Knockdown of ZEB1 reverses cancer stem cell properties in prostate cancer cells

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Received August 10, 2020; Accepted January 26, 2021

DOI: 10.3892/or.2021.8009

Abstract. Prostate cancer (PCa) is the second most diagnosed type of cancer in men worldwide. Advanced PCa is resistant to conventional therapies and high recurrence has been associated with high rates of metastasis. Cancer stem cells (CSCs) have been proposed to be responsible for this, due to their ability of self-renewal and differentiation into other cell types. Zinc finger E-box-binding homeobox 1 (ZEB1), a transcription factor involved in the regulation of epithelial-mesenchymal transition (EMT), has been associated with the activation of several mechanisms that lead to resistance to treatment. As recent evidence has shown that CSCs may originate from non-CSCs during EMT, it was hypothesized that knocking down ZEB1 expression in PCa cell lines could revert some properties associated with CSCs. Using lentiviraltransduction, ZEB1 expression was silenced in the PCa DU145 and LNCaP cell lines. The mRNA and protein expression levels of key canonical CSC markers (Krüppel-like factor 4, SOX2, CD44 and CD133) were determined using reverse transcription-quantitative PCR and western blot analysis, respectively. In addition, the colony forming ability of the ZEB1-knockdown cells was evaluated, and the type of colonies formed (holoclones, paraclones and meroclones) was also characterized. Finally, the ability to form prostatospheres was evaluated *in vitro*. It was found that in ZEB1-knockdown DU145 cells, the expression levels of CSC phenotype markers (CD44, CD133 and SOX2) were decreased compared with those in the control group. Furthermore, ZEB1-knockdown cells exhibited a lower ability to form prostatospheres and to generate colonies. In conclusion, stable silencing of ZEB1 reversed CSC properties in PCa cell lines. Since ZEB1 is associated with malignancy, therapy resistance and a CSC phenotype in PCa cell lines, targeting ZEB1 may be a key factor to eradicate CSCs and improve the prognosis of patients with advanced PCa.

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Key words: cancer stem cells, epithelial-mesenchymal transition, zinc finger E-box-binding homeobox 1, prostate cancer

Introduction

Prostate cancer (PCa) is the second most common type of cancer in men and the fourth leading cause of cancer-associated death worldwide, according to GLOBOCAN 2018 statistics (1). In addition, mortality is mainly associated with the age of diagnosis, degree of histological differentiation, metastasis and resistance to hormonal therapy, with the latter being one of the greatest challenges in PCa management (2,3). Androgen deprivation therapy (ADT), mainly achieved with gonadotropin-releasing hormone analogs, is the first-line therapy to treat metastatic PCa. Patients with advanced PCa initially respond to ADT, showing decreased tumor size and blood prostate-specific antigen levels (4,5). However, after two or three years, nearly all patients relapse, progressing to castration-resistant PCa (CRPC) (3,6). Increasing evidence has indicated that cancer stem cells (CSCs), a small subpopulation of malignant cells with stem-like properties, may serve an important role in the progression of CRPC (7-11).

Functionally, CSCs are defined by their ability of self-renewal and asymmetric division, giving rise to heterogeneous cell lines (12). Prostate CSCs are able to initiate the development of tumors in the metastatic niche and to differentiate into cancer cells with highly aggressive phenotypes, contributing to the progression of the disease (13,14). From a molecular standpoint, CSCs are characterized by the expression of specific surface markers; in particular, prostate CSCs express CD133 (also known as prominin-1) and CD44, and high levels of the multidrug resistance pump ATP binding cassette subfamily G member 2 and integrin α2β1 (10,15,16).

CSCs contribute to cancer progression due to their resistance to different therapeutic approaches (17-19). Prostate CSCs are not sensitive to ADT, due to not expressing the

androgen receptor (AR) (9,10,16). Furthermore, CSCs exhibit abnormal activation of DNA repair pathways, low proliferation rates and high expression levels of multidrug resistance efflux pumps (15-17,19). These intrinsic characteristics of CSCs may account for the failure of radio- and chemotherapy in patients with CRPC (16,20,21).

Due to their importance in PCa progression, CSCs have become a potential target for advanced PCa. However, the origin and molecular mechanisms of CSCs are not fully understood. It has been suggested that CSCs may originate from the malignant transformation of normal prostate stem cells (22); however, several studies have indicated that CSCs may originate from non-CSCs (23-25). Notably, numerous molecular networks have been found to induce reprogramming of non-CSCs into CSCs and have been involved in epithelial-mesenchymal transition (EMT) (14,26,27).

EMT is a trans-differentiation mechanism, in which epithelial cells change their phenotype and acquire mesenchymal features. The main change that occurs during EMT is the loss of E-cadherin expression, resulting in loss of apicobasal polarity and cell-cell contact, and increased migration and invasion (28). In cancer cells, several signaling pathways can initiate and maintain EMT, including the transforming growth factor-β, Wnt/β-catenin and integrin/integrin-linked kinase signaling pathways (28-30). These signaling pathways converge on the activation of EMT transcription factors, which directly inhibit E-cadherin expression (31,32). Among these transcription factors, zinc finger E-box-binding homeobox 1 (ZEB1) has been associated with the activation of several mechanisms leading to resistance to treatment (33-36). It has been previously demonstrated that ZEB1 induces EMT in PCa cell lines, promoting the loss of E-cadherin expression, and increases migration and invasion, resulting in an aggressive phenotype (37-39). Accordingly, ZEB1 is expressed at higher levels in the highly aggressive DU145 cell line compared with in other PCa cell lines, such as PC3, LNCaP and 22Rv1 cells (38). Knocking down ZEB1 expression in the DU145 cell line increases E-cadherin expression and decreases invasion and migration (38). Furthermore, DU145 cells exhibit some characteristics of CSCs, such as chemotherapy resistance, and the stable knockdown of ZEB1 sensitizes these cells to docetaxel, a taxane widely used for the treatment of CRPC (37). Based on the aforementioned studies, the present study hypothesized that knocking down ZEB1 in PCa cells could also revert some features associated with CSCs.

Materials and methods

Cell culture. The PCa DU145 (ATCC®HTB81™) and LNCaP clone FGC (ATCC®CRL1740™) cell lines were purchased from the American Type Culture Collection. DU145 cells were originally obtained from a brain metastasis of PCa and are insensitive to androgens, resembling CRPC (40). LNCaP cells were originally obtained from a lymph node metastasis of PCa and are responsive to androgens (41). DU145 cells were maintained in DMEM F12 medium and LNCaP cells were maintained in RPMI-1640 medium (both Gibco; Thermo Fisher Scientific, Inc.). Both culture media were supplemented with 10% fetal bovine serum (Corning Life Sciences), streptomycin-penicillin and amphotericin B (Corning, Inc.). Cell cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Lentiviral transduction. Knockdown of ZEB1 expression in DU145 and LNCaP cells was achieved using transduction with lentiviral vectors containing a short hairpin (sh)RNA against ZEB1 [pLenti-U6-shRNA (hZEB1)-Rsv(RFP-Puro)], or a scrambled shRNA used as a negative control [pLenti-U6-shRNA (Neg-control)-Rsv(RFP-Puro)]. Pre-packaged lentiviral particles were purchased ready to use from GenTarget, Inc., and cells were infected using a standard procedure. Briefly, 10^6 cells/well were seeded in 6-well plates. After 24 h at 37˚C, cells were incubated with lentiviral particles at a multiplicity of infection of 3, with 6 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) in 1 ml culture medium for 24 h at 37˚C. Subsequently, cells integrating the vectors were selected using 2 μg/ml puromycin for 24 h at 37˚C.

Western blotting. Whole-cell protein was extracted from cells using RIPA buffer with cOmplete™ Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics), and protein concentration was determined using a Bradford protein assay. A total of 50 μg protein/lane was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked for 1 h at room temperature with 5% BSA (Winkler Ltda) in 0.2% TBS-Tween and incubated overnight at 4˚C with primary antibodies diluted in blocking buffer. After washing three times in 0.2% TBS-Tween, bound primary antibodies were detected with HRP-conjugated secondary antibodies incubated for 1.5 h at room temperature, and revealed with an enhanced chemiluminescence detection kit for HRP (EZ-ECL; Biological Industries). Chemiluminescence was detected using the Fusion FX image system (VilberLourmat) and the optical density of the bands was analyzed using the software ImageJ v1.51 (National Institutes of Health).

The antibodies used were as follows: SOX2 (1:1,000; cat. no. ab92494; Abcam), Krüppel-like factor 4 (KLF4; 1:1,000; cat. no. ab215036; Abcam), CD44 (1:5,000; cat. no. ab51037; Abcam), CD133 (1:500; cat. no. Pas-38014; Thermo Fisher Scientific, Inc.), ZEB1 (1:1,000; cat. no. Pa5-28221; Thermo Fisher Scientific, Inc.), E-cadherin (1:1,000; cat. no. 610181; BD Biosciences), β-actin (1:5,000; cat. no. 691002; MP Biomedicals, LLC), anti-mouse HRP (1:10,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.) and anti-rabbit HRP (1:10,000; cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.).

RNA extraction and reverse transcription-quantitative (q)PCR. Total RNA was extracted from cells using TRIzol (Ambion; Thermo Fisher Scientific, Inc.). A total of 3,000 ng cDNA was synthesized using the cDNA Affinity Script QPCR kit (Agilent Technologies, Inc.), according to the manufacturer's protocol, and 100 ng cDNA was amplified by qPCR using the Brilliant II SYBR Green qPCR Master Mix kit (Agilent Technologies, Inc.) according to the manufacturer's protocol. For qPCR, the thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 15 sec. The housekeeping

Table I. Primer sequences used for quantitative PCR.

Gene	Forward primer	Reverse primer
ZEB1	5'-TTCACAGTGGAGAGAAGCCA-3'	5'-GCCTGGTGATGCTGAAAGAG-3'
CD44	5'-CACGTGGAATACACCTGCCA-3'	5'-GACAAGTTTTGGTGGCAGGT-3'
CD133	5'-TCAATTTTGGATTCATATTT-3'	5'-ACTCCCATAAAGCTGGACCC-3'
SOX2	5'-GTCTAGCCTCGTCGATGAAC-3'	5'-AACCCCAAGATGCACAACTC-3'
KLF4	5'-CCCCGTGTGTTTACGGTAGT-3'	5'-AGAGTTCCCATCTCAAGGCA-3'
PUM1	5'-CGTACGTGAGGCGTAAGTAA-3'	5'-CGGTCGTCCTGAGGATAAAA-3'

ZEB1, zinc finger E-box-binding homeobox 1; KLF4, Krüppel-like factor 4; PUM1, pumilio RNA binding family member 1.

gene pumilio RNA binding family member 1 was used as a normalizer (42) and the results were analyzed using the $2^{-\Delta\Delta Cq}$ method (43). The primer sequences used for qPCR are presented in Table I.

Colony formation assay. Cells cultured under adherent conditions were detached using 0.25% trypsin at 37˚C for 10 min, seeded at $2x10^4$ cells/plate in 6-well plates and incubated at 37˚C in a humidified atmosphere with 5% CO_2. After 15 days, cells were fixed for 10 min at room temperature with cold 100% methanol, stained with crystal violet (0.5% crystal violet in 25% methanol) for 10 min at room temperature, washed and air-dried at room temperature. The resulting colonies were photographed using an Olympus SZ60 stereoscopic light microscope (Olympus Corporation), and the images were analyzed using ImageJ v1.51 (National Institutes of Health). Groups of ≥50 cells were considered as colonies, and the types of colonies formed were classified according to their morphology in holoclones, meroclones and paraclones, as previously described by Barrandon and Green (44).

Prostatosphere formation assay. Cells from cultures maintained under adherent conditions were washed with PBS and detached using acutase (eBioscience; Thermo Fisher Scientific, Inc.) for 7 min at 37˚C. The collected cells were centrifuged at 300 x g for 5 min at room temperature, and the pellet was mechanically disaggregated using a micropipette and collected through a 40-μm cell strainer (BD Falcon; Becton, Dickinson and Company). Cells ($1x10^5$) were seeded in 6-cm dishes coated with 1% agarose in a culture medium suitable for inducing cell growth under non-adherent conditions, as previously described (15). Prostatospheres were photographed every other day for a total of 7 days using an Olympus SIG60 stereoscopic light microscope (Olympus Corporation; magnification, x100) and analyzed using the software AxioVision v4.8.1 (Carl Zeiss AG), as described by Acikgoz *et al* (45). Prostatosphere 3D volume was calculated from 2D images using the following formula: (length x width x width) x (3.1416/6).

Statistical analysis. Data analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation of at least three independent experiments, and the Mann-Whitney U test was used to analyze differences between groups. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of ZEB1 in the PCa DU145 cell line. To determine whether ZEB1 could revert some of the CSC features in PCa cells, knockdown of ZEB1 expression in the PCa DU145 cell line was performed using transduction with lentiviral vectors expressing a shRNA targeting ZEB1 (DU145 sh-ZEB1). Scramble shRNA was used as a control (DU145 sh-Scr). The cells transduced with ZEB1 shRNA formed cohesive groups in adherent conditions (Fig. 1A) and sh-ZEB1 significantly decreased ZEB1 mRNA (Fig. 1B) and protein expression (Fig. 1C and D) compared with sh-Scr. ZEB1 is a known transcriptional repressor of E-cadherin. Therefore, to evaluate whether knocking down ZEB1 expression in these cells modified E-cadherin expression, E-cadherin protein expression was determined using western blot analysis. As shown in Fig. 1C and D, ZEB1-knockdown significantly increased E-cadherin expression.

Knockdown of ZEB1 in DU145 cells decreases the expression levels of the CSC markers CD44 and CD133. In the established ZEB1-knockdown cell line, the expression levels of the CSC markers CD44 and CD133 were evaluated. Reverse transcription-qPCR revealed that DU145 cells transduced with sh-ZEB1 exhibited significantly decreased mRNA expression levels of CD44 and CD133 compared with DU145 cells transduced with sh-Scr (Fig. 2A). These results were verified using western blot analysis (Fig. 2B), revealing that ZEB1-knockdown induced a significant decrease of ~75 and 20% of CD44 and CD133 protein expression, respectively (Fig. 2C).

Knockdown of ZEB1 in DU145 cells decreases SOX2 expression. Since ZEB1-knockdown decreased the expression levels of the CSC markers CD44 and CD133, which in turn are controlled by CSC transcription factors, such as KLF4 and SOX2, the present study further investigated whether ZEB1-knockdown affected the expression levels of these transcription factors. ZEB1-knockdown in the DU145 cell line significantly decreased the expression levels of SOX2, at both the mRNA and protein level; however, the expression levels of KLF4 were not significantly changed at the mRNA level, but were significantly decreased at the protein level (Fig. 3).

Effect of ZEB1-knockdown on the colony forming ability of DU145 cells. One of the main characteristics of CSCs is the

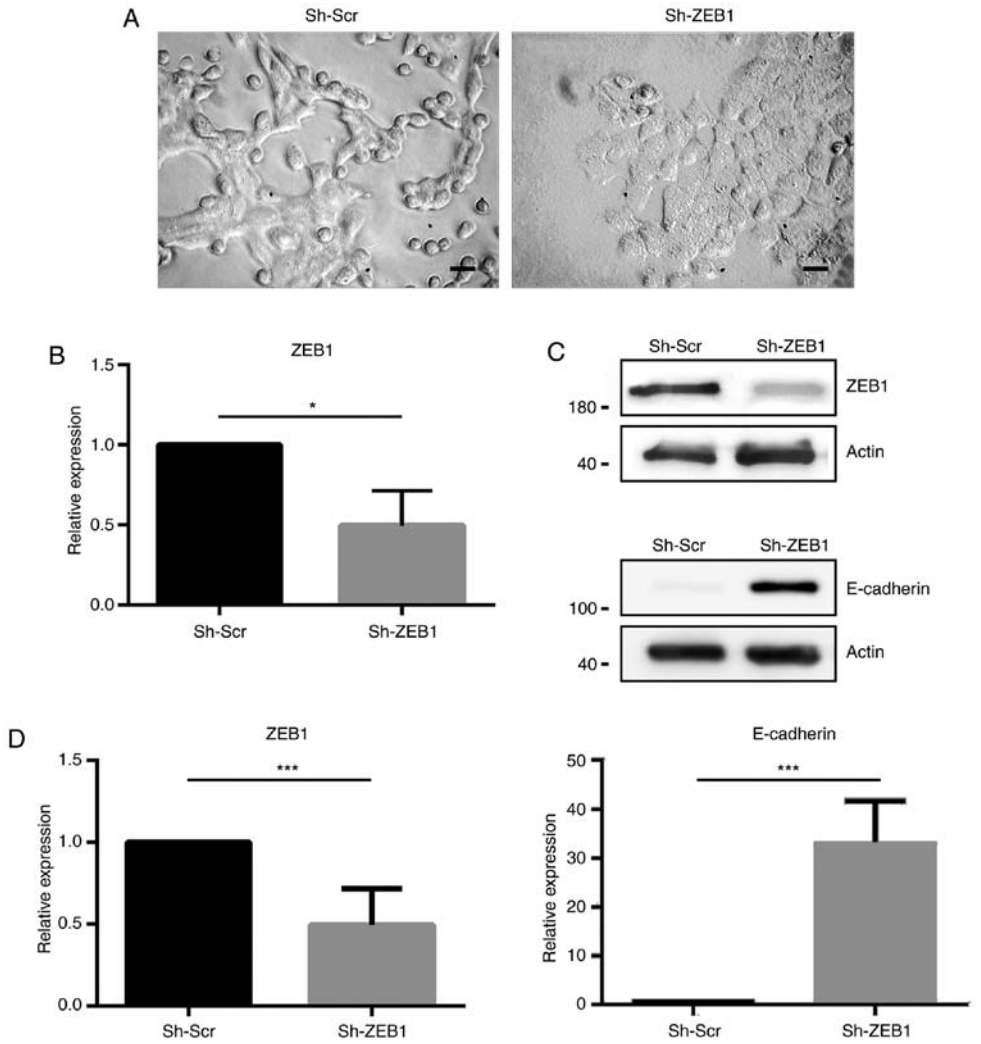

Figure 1. ZEB1-knockdown in the prostate cancer DU145 cell line. (A) Representative microphotographs of DU145 cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. Scale bar, 20 μm. (B) Relative ZEB1 mRNA expression measured via reverse transcription-quantitative PCR. (C) Representative western blots for ZEB1 and E-cadherin protein expression. (D) Quantification of the optical density from the western blots for ZEB1 and E-cadherin protein expression. Data are expressed as the mean ± SD (n=3). *P≤0.05 and ***P≤0.001 (Mann-Whitney U test). ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA.

ability of self-renewal. To determine if ZEB1-knockdown in PCa cells affected this ability, a colony formation assay was performed, in which single cells that self-renew will form colonies of the clones. ZEB1-knockdown significantly decreased the number of colonies formed by DU145 cells (Fig. 4A and B). Furthermore, the types of colonies formed by DU145 cells transduced with sh-ZEB1 were different compared with those in cells transduced with sh-Scr, with respect to size and morphology. The colonies were classified into holoclones, meroclones and paraclones (44): Holoclones are colonies of small and densely compact cells, with regular edges, paraclones consist of larger and elongated cells that grow in a scattered way, with irregular edges, and meroclones have an intermediate morphology, between a paraclone and a holoclone. DU145 cells transduced with either sh-Scr or sh-ZEB1 formed all three types of the colonies (Fig. 4C); however, cells transduced with sh-Scr formed mainly holoclones, whereas cells transduced with sh-ZEB1 formed a high percentage of paraclones (Fig. 4D). In all cases, the mean number of cells per colony was significantly higher in the colonies formed by cells transduced with sh-Scr compared with that in colonies with cells transduced with sh-ZEB1 (Fig. 4E).

Effect of ZEB1-knockdown on the anchorage-independent growth ability of DU145 cells. Prostate CSCs can grow in an anchorage-independent manner forming prostatospheres when cultured in soft agar (46). Prostatospheres formed by cells transduced with sh-Scr and sh-ZEB1 were obtained following 7 days of anchorage-independent growth. There was a higher number of prostatospheres formed by cells transduced with sh-Scr compared with cells transduced with sh-ZEB1 after 1 and 3 days (Fig. 5A and B). Furthermore, the prostatospheres formed by cells transduced with sh-Scr were bigger compared with those formed by cells transduced with sh-ZEB1 after 5 and 7 days (Fig. 5C and D). Therefore, ZEB1-knockdown in DU145 cells affected their ability to generate prostatospheres in anchorage-independent cultures.

Effect of ZEB1-knockdown on the expression levels of CSC markers, clonogenicity and prostatosphere forming ability

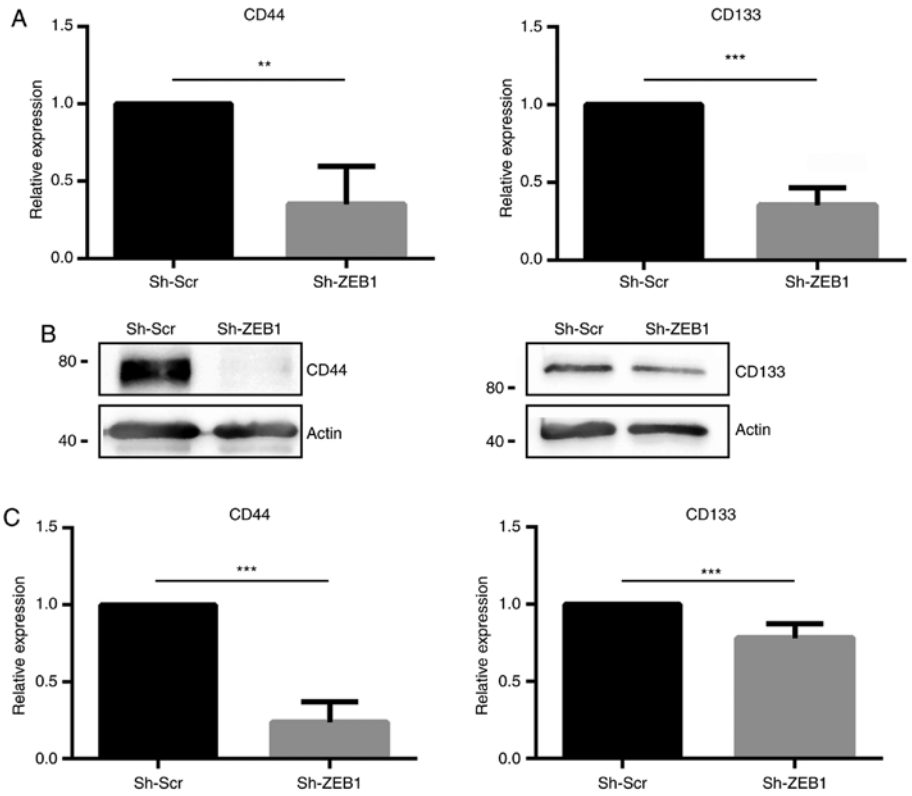

Figure 2. ZEB1-knockdown decreases the expression levels of CSC markers in the prostate cancer DU145 cell line. (A) Relative mRNA expression levels of the CSC markers CD44 and CD133 measured via reverse transcription-quantitative PCR in DU145 cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. (B) Representative western blots for CD44 and CD133 protein expression, and (C) their quantification. Data are expressed as the mean ± SD (n=3). **P≤0.01 and ***P≤0.001 (Mann-Whitney U test). ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA; CSC, cancer stem cell.

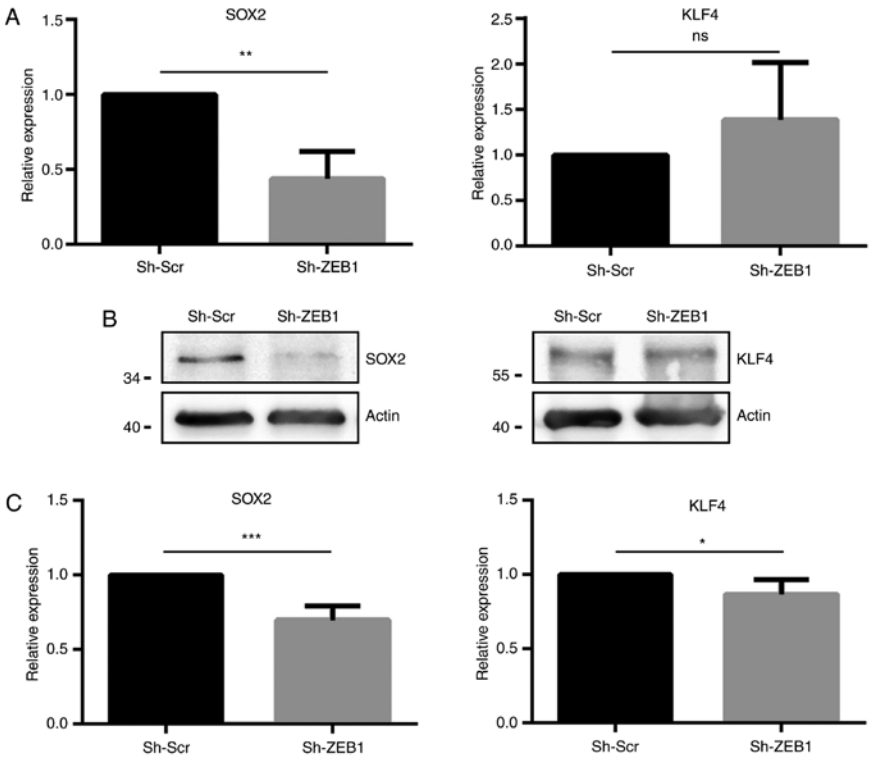

Figure 3. ZEB1-knockdown decreases the expression levels of the CSC transcription factor SOX2 in the prostate cancer DU145 cell line. (A) Relative mRNA expression levels of the CSC transcription factors SOX2 and KLF4 measured via reverse transcription-quantitative PCR in DU145 cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. (B) Representative western blots for SOX2 and KLF4 protein expression, and (C) their quantification. Data are expressed as the mean ± SD (n=3). *P≤0.05, **P≤0.01 and ***P≤0.001 (Mann-Whitney U test). ns, not significant; ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA; CSC, cancer stem cell; KLF4, Krüppel-like factor 4.

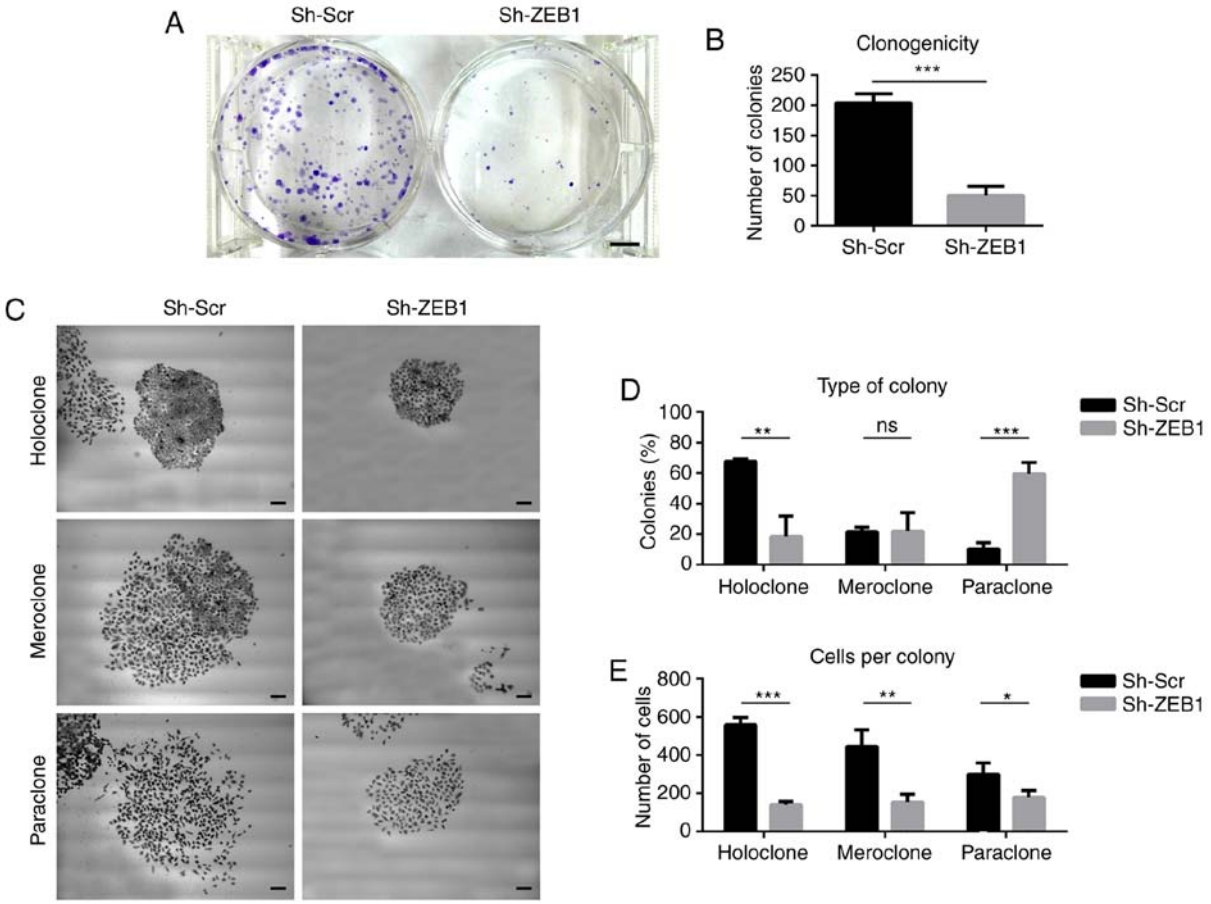

Figure 4. ZEB1-knockdown decreases the clonogenic capacity of the prostate cancer DU145 cell line. (A) Representative image of the colony formation assay using DU145 cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. Scale bar, 5 mm. (B) Number of colonies formed after 2 weeks of growth under limiting dilution conditions. (C) Representative images of the type of colonies formed in each condition. Scale bar, 50 μm. (D) Quantification of the different types of colonies formed. (E) Quantification of the cell number of each type of colony. Data are expressed as the mean ± SD (n=3). *P≤0.05, **P≤0.01 and ***P≤0.001 (Mann-Whitney U test). ns, not significant; ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA.

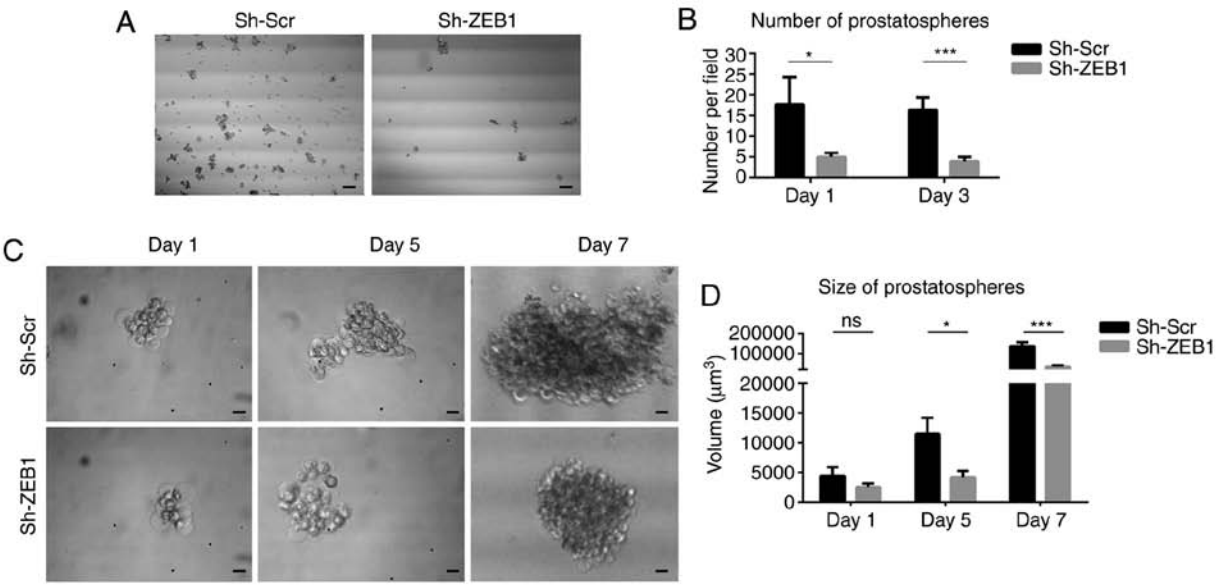

Figure 5. ZEB1-knockdown decreases the prostatosphere formation capacity of the prostate cancer DU145 cell line. (A) Representative images of the prostatosphere formation assay using DU145 cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. Scale bar, 100 μm. (B) Number of prostatospheres formed after 1 and 3 days of growth under non-adherent conditions. (C) Representative images of the prostatospheres formed in each condition at days 1, 5 and 7 after seeding. Scale bar, 20 μm. (D) Quantification of the size of the prostatospheres formed by each cell line at days 1, 5 and 7 after seeding. Data are expressed as the mean ± SD (n=4). *P≤0.05 and ***P≤0.001 (Mann-Whitney U test). ns, not significant; ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA.

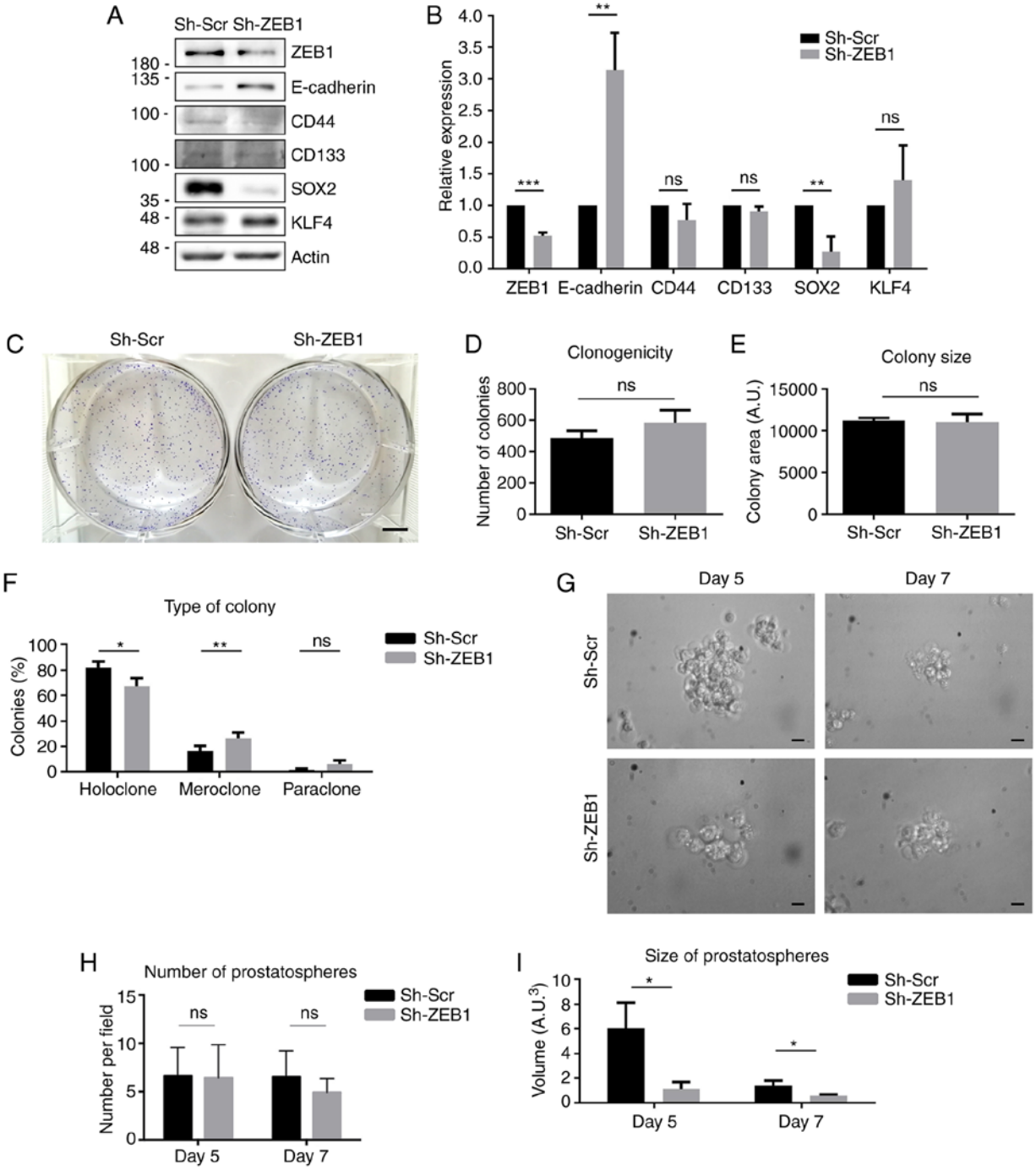

Figure 6. Effect of ZEB1-knockdown in the prostate cancer LNCaP cell line. (A) Representative western blots for ZEB1, E-cadherin, CD44, CD133, SOX2 and KLF4 protein expression in LNCaP cells transduced with a lentiviral vector carrying Sh-ZEB1 or Sh-Scr. (B) Quantification of optical density of western blots. (C) Representative image of colony formation assay using LNCaP Sh-ZEB1 and Sh-Scr cells. Scale bar, 5 mm. (D) Number of colonies formed after 2 weeks of growth under limiting dilution conditions. (E) Size of the colonies formed by each cell condition. (F) Quantification of the different types of colonies formed. (G) Representative images of the prostatospheres formed in each condition at days 5 and 7 after seeding. Scale bar, 20 μm. (H) Number of prostatospheresformed after 5 and 7 days of growth under non-adherent conditions. (I) Quantification of the size of the prostatospheres formed by each cell line at 5 and 7 days after seeding. Data are expressed as the mean ± SD (n=3). *P≤0.05, **P≤0.01 and ***P≤0.001 (Mann-Whitney U test). ns, not significant; ZEB1, ZEB1, zinc finger E-box-binding homeobox 1; Sh-ZEB1, short hairpin RNA against ZEB1; Sh-Scr, scrambled short hairpin RNA; KLF4, Krüppel-like factor 4; A.U., arbitrary units.

in LNCaP cells. The DU145 cell line is characterized by a lack of the AR and by their high aggressiveness (47), which are intrinsic characteristics of CRPC. To evaluate if knocking down ZEB1 exerted the same effects in androgen-sensitive cells, ZEB1 expression was knocked down in another PCa cell line, LNCaP. In these cells, the expression levels of E-cadherin, CD44, CD133, SOX2 and KLF4 were investigated, and the results revealed that ZEB1-knockdown significantly increased E-cadherin protein expression and significantly decreased SOX2 protein expression; however, no significant changes were observed in the expression levels of CD44, CD133 and KLF4 (Fig. 6A and B). To assess the

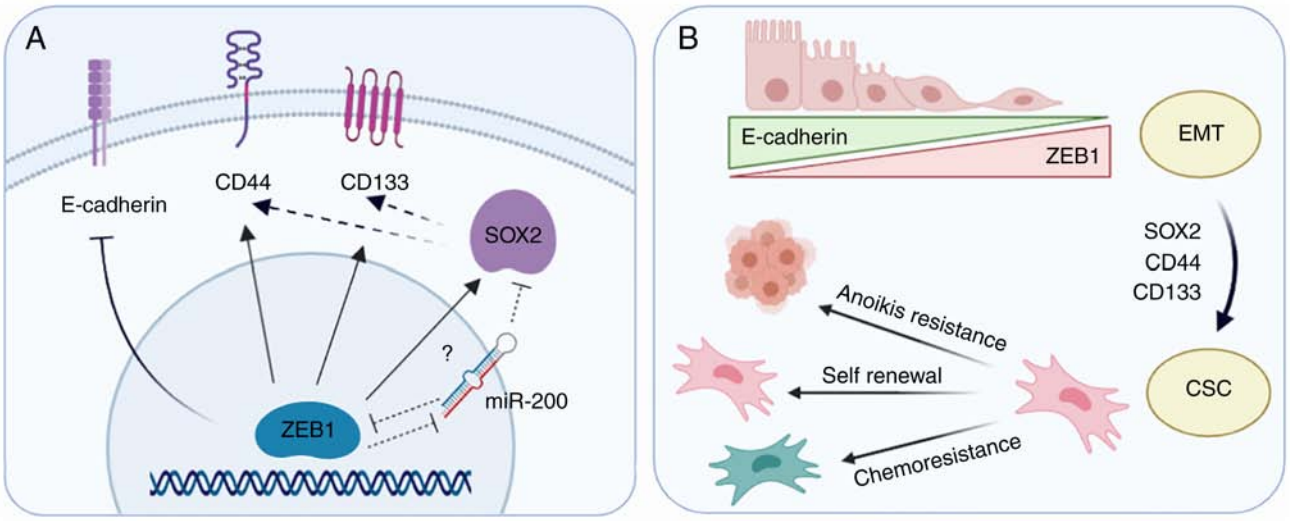

Figure 7. Representation of the effect of ZEB1 expression on EMT and CSC markers in prostate cancer cells. (A) ZEB1 transcription factor inhibits E-cadherin expression, inducing EMT. ZEB1-knockdown downregulates the stemness transcription factor SOX2 and decreases the expression levels of the prostate CSC markers CD44 and CD133, indicating that ZEB1 may be promoting the expression of these proteins directly (arrows) or indirectly (dashed arrows). A possible mediator may be miR-200, as previous studies (75,76) have shown that it is directly repressed by ZEB1, and in turn, miR-200 directly represses SOX2. (B) Upregulation of SOX2, CD44 and CD133 by ZEB1 may lead to a CSC phenotype. Targeting ZEB1 with small interfering RNAs may reverse this process, decreasing anoikis resistance, self-renewal capacity and chemoresistance of androgen-independent prostate cancer cells. EMT, epithelial-mesenchymal transition; CSC, cancer stem cell; miR, microRNA; ZEB1, zinc finger E-box-binding homeobox 1.

ability of self-renewal of the LNCaP ZEB1-knockdown cells, a colony formation assay was performed. ZEB1-knockdown in LNCaP cells did not affect the number or size of the colonies formed (Fig. 6C-E). However, it modified the morphology of the colonies, forming significantly fewer holoclones and more meroclones compared with the control cells (Fig. 6F). Finally, to evaluate the ability of these cells for anchorage-independent growth, a prostatosphere formation assay was performed. As shown in Fig. 6G-I, the LNCaP control cells cultured in soft agar formed bigger prostatospherescompared with the LNCaP cells transduced with sh-ZEB1 after 5 and 7 days, although by day 7 the prostatospheres were smaller compared with day 5 as the LNCaP cells disaggregated.

Discussion

The amount of CSCs within a tumor varies and may be important for the prognosis of the disease (16,48-50). Studies in several types of cancer, such as melanoma, breast, colon and prostate cancer, have described CSCs as tumor-initiating cells, as they can generate a new tumor in distant organs in an appropriate cellular environment and contribute to cancer aggressiveness due to their radio- and chemo-resistance, driving recurrence following conventional therapy (17,18,51).

EMT is accompanied by a reactivation of signaling pathways involved in self-renewal, such as the Wnt and Notch signaling pathway, which facilitate changes in the phenotypic profile of cells, with some of them acquiring a more aggressive and/or mesenchymal phenotype, favoring metastasis and invasiveness (52,53). ZEB1 is a transcription factor that modulates EMT, repressing E-cadherin expression and favoring the expression of mesenchymal markers, in coordination with other transcription factors from the SNAIL and TWIST family (38,54,55).

In the present study, the effect of ZEB1-knockdown on the expression levels of E-cadherin and pluripotency genes commonly expressed in embryonic cells, SOX2 (56,57) and KLF4 (58,59), was investigated. It was found that silencing ZEB1 in DU145 cells induced an increase in E-cadherin and a decrease in SOX2 expression. However, ZEB1 silencing did not regulate the expression levels of KLF4. Stoichiometric SOX2 and KLF4 expression is sufficient for pluripotency in the absence of OCT4 (60). However, knocking down SOX2 by itself results in a decrease of stemness and tumor growth, and induces tumor regression in several types of cancer, such a colorectal, breast and lung cancer (61). Considering that SOX2 has been described as one of the transcription factors that is overexpressed in more aggressive cancer cells (62,63), targeting ZEB1 and the consequent decrease of SOX2 expression may impair CSC self-renewal and maintenance in a variety of tumors, including PCa. However, it is not clear whether this effect may be a result of the EMT process or be EMT-independent. Previous studies have reported that SOX2 increases cell proliferation and survival by inducing EMT (64,65). Forced SOX2 expression increases the expression levels of the EMT transcription factors TWIST, SNAI1 and SNAI2 in pancreatic cancer cell lines (66). In PCa, SOX2-knockdown decreases the expression levels of SNAI1 and SNAI2, and inhibits migration and prostatosphere formation (67). Furthermore, knocking down SNAI1 in pancreatic cancer cells increases E-cadherin expression and downregulates SOX2 expression, as well as decreases tumor size *in vivo* (68). In the PCa PC3 cells, silencing E-cadherin increases the formation of prostatospheres and the expression levels of CD44 and SNAI1 (69). Notably, knocking down SNAI1 in these cells results in a decrease in prostatosphere formation and clonogenicity (69), which is similar to the observed phenotype of the DU145 cells with ZEB1-knockdown in the present study. This suggested that transcriptional factors involved in EMT may be key for

the induction of CSC features. On the other hand, a previous study has reported temporary SOX2 and KLF4 expression, mainly during colonization in the metastatic niche by CSCs, which was absent or low during EMT (70).

Consistent with the results of the present study in DU145 cells, knockdown of ZEB1 in LNCaP cells increased E-cadherin and decreased SOX2 expression. However, in LNCaP cells, no changes were observed in the expression levels of the CSC markers, CD44 and CD133. Prostate CSCs are characterized by a molecular signature that includes positive expression of CD44 and CD133 (15). In the present study, it was found that LNCaP cells expressed very low levels of CD44 and CD133. By contrast, DU145 cells were positive for CD44 and CD133 expression, and their expression levels were downregulated by ZEB1-knockdown. A possible explanation for the different results observed may be due to the intrinsic characteristics of both cell lines. LNCaP cells are androgen-sensitive cells derived from a lymph node metastasis (41), representing an earlier stage of PCa, whereas DU145 are androgen-insensitive cells, derived from a brain metastasis (40); therefore, they are more representative of CRPC. Androgen sensitivity of PCa cells serves a role in CSC phenotype and ZEB1 expression. In androgen-sensitive cells, androgens promote the expression levels of ZEB1 via the binding of the AR to androgen response elements present in the ZEB1 promoter (71). Prostate CSCs do not express AR and other prostate epithelial differentiation markers (9,16). However, it has been demonstrated that ZEB1 expression may be induced in AR-null cells (72). Furthermore, in our previous study, ZEB1 expression in PCa cell lines was characterized, revealing that DU145 cells expressed higher levels of ZEB1 compared with LNCaP cells (38). The aforementioned studies, together with the results of the present study, indicated that DU145 cells may be enriched in cell populations that display CSC properties, such as chemotherapy and anoikis resistance, whereas LNCaP cells did not display these characteristics.

Increasing evidence has revealed that ZEB1 is a key factor for the transition between non-CSCs and CSCs in other types of cancer, such as pancreatic and breast cancer (55,73,74). This effect may be mediated by non-coding RNAs inhibiting the expression levels of stemness genes. Several stemness-repressing microRNAs (miRs) have been described (75). Among them, miR-200 may represent the link between ZEB1 and CSCs. It has been demonstrated that ZEB1 and miR-200 are associated with a double-negative feedback loop: ZEB1 inhibits miR-200 expression, which in turn suppresses the translation of ZEB1 mRNA (76). On the other hand, miR-200 also represses the expression levels of SOX2 and KLF4 (75). Therefore, one of the limitations of the present study was the lack of analyzing miR-200 expression following ZEB1-knockdown. It would be interesting to determine whether the downregulation of SOX2 by ZEB1-knockdown would result in the loss of direct interaction between ZEB1 and the SOX2 promoter or through the lack of ZEB1 inhibition on miR-200.

Knocking down ZEB1 in the present study decreased the expression levels of SOX2, as well as the number of colonies formed *in vitro* and the proportion of holoclones, and increased the number of paraclones. Cells that form holoclones and express CSC markers, such as CD44 and CD133, have the ability of self-renewal, generate cultures in non-adherent conditions and have highly tumorigenic abilities when injected into immunodeficient mice (77,78). On the other hand, paraclones can proliferate, but not self-renew (77). The results in the present study are consistent with the study by Knaack *et al* (79), which found higher expression levels of ZEB1 in holoclones in pancreatic cancer cells compared with those in paraclones. Moreover, holoclones of pancreatic cancer cells had increased expression levels of TNFα and other pro-inflammatory genes acting as EMT inducers compared with paraclones, which is consistent with the higher expression levels of ZEB1 (79).

The present study also revealed that there was a decrease in the expression levels of CD44 and CD133 in DU145 cells following ZEB1-knockdown. Overexpression of CD44 and SOX2 in PCa cells results in the upregulation of the SNAI1 and SNAI2 transcription factors leading to EMT (67). Overexpression of CD133 in PCa cells increases the expression levels of other CSC markers, decreases E-cadherin expression and enhances migration and bone metastasis formation (80). Furthermore, the presence of CD44 and CD133 have been identified as important factors in the formation of prostatic spheroids, which is directly associated with the ability of self-renewal and anoikis resistance in PCa cells (81). In the present study, knocking down ZEB1 decreased the number of colonies formed in adherent conditions, as well as the number and size of the prostatospheres generated, which is consistent with the observed downregulation of CD44, CD133 and SOX2 expression. Pluripotency genes are important factors in chemoresistance and apoptosis evasion. Overexpression of SOX2 and OCT4 in gastric cancer cells increases their resistance to oxaliplatin and fluorouracil (82). In PCa, $CD133^+$ cells, sorted from human 22Rv1 PCa cells, are highly resistant to γ-radiation and docetaxel (83). In T-cell acute lymphoblastic leukemia, CD44 enhances the activity of ATP-binding cassette multidrug efflux transporters, inducing resistance to doxorubicin (84). In agreement with this, our previous study revealed that knocking down ZEB1 in DU145 cells decreases the expression levels of multidrug resistance-associated protein 1 and ATP-binding cassette subfamily C member 4, and enhances their sensitivity to docetaxel (37). This suggested that ZEB1, SOX2, CD44 and CD133 may participate together to promote chemoresistance. Overall, the results of the present study indicated that targeting ZEB1 in PCa decreased the expression levels of CSC markers and affected their function; thus, this may directly impact tumor resistance and recurrence (Fig. 7).

In conclusion, knocking down ZEB1 in aggressive PCa cells decreased the expression levels of the CSC markers CD44 and CD133, and of the transcription factor SOX2. Additionally, compared with the control cells, cells with ZEB1-knockdown exhibited a lower capacity for anchorage-independent growth and self-renewal, important characteristics for metastasis and recurrence. As a future therapy, targeting ZEB1 may reprogram CSCs into non-CSCs, decreasing their number within a tumor, and therefore improving the response to therapy and prognosis of patients with advanced PCa.

Acknowledgements

The authors would like to thank Ms. Graciela Caroca and Ms. Catherine Gatica from the Laboratory of Cellular and

Molecular Oncology, Department of Basic and Clinical Oncology, University of Chile (Santiago, Chile), for their technical assistance.

Funding

The present study was supported by grants from the FondoNacional de Ciencia y Tecnología (grant nos. 1151214 and 1201704) and U-Redes (grant no. 007/17).

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

GP and FLM designed and performed the experiments, conducted the statistical analysis and wrote the manuscript. SI participated in the design, experimental work and data analysis of LNCaP cells. MJT analyzed the data. EAC and HRC conceived the study, participated in its design and coordination, wrote the manuscript and are responsible for confirming 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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