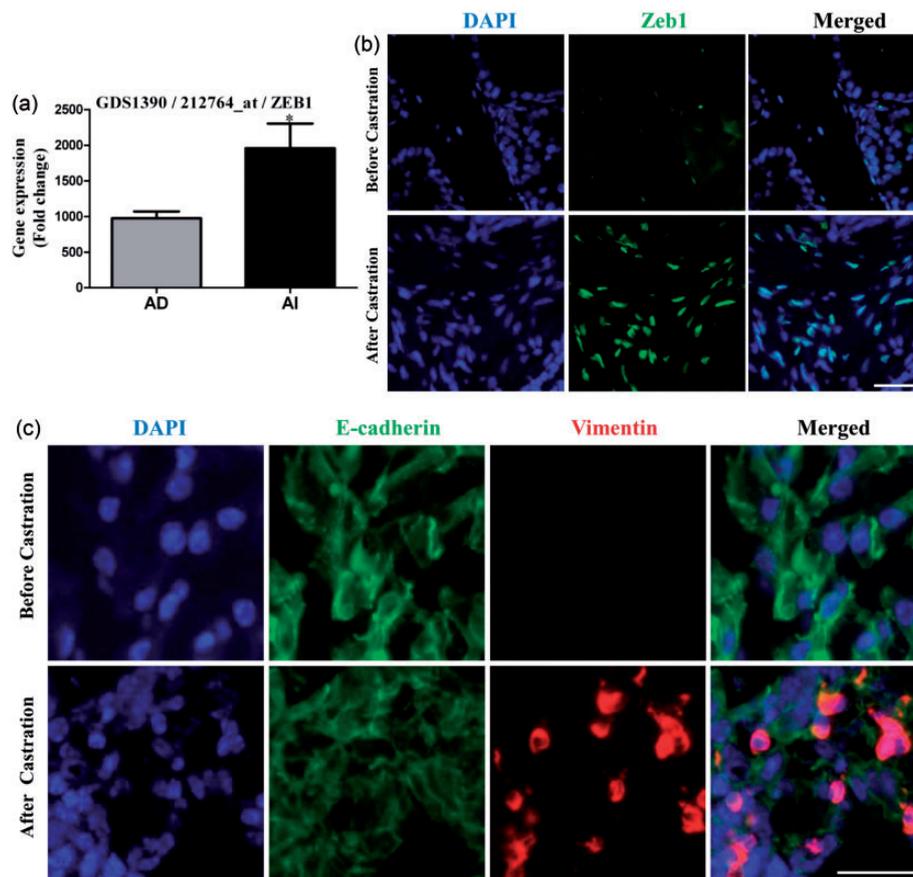


Figure 2 Zeb1 is increased in AIPC clinical specimens or prostate tumors in castrated PTEN conditional knockout mice. (a) Zeb1 expression levels in androgen-dependent and androgen-independent patients. The data are based on the analysis of GDS1390 from the NCBI/GEO database. (b) and (c) Zeb1, Vimentin, and E-cadherin expression in the intact and castrated PTEN conditional knockout mice by immunofluorescent staining. Note that higher level of Zeb1 is seen in castrated mouse prostate tumors. E-cadherin/Vimentin co-localization is observed after castration. Bars, 50 μ m; **, $P < 0.01$.

To determine whether Zeb1 knockdown could inhibit the tumorigenicity of the prostate cancer cells *in vivo*, we performed xenograft experiments using androgen-independent DU145 prostate cancer cells. Compared with DU145 vector control, DU145 sh-Zeb1 significantly suppressed tumorigenesis capacity in the xenograft mouse model *in vivo* (Figure 5(e), $P < 0.01$). The average size of the tumors decreased approximately 1.5-fold when Zeb1 was knockdown. Consistent with these results, cell proliferation assays in DU145 *in vitro* with sh-Zeb1 or LNCaP with Zeb1 also showed an inhibitory or stimulatory effect, respectively (Supplementary Figure 4(a) and (b)). Collectively, these results indicate that knockdown of Zeb1 attenuates androgen independence and inhibits migration and tumorigenesis ability of prostate cancer cells.

Zeb1 enhances stem cell properties including up-regulation of Sox2, colonogenesis and sphere formation of prostate cancer cells

As Zeb1 is an activator enhancing EMT, and EMT has been implicated in the acquisition of cancer stem cell properties in pancreatic cancer⁴ and breast cancer¹¹, we hypothesized

that Zeb1 expression may affect the traits of CSCs in prostate cancer cells. To test this hypothesis, we analyzed the CSCs properties in prostate cancer cells in which Zeb1 was up-regulated or down-regulated, respectively. The characteristics of CSCs include the ability of self-renewal, differentiation, and homeostatic control as well as the expression of pluripotent stem cell genes such as Sox2 and resistance to apoptosis.^{4,12} As shown in Figure 6(a) and (b), overexpression of Zeb1 in LNCaP cells resulted in elevated expression of stem cell markers such as Sox2 at mRNA and protein levels. In contrast, knockdown of Zeb1 led to a reduced expression of Sox2 in DU145 cells (Figure 6(c)). Interestingly, after analyzing the microarray data in the GEO database of mouse prostate tissues, we found that both Zeb1 and Sox2 levels were increased two weeks after castration and were reversed to a relatively normal level following androgen replacement ($P < 0.01$, GDS 2562) (Figure 6(d) to (e)), suggesting a positive correlation between Zeb1 and Sox2. Moreover, IP and Western blot analyses revealed that Zeb1 protein might bind to Sox2 (Figure 6(f)). Support for this notion comes from the work of Erik Engelen¹³ using mass spectrometry analysis.

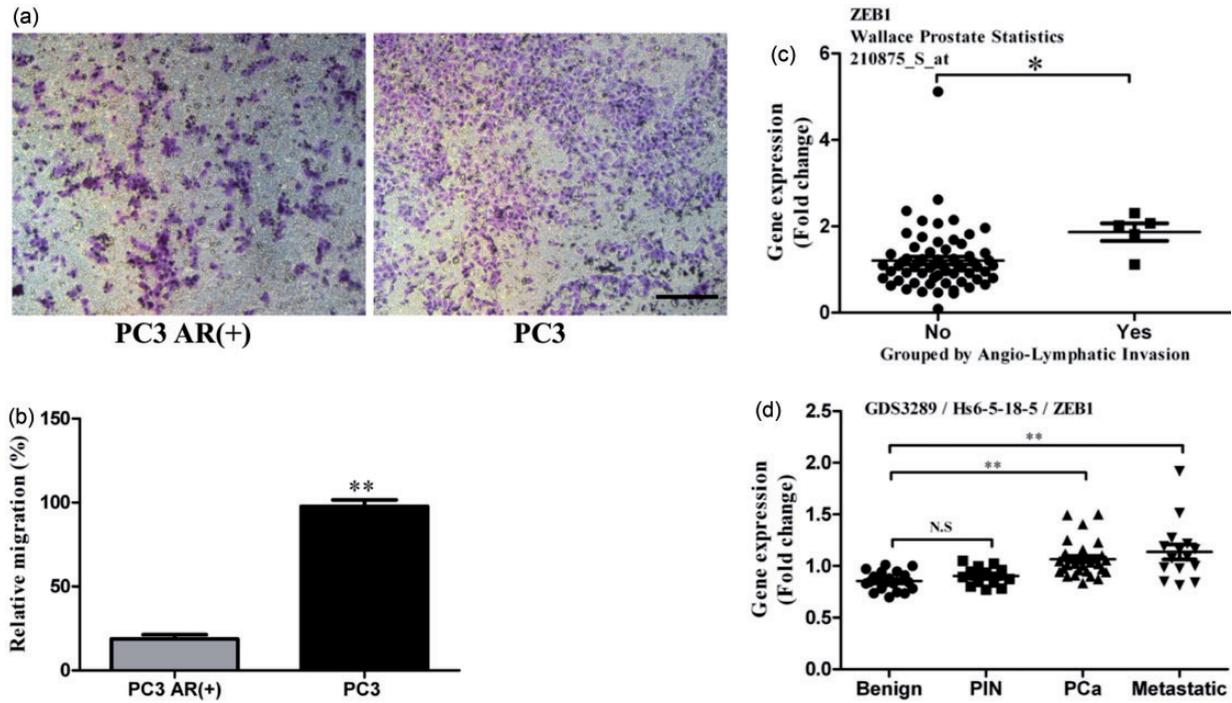


Figure 3 Elevated Zeb1 expression in AIPC cell lines shows higher migration ability and is associated with angio-lymphatic invasion. (a) and (b) Representative transwell migration assays display a higher migration rate in AIPC cells (PC3) compared with the ADPC cells (PC3 AR+). (c) Zeb1 expressions between specimens with or without angio-lymphatic invasion. The data are based on the Wallace study. (d) Differential Zeb1 expressions in the benign, prostate intraepithelial neoplasia (PIN), prostate cancer and metastatic prostate cancer tissues. The data are based on analysis of GDS3289 from the NCBI/GEO database. Bars, 50 μ m; *, $P < 0.05$; **, $P < 0.01$. (A color version of this figure is available in the online journal.)

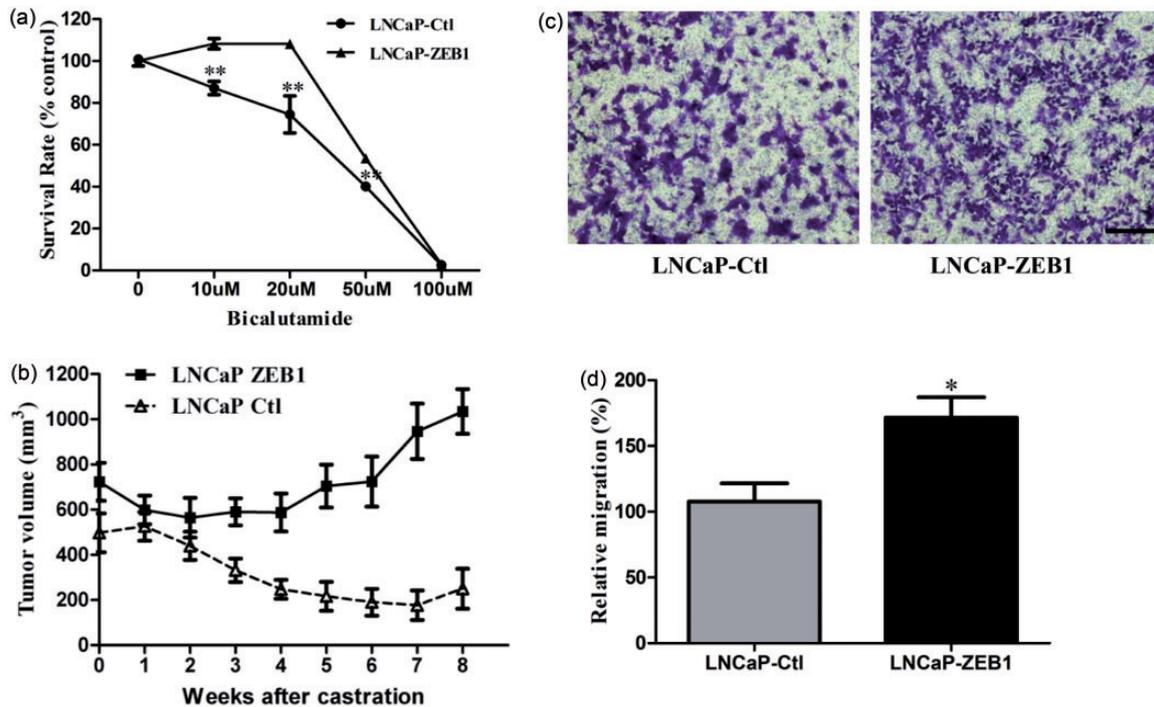


Figure 4 Ectopic expression of Zeb1 promotes androgen-independent and migration in prostate cancer cell lines. (a) A dose-response curve of LNCaP cells transfected with control or Zeb1 vectors displays relative bicalutamide sensitivity by CCK8 assay. (b) Response of LNCaP-Zeb1 cell growth in athymic male nude mice to castration. LNCaP-Ctl and LNCaP-Zeb1 cells mixed with Matrigel are injected into the flank of the mice. When the volume of the tumors reached approximately 600 mm^3 , the mice were castrated and the size of the tumors was monitored by weekly measurements with a caliper. ■, average size of LNCaP-Zeb1 tumors ($n = 5$); △, average size of LNCaP-Ctl tumors ($n = 6$). The growth curves are significantly different ($P < 0.05$). Data are expressed as Mean \pm SE. (c) and (d) Representative transwell migration assays display a higher migration rate in prostate cancer cells transfected with the Zeb1 vector compared with the control vector. Bars, 50 μ m; *, $P < 0.05$; **, $P < 0.01$. (A color version of this figure is available in the online journal.)

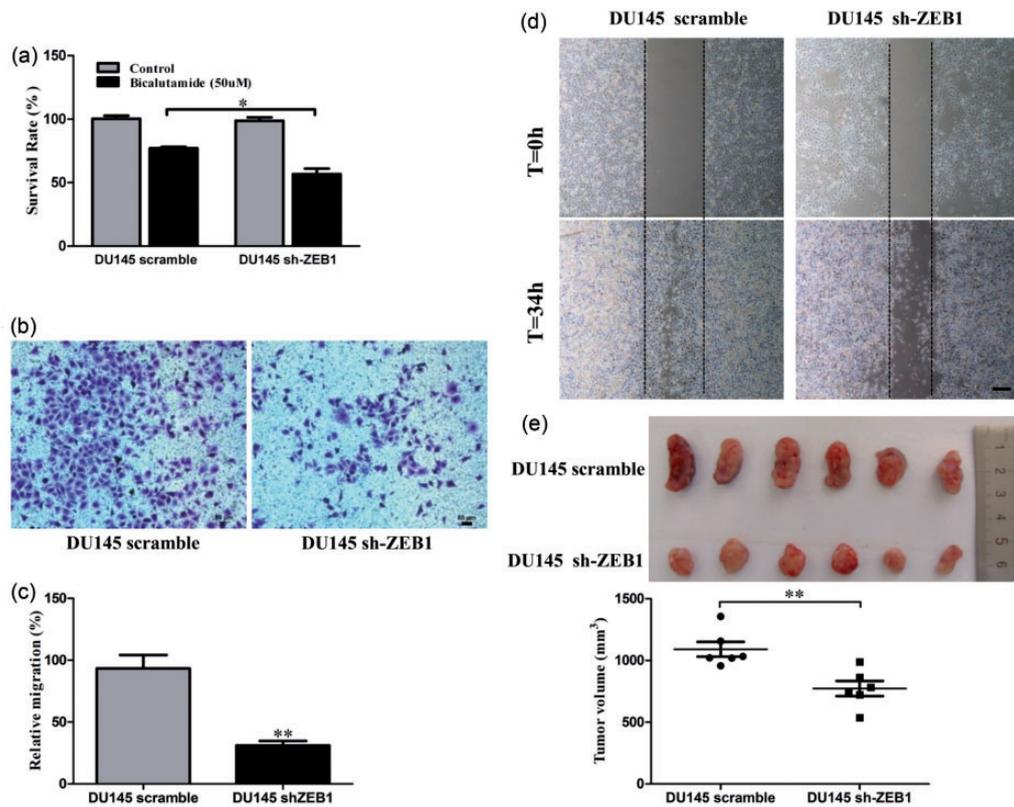


Figure 5 Zeb1 knockdown attenuates androgen-independence and inhibits migration and tumorigenesis ability in prostate cancer cell lines. (a) Knockdown of Zeb1 induces prostate cancer cells to become more sensitive to bicalutamide treatment by CCK8 assays. (b) to (d) Representative transwell migration assays and wound closure assays display a lower migration rate in prostate cancer cells transfected with sh-Zeb1 compared with the control vector. (e) An *in vivo* xenograft model shows an inhibition of growth in DU145 cells transfected with sh-Zeb1 as compared to the scramble shRNA. Bars, 50 μ m; *, $P < 0.05$; **, $P < 0.01$. (A color version of this figure is available in the online journal.)

To provide further direct evidence for the correlation of Zeb1 and stem cell-like properties, we examined the ability of self-renewal using the colony formation and sphere formation assays. Compared to the control group, reduced colonogenesis (Figure 6(g) and (h)) and decreased number of prostasphere (Figure 6(k) and (l)) were observed in sh-Zeb1 transfected group under the same culture conditions. In contrast, enhanced colonogenesis (Figure 6(i) and (j)) and increased number of prostasphere (Figure 6(m) and (n)) were observed in Zeb1 transfected group than the control group. These results support the proposal that Zeb1 presumably enhances stemness of the prostate cancer cells.

Discussion

Although our previous study² reports that castration induces EMT in the normal prostate and prostate cancer, whether an EMT regulator can influence the androgen independence and metastasis via gene silencing and overexpression approaches is undetermined. The present study demonstrates clearly that an important EMT modulator Zeb1 promotes androgen independence, migration and invasion of prostate cancer. Overexpression of Zeb1 causes the ADPC cells to become more androgen-independent and enhances migration. Conversely, interference of Zeb1 induces an opposite effect, that is, more sensitive to

androgen deprivation and reduced migration and tumor formation abilities.

The role of Zeb1 in the development of therapeutic resistance has been implicated in pancreatic cancer¹⁴ and glioblastoma cells¹⁵, however, to the best of our knowledge, our finding is the first to provide direct evidence that Zeb1 could influence androgen independence in prostate cancer. Firstly, the present experiments extend the observations reported in the previous study indicating the differential expression of EMT molecules including Zeb1 between androgen-dependent LNCaP and androgen-independent PC3 prostate cancer cells.² In the present experiments we examine three better paired androgen-dependent and independent prostate cancer cells: LNCaP vs. C2-4B; PC3 (AR+) vs. PC3; CW22-RV1 vs. DU145. We find that all three androgen-independent prostate cancer cells express higher levels of Zeb1 than those androgen-dependent prostate cancer cells. Secondly, clinical AIPC samples display a higher level of Zeb1 than those ADPC samples. Thirdly, in the PTEN conditional knockout mice model, castration causes a significant elevation in Zeb1 expression with co-expression of E-cadherin and Vimentin. Although it is possible that castration or androgen deprivation might induce production of EMT-inducing growth factors such as TGF- β in the prostate tissue, which in turn causes the EMT process, future experiments are required to verify

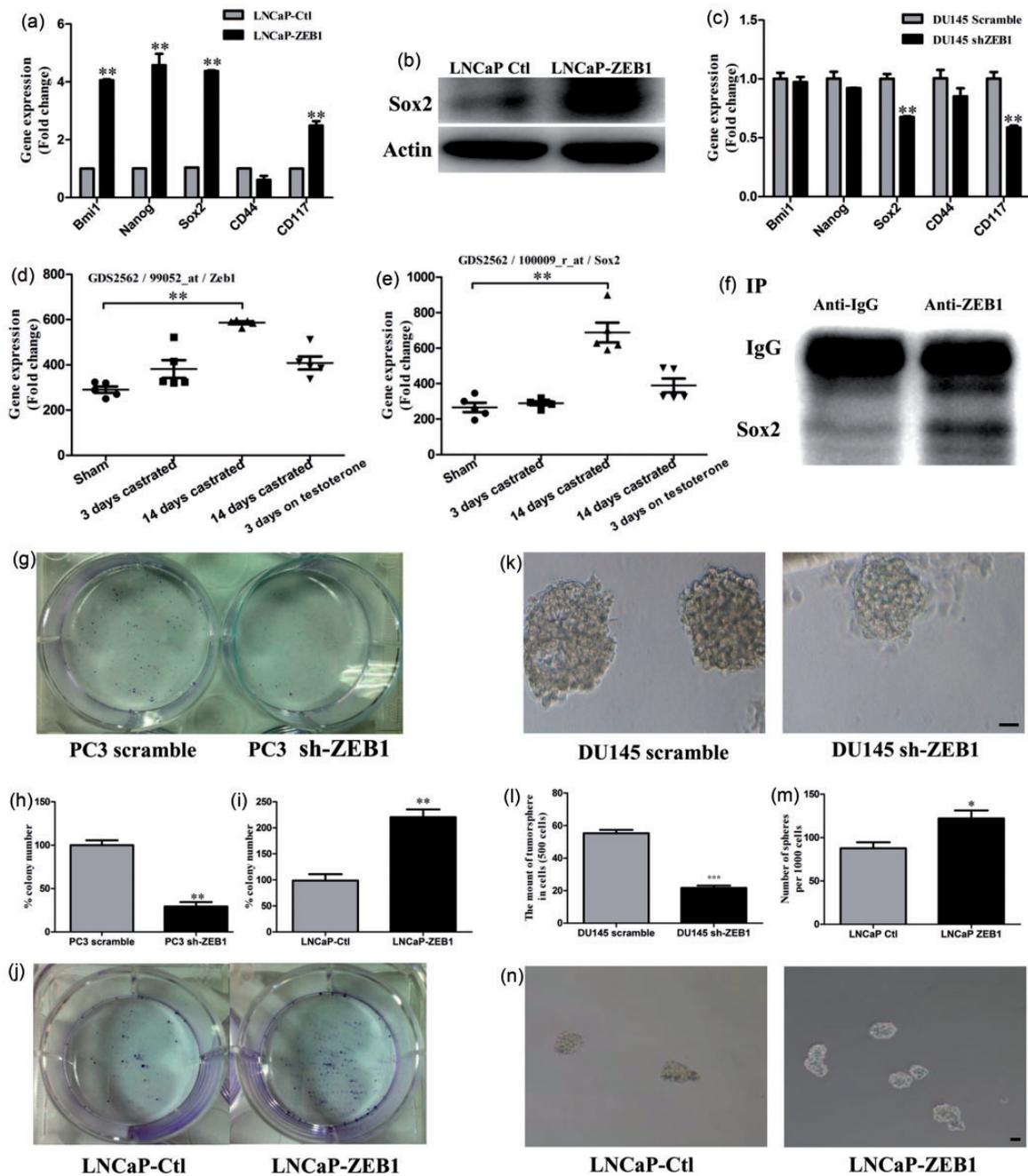


Figure 6 Zeb1 induces stem cell-like properties of prostate cancer cells. (a) to (c) Stem cell marker expression in cells transfected with ectopic Zeb1 and sh-Zeb1. Note that the alteration of Sox2 mRNA and protein levels is positively associated with Zeb1 expression. (d) and (e) Zeb1 and Sox2 expression changes after two weeks post castration and three days of treatment with testosterone. The data are based on the analysis of GDS2562 from the NCBI/ GEO database. (f) IP assay and Western blot analyses revealed that Zeb1 protein might bind to Sox2. (g) and (h) Colony formation assays display a lower clonogenic rate in prostate cancer cells transfected with sh-Zeb1 compared with the control vector. (i) and (j) Colony-formation assays show that cells transfected with the Zeb1 vector grow faster than those transfected with the control vector. (k) and (l) Sphere assays show reduced sphere-forming ability in the sh-Zeb1 transfectants compared with vector control cells. (m) and (n) Sphere assays show enhanced sphere-forming ability in the Zeb1 transfectants than those cells transfected with a control vector. Bars, 50 μ m; *, $P < 0.05$; **, $P < 0.01$. (A color version of this figure is available in the online journal.)

this possibility. Fourthly, overexpression of Zeb1 in ADPC cells leads to increased resistance to bicalutamide (a common anti-androgen drug) *in vitro* and continued tumor growth even after castration *in vivo*. Finally, knock-down of Zeb1 elicits opposite effects. These data together support the idea that Zeb1 can act as an important modulator for the development of AIPC.

The present study reinforces the notion that Zeb1 promotes invasion and migration of prostate cancer cells. Firstly, knockdown of Zeb1 in PC3 cells reverses EMT or causes MET with increased E-cadherin expression and epithelial morphology. As a result, Zeb1 knockdown leads to a reduced capacity of migration in both PC3 and DU145 cell lines. Secondly, overexpression of Zeb1 elicits opposite

effects. Finally, our data analyses from the GEO and Oncomine databases provide further evidence that Zeb1 plays an important role in prostate tumorigenesis and invasion, that is, high level of Zeb1 has been observed in both prostate tumors and the tumors with angio-lymphatic invasion. Combining with our previous study,² these data together suggest that Zeb1 is a promoting factor for prostate tumorigenesis and invasion.

Increasing evidence suggest that EMT is often activated during cancer invasion and metastasis with the acquisition of stem cell-like properties.¹¹ In the present study, we investigate whether Zeb1 expression influences acquisition of stem cell characteristics by prostate cancer cells. We find that over-expression of Zeb1 increases the expression of the pluripotent stem cell markers such as Sox2, accompanied by enhanced colonogenesis and sphere-forming capacities, common properties of CSCs. Furthermore, IP assay suggests that Zeb1 protein might bind to Sox2, although whether such binding is specific and whether Zeb1 can directly regulate Sox2 remain to be verified in future studies. Together, these results suggest that the EMT-activator Zeb1 is strongly correlated with stem-cell like properties possibly through binding to Sox2 protein. Our findings are not only consistent with the previous studies indicating that normal prostate stem cells display higher expression levels of Zeb1 than those non-stem cells² but also are in agreement with the work done in pancreatic cancer,⁴ glioblastoma,¹⁵ and breast cancer.³ For example, a recent study linking EMT and CSCs by Weinberg and colleagues demonstrates that Zeb1 is capable to convert non-CSCs to a CSC status and functions as an important modulator in the stemness maintenance of breast cancer cells.³ Thus, these data together support the notion that high expression levels of Zeb1 facilitate the induction of stemness of CSCs in prostate cancer cells.

Taken together, the present study provides direct evidence that Zeb1, an important EMT modulator, promotes prostate cancer progression including androgen independence, invasion, and metastasis. Such progression-promoting effects by Zeb1 appear to be mediated via induction of stem cell-like properties. Therefore, reversing the EMT by targeting Zeb1 in combination with the traditional androgen deprivation therapy might be a conceivable approach in the war against AIPC.

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