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ORIGINAL ARTICLE

Prostate Cancer

The transcription factor ZEB1 promotes an aggressive phenotype in prostate cancer cell lines

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It has been reported that one of the factors that promotes tumoral progression is the abnormal activation of the epithelial–mesenchymal transition program. This process is associated with tumoral cells acquiring invasive and malignant properties and has the transcription factor zinc finger E-box-binding homeobox 1 (ZEB1) as one of its main activators. However, the role of ZEB1 in promoting malignancy in prostate cancer (PCa) is still unclear. Here, we report that ZEB1 expression correlates with Gleason score in PCa samples and that expression of ZEB1 regulates epithelial–mesenchymal transition and malignant characteristics in PCa cell lines. The results showed that ZEB1 expression is higher in samples of higher malignancy and that overexpression of ZEB1 was able to induce epithelial–mesenchymal transition by upregulating the mesenchymal marker Vimentin and downregulating the epithelial marker E-Cadherin. On the contrary, ZEB1 silencing repressed Vimentin expression and upregulated E-Cadherin. ZEB1 expression conferred enhanced motility and invasiveness and a higher colony formation capacity to 22Rv1 cells whereas DU145 cells with ZEB1 silencing showed a decrease in those same properties. The results showed that ZEB1 could be a key promoter of tumoral progression toward advanced stages of PCa.

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Keywords: epithelial–mesenchymal transition; prostate cancer; transcriptional repression; ZEB1

INTRODUCTION

Prostate cancer (PCa) is a main problem of health worldwide, representing the second most diagnosed cancer in men and fifth in cancer-associated mortality.¹ The major risk factor is age, with an average age of diagnostic of 66 years, while other factors can include obesity and family history. At its early stages, PCa is usually asymptomatic causing that in many cases the disease has already reached an advanced stage when diagnosed and treatment is less or even noneffective.²

In the last years, it has been reported that during tumoral progress there is an abnormal activation of the epithelial–mesenchymal transition program (EMT).³ EMT is a reversible process normally activated during the development in which epithelial cells lose their apical–basal polarity and cell-to-cell contacts acquiring a mesenchymal-like phenotype that makes them more motile and invasive.⁴ The activation of EMT during tumoral progress leads to the acquisition of a more malignant and aggressive phenotype promoting metastasis in epithelial tumors.⁵ The main event in this process is the repression of the epithelial marker E-cadherin and the induction of mesenchymal genes such as Vimentin or fibronectin.⁶ These changes are controlled by several transcription factors such as E-box-binding homeobox 1 (ZEB1), Snail, Slug, and Twist.⁷

ZEB1 belongs to the zinc finger family of homeodomain transcription factors and contains regions that bind to DNA, to co-activator/repressors, and to other transcription factors, thus being able to activate or repress genes by different means and in different contexts.^{8,9} As an EMT activator, its best known role is to directly repress E-cadherin expression,¹⁰ which is a crucial step in EMT activation.

It has also been reported that ZEB1 can control the expression of genes involved in other aspects of the EMT switch, such as the direct activation of the Vimentin gene, repression of Pals1 (part of the adherens junctions), repression of Crumbs3 (a controller of cell polarity),¹¹ and activation of metalloproteinases such as MT1-MMP,¹² thus contributing to tumoral malignancy from various angles.

Moreover, recent reports in different types of cancer have correlated a higher ZEB1 expression with late stages of the disease. In uterine cancer, ZEB1 is expressed almost exclusively in more aggressive classes,¹³ where its expression is related to poorer clinical outcome in breast cancer¹⁴ and other types of epithelial tumors.¹⁵ A recent investigation showed that ZEB1 appeared to be overexpressed in PCa samples of high Gleason compared to samples of lower malignancy.¹⁶ Even though there is information regarding ZEB1 and its effects on tumoral cells, we still lack information about the role that it could be playing in PCa progress. In the present study, we show that ZEB1 overexpression leads to the activation of the EMT program and increases clonogenicity, motility, and invasiveness in PCa cell lines and that silencing of ZEB1 can lower these malignant properties.

MATERIALS AND METHODS

Cell cultures

The human prostate carcinoma cell lines PC3, LnCaP, 22Rv1, and DU145 and the prostate epithelial line RWPE1 were obtained from the American Type Culture Collection (Rockville, MD, USA). PC3 and LnCaP cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) while DU145 and 22Rv1 cells were cultured in RPMI 1640 medium. Both DMEM and Roswell Park Memorial

Institute medium (RPMI) 1640 were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. RWPE1 cells were grown in keratinocyte serum-free medium (KFSM) supplemented with 0.05 mg ml⁻¹ bisphenol A ethoxylate (BPE), 5 ng ml⁻¹ epidermal growth factor (EGF) and 1% penicillin and streptomycin. All cell lines were maintained under standard cell culture conditions at 37°C in 5% CO₂ in a humid environment.

Lentiviral infection for stable overexpression and silencing of ZEB1

5.5 × 10⁴ 22Rv1 or DU145 cells were seeded on a 6-well plate for 24 h before infection. Then, cells were transduced with different lentiviruses at a multiplicity of infection of 5 in the presence of 8 µg ml⁻¹ polybrene (Sigma-Aldrich, St. Louis, MO, USA). To determine the stable expression of the used vectors, cells were selected for 48 h with 1.5 µg ml⁻¹ of puromycin. Afterward, mRNA levels of ZEB1 were analyzed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For ZEB1 overexpression, the pLenti-suCMV (ZEB1)-Rsv (RFP-Puro) vector was used with the pLenti-suCMV-Rsv (RFP-Puro) as control. For ZEB1 silencing, the pLenti-U6-shRNA (h ZEB1)-Rsv (RFP-Puro) vector was used with pLenti-U6-shRNA (neg-control)-Rsv (RFP-Puro) as control. All lentiviruses used in this work were purchased from Gen Target Inc. (San Diego, CA, USA).

Immunohistochemistry

Formalin-fixed and paraffin embedded prostate specimens were obtained from the archives of the Pathological Anatomy Service, Clinic Hospital of the University of Chile, with the corresponding authorization. All samples were evaluated by an expert pathologist and classified according to their Gleason scores. The methodology was carried out as previously described in our laboratory.¹⁷ Briefly, following dewaxing and rehydration, samples were incubated for 10 min at 95°C–100°C in antigen retrieval buffer. After cooling down, the endogenous peroxidase was inhibited and samples were blocked with PBS (2% BSA). The sections were incubated overnight at 4°C with the primary antibody, and a secondary antibody was added to the sections for 30 min at 37°C. Samples were also counterstained with hematoxylin. ZEB1 staining intensity was scored by the pathologist as follows: negative (0), weak (1), moderate (2), and strong (3).

qRT-PCR

Cells were grown to confluence and lysed by adding 1 ml of TRIZOL (Thermo Fisher, Waltham, MA, USA; Cat. 15596-026) directly in the culture dish (1 ml per 3.5 cm diameter dish) and scraping. RNA was extracted and quantified using a Synergy HT Multi-Detection Microplate Reader (BIOTEK, Winooski, VT, USA). cDNA was synthesized using the AFFINITYSCRIPT QPCR CDNA synthesis kit (Agilent Tech., Santa Clara, CA, USA; Cat. 600559) following the manufacturer's protocol. qRT-PCR was performed in triplicate using the Brilliant II SYBR green QPCR Master Mix kit (Agilent Tech., Cat. 600828) in an Aria Mix Real-Time PCR system (Agilent Tech., 68830A model). Data were analyzed using the AriaMX 1.0 program (Agilent Tech.). The following sets of primers were used: ZEB1 (s): 5'-GTA AGA GGC CTC ACG AGT GT-3', (as): 5'-GCA GTA GGA GTA GCG GTG AT -3', CDH1 (E-Cadherin) (s): 5'-GAA CGC ATT GCC ACA TAC AC-3', (as): 5'-ATT CGG GCT TGT TGT CAT TC-3', KRT18 (Cytokeratin 18) (s): 5'-ACA GAG TGA GGA GCC TGG AGA CCG A-3', (as): 5'-CAG TAT TTG CGA AGA TCT GAG CCC TC-3', Cdh2 (N-Cadherin) (s): 5'-GGA CAG TTC CTG AGG GAT CA-3', (as): 5'-GGA TTG CCT TCC ATG TCT GT-3', VIM (Vimentin) (s): 5'-GCC AAG GCA AGT CGC G-3', (as): 5'-CA TTT CAC GCA TCT GGC G-3'. The mRNA levels were normalized using the housekeeping gene

PUM1 (Pumilio) (s): 5'-CGG TCG TCC TGA GGA TAA A-3', (as): 5'-CGT ACG TGA GGC GTG AGT AA-3'.

Western blot analysis

Western blot studies were carried out as previously described in our laboratory.¹⁸ Briefly, cells were grown to confluence and then proteins were extracted using RIPA buffer and quantified by the Bradford method. For the analysis, 50 µg of protein was resolved over 10% polyacrylamide gels and electrotransferred onto a nitrocellulose membrane. The membranes were blocked with a blocking buffer for 1 h at room temperature and then incubated overnight at 4°C with the corresponding primary antibody in blocking buffer, followed by incubation with the appropriate secondary antibody for 1 h and detection by chemiluminescence. Bands were quantified using the ImageJ photo analyzing program (US National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used: ZEB1 (Millipore, Villerica, MA, USA; Cat. ABN285), E-Cadherin (BD Transduction Laboratories, San Jose, CA, USA; Cat. 610181), N-Cadherin (Abcam, Cambridge, UK; Cat. ab12221), Vimentin (Abcam; Cat. ab8978), CK-18 (Abcam; Cat. ab7797), and β-actin as loading control (MP Bio, Santa Ana, CA, USA; Cat. 69100).

Migration and invasion assay

The migration and invasion assays were conducted using the CytoSelect™ 96-Well Cell Migration and Invasion kit (Cell Biolabs, San Diego, CA, USA, Cat. CBA-106-C), which is based on the Boyden chamber method. Briefly, 22Rv1 cells (control or ZEB1 overexpressing) and DU145 cells (transfected with a ZEB1-shRNA or scramble-shRNA) were harvested and resuspended in serum free RPMI medium at a density of 0.5 × 10⁶ cells for the migration assay and 1 × 10⁶ for the invasion assay. Next, 100 µl of the cell suspension was added to the upper chamber and 150 µl of RPMI (10% fetal bovine serum) to the lower chamber. In the case of the migration assay, the membrane separating both chambers was a polyvinyl chloride (PVC) filter with 8.0-µm pores, while in the invasion assay, it was covered in basement membrane. The chamber was incubated for 24 h at 37°C after which the cells that had migrated or invaded to the lower surface of the filter were lysed and quantified according to the kit manufacturer's instructions in a fluorescence plate reader at 480 nm/520 nm (Biotek Synergy HT Multi-Mode Microplate Reader).

Wound-healing assay

DU145 cells (transfected with ZEB1-shRNA or scramble-shRNA) were seeded in 6-well plates and cultured until confluence using RPMI supplemented with 10% fetal bovine serum. Next, using a pipette tip, a scratch was made on the plate, creating a straight line. Cells were washed with PBS afterward to remove detached cells, left in culture medium, and placed at 37°C under a camera focused in the scratch using a CytoSMART™ Lux 10X System (Lonza Group Ltd., Basel, Switzerland) taking photographs every 15 min. The experiment was ended after the cells finish closing the wound, and the gap distance at different times was measured using the ImageJ photo analyzing program.

Clonogenic assay

Dilutions consisting of 1 × 10³ cells were seeded in each well of a 6-well plate for each type of cell studied, covered with their correspondent culture medium, and then placed in an incubator at 37°C with 5% CO₂ in humidified air. The 6-well plates containing the cells were incubated for 12 days until large colonies were formed. Then, the medium was removed and cells were rinsed with cold PBS three times. The resulting colonies were fixed for 10 min with cold methanol and stained with

crystal violet (0.5% crystal violet and 25/75 methanol/water) for 30 min. Later, dishes were rinsed and dried at room temperature. For the colony counting, a microscope (Leica, Wetzlar, Germany; model DM2500) was used and only those colonies containing 50 or more cells were considered.

Proliferation assay

Cells were seeded in 6-well plates (2×10^4 cells per well) and then 3 wells were counted every 24 h for 6 days. A cell suspension of every well in 1 ml of medium was obtained, and a 1:2 dilution was made with trypan blue stain (10 μ l of cell suspension and 10 μ l of trypan blue 0.4%), and then 10 μ l of this mix was placed between the cover slip and chamber of a hemocytometer. Viable cells in the four corner squares were counted (nonviable cells would stain blue), and the number of cells per ml was obtained from the average count per square multiplied by 2×10^4 .

Statistical analysis

Data were expressed as mean \pm s.d. All experiments were repeated at least three times and the significance between the control and transduced cells was calculated using an unpaired Student's *t*-test for *P* value. *P* < 0.05 was considered as statistically significant difference. Statistical analysis for the association between ZEB1 expression and Gleason score in PCa samples was carried out using Fisher's exact test with 4×2 contingency table. The GraphPad prism 5.0 program was used (GraphPad Software Inc., San Diego, CA, USA) for the statistical analysis.

Ethical approval

All procedures were approved by the Bioethics Committee for Research on Human Beings and the Risk Prevention and Biosafety Unit of the Faculty of Medicine of the University of Chile.

RESULTS

ZEB1 expression in human PCa samples and cell lines

Graham *et al.*¹⁶ reported some years ago that ZEB1 expression correlated with tumor grade and aggressiveness in PCa samples.¹⁶ To confirm their results in our patient series (and in a national context), we analyzed a tissue microarray containing samples of different Gleason scores using immunohistochemistry. As shown in **Figure 1a** and **1b**, all PCa samples showed staining in the stroma, but samples of high Gleason score (≥ 8) also showed high nuclear staining in tumoral epithelial cells whereas samples of low score (≤ 6) have minimal-to-negative nuclear staining in these cells (Fisher's exact test, *P* < 0.05). Next, basal levels of ZEB1 and classical epithelial (E-Cadherin, Cytokeratin 18 (CK18)) and mesenchymal (Vimentin, N-Cadherin) markers were analyzed in different PCa cell lines using Western blot analysis in order to better characterize these cells regarding their epithelial phenotype. **Figure 1c** and **1d** show the different expressions of these markers in the different PCa cell lines compared to the prostate epithelial line RWPE1. The highly aggressive and metastatic DU145 cell line showed high expression of ZEB1 and mesenchymal markers with a low expression of epithelial markers while the poorly tumorigenic 22Rv1 cell line presented low expression of ZEB1 and mesenchymal markers with a high expression of epithelial markers (*t*-test, *P* < 0.05). These results led us to choose the 22Rv1 line as a model for the study of ZEB1 overexpression and the DU145 line for the silencing of this transcription factor.

Effect of ZEB1 overexpression and silencing on EMT markers

Using lentiviral transduction, we obtained stable cell lines with overexpressed (22 ZEB1) and silenced (DU sh) ZEB1 and their respective controls (22 null and DU scr). **Figure 2a** shows the

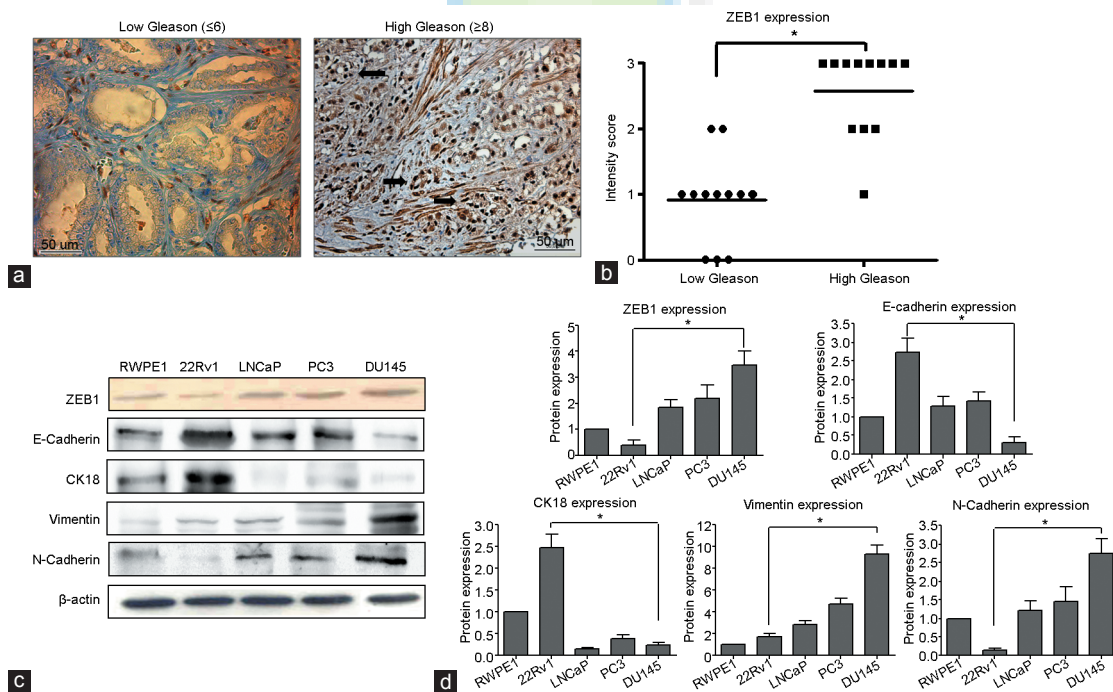


Figure 1: ZEB1 expression in human prostate cancer samples, cell lines, and basal levels of EMT markers in cell lines. (a) Representative pictures of immunohistochemical staining for ZEB1 in different samples of low (≤ 6) and high (≥ 8) Gleason score ($\times 40$). Scale bars = 50 μ m. (b) Summary of the differential expression of ZEB1 between samples of low and high Gleason score. (c) Western blot analysis of ZEB1 and EMT markers in different prostate cancer cell lines. (d) Graphical representation of the Western blot results. All experiments were carried out three times with similar results (*n* = 3). **P* < 0.05 in all cases. EMT: epithelial–mesenchymal transition.

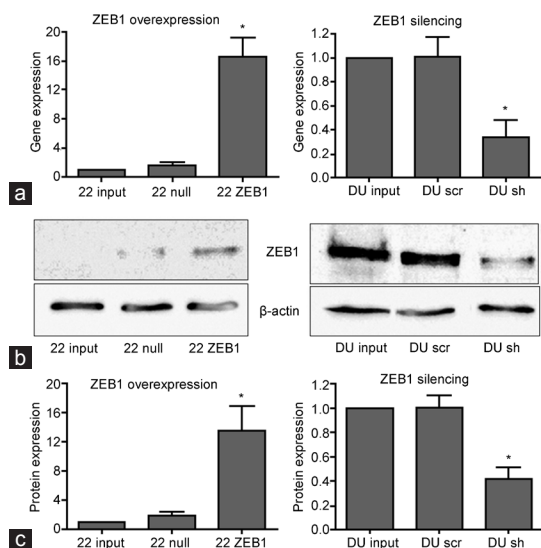


Figure 2: Results for the stable ZEB1 silencing and overexpression in prostate cancer cell lines 22RV1 and DU145. (a) qRT-PCR analysis for the overexpression and silencing of ZEB1 in cell lines 22RV1 and DU145, respectively. (b) Western blot analysis for the overexpression and silencing of ZEB1 in cell lines 22RV1 and DU145, respectively. (c) Quantification of the Western blot results. Each image shows a representative experiment repeated three times with similar results ($n = 3$). * $P < 0.05$ in all cases. qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

results of the overexpression and silencing through qRT-PCR while **Figure 2b** and **2c** shows the protein levels using Western blot analysis.

EMT is characterized for a downregulation of key epithelial markers (being E-Cadherin the most important) and an upregulation of mesenchymal markers. In the present work, we used qRT-PCR and Western blot analysis to study the effect of ZEB1 expression on this switch in markers. The qRT-PCR analysis showed that overexpression of ZEB1 led to a decrease in *CDH1* (E-Cadherin) expression and an increase in *VIM* (Vimentin) expression in 22RV1 cells; in contrast, silencing of ZEB1 caused an increase in *CDH1* expression and a decrease in *VIM* in DU145 cells when compared with their respective controls (**Figure 3a**, t -test, $P < 0.05$). The Western blot analysis demonstrated similar results at the protein level, with 22 ZEB1 cells showing lower E-Cadherin expression and higher Vimentin, and the DU sh cells showing higher E-Cadherin and lower Vimentin levels (**Figure 3b** and **3c**, t -test, $P < 0.05$) compared to their controls. Gene and protein expressions of other EMT markers such as Cytokeratin 18 and N-Cadherin were also studied, but no changes in either of these markers were found in the 22 ZEB1 cells or in the DU sh cells using qRT-PCR or Western blot analysis (**Figure 3a-3c**) when compared to their respective controls (t -test, $P > 0.05$).

Effect of ZEB1 on cell proliferation and clonogenic capacities

One of the hallmarks of cancer cells is their ability to proliferate indefinitely.¹⁹ To assess the effect of ZEB1 on this aspect of tumoral malignancy, a clonogenic assay was used. **Figure 4a** and **4b** shows that overexpression of ZEB1 conferred 22RV1 cells with a higher clonal capacity when compared to their control (t -test, $P < 0.05$). On the other hand, silencing of ZEB1 in DU145 cells lowered their clonogenic capacities in a significant percentage (t -test, $P < 0.05$). To corroborate that these results were due to changes in the clonogenic properties of the cells and not due to a possible change in their proliferation rate,

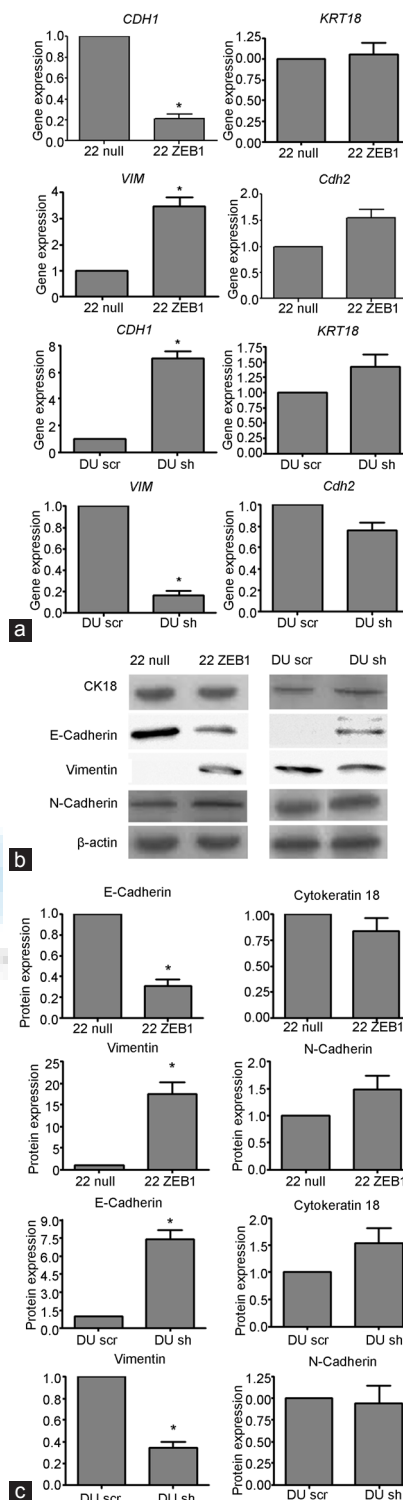


Figure 3: Analysis of EMT markers. (a) qRT-PCR analysis of EMT markers in 22RV1 cells with ZEB1 overexpression and DU145 cells with ZEB1 silencing. (b) Western Blot analysis of EMT markers in 22RV1 cells overexpressing ZEB1 and DU145 with ZEB1 silencing. (c) Quantification of the Western blot results. Each image shows a representative experiment repeated three times with similar results ($n = 3$). * $P < 0.05$ in all cases. qRT-PCR: quantitative reverse transcription-polymerase chain reaction; EMT: epithelial-mesenchymal transition.

we analyzed cell growth. This was studied using the common trypan blue exclusion method and a hemocytometer. As shown in **Figure 4c**,

there were no significant differences in cell proliferation in the 22 ZEB1 cells or in the DU sh cells when compared with their respective controls (*t*-test; $P > 0.05$).

Effect of ZEB1 on cell migratory and invasive properties

To examine the effect of ZEB1 expression on the cell migratory and invasive properties, we used a modified Boyden chamber assay. In the migration assay, 22 ZEB1 cells showed a higher percentage of migratory cells while DU sh cells had a lower percentage of migratory cells when we compared them with their respective controls (Figure 5a, *t*-test, $P < 0.05$). In a similar manner, in the invasion assays, 22 ZEB1 cells demonstrated to have a higher invasion rate while DU sh cells had a lower rate when comparing both to their respective controls (Figure 5b, *t*-test, $P < 0.05$). Interestingly, DU145 cells were able to both migrate and invade more than 22Rv1 cells (data not shown), which is in accordance with data reported by the ATCC that has DU145 cells as being very aggressive and metastatic and 22Rv1 being less aggressive.

To confirm these findings, we carried out a wound-healing assay using DU145 cells. Figure 6 shows that silencing of ZEB1 in DU145 cells lowered cell migration towards the nude area of the wound when compared to the control cells (*t*-test, $P < 0.05$), results that are congruent with those found with the Boyden chamber assays.

DISCUSSION

In the last decade, it has been reported that the abnormal activation of the EMT program is a key event during the different stages of tumoral growth, especially for the formation of local and distant metastasis.²⁰ In this context, the association between tumoral progress and the transcription factors that induce EMT (and more specifically ZEB1) has been highlighted by recent articles.^{21,22} Aigner *et al.*¹¹ reported that ZEB1 repressed several genes involved in epithelial differentiation and cell-cell adhesion such as Crumbs3 and HUGL2 in immortalized human mammary epithelial cells. Interestingly, in this study, it was demonstrated that knockdown of Snail (another EMT activator) was not sufficient to reestablish the expression of Crumbs3, HUGL2, or E-cadherin.

In the present research, it is showed that overexpression of the transcription factor ZEB1 leads to the activation of the EMT program in the PCa cell line 22Rv1, this was evidenced by changes in some of the typical EMT markers such as E-Cadherin and Vimentin and in functional properties such as in migration and invasiveness. Interestingly, no significant changes in other EMT markers such as N-Cadherin or Cytokeratin 18 when overexpressing or silencing ZEB1 were found. This is in accordance with the findings of Aigner *et al.*¹¹ who reported that repression of a single EMT-inducing factor was not able to revert all changes associated with the EMT process. One of the possible explanations for this finding is that, as was reported by De Herreros *et al.*,²³ there would be a temporality in the activation of the different factors inducing EMT; for example, Snail may be important at the start of the program, but then, ZEB1 would take over, sustaining it in time. Garg²⁴ even reports that Snail and Twist could act in conjunction to upregulate ZEB1 expression during the early stages of EMT. Supporting this idea, in our model, ZEB1 silencing was only able to revert the expression of E-Cadherin and Vimentin but not other classic EMT markers such as N-Cadherin or Cytokeratin 18. Dave *et al.*²⁵ reported that there exists cooperation among the different EMT promoters. Then, it is possible that ZEB1 requires the action of other transcription factors such as Snail or Twist to carry out a complete switch in EMT markers.

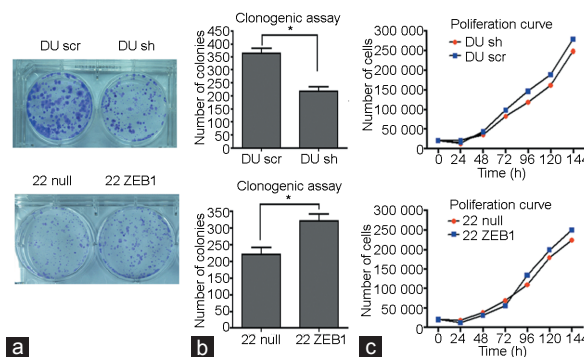


Figure 4: Proliferation and clonogenic assays. (a) Clonogenic assay of 22Rv1 cells with ZEB1 overexpression and DU145 cells with ZEB1 silencing compared to their controls. Photographs from representative experiments are shown. (b) Graphical representation from the results of the clonogenic assays in (a). (c) Proliferation curves of 22Rv1 cells with overexpressing ZEB1 and DU145 with ZEB1 silencing. $n = 3$, $*P < 0.05$ in all cases.

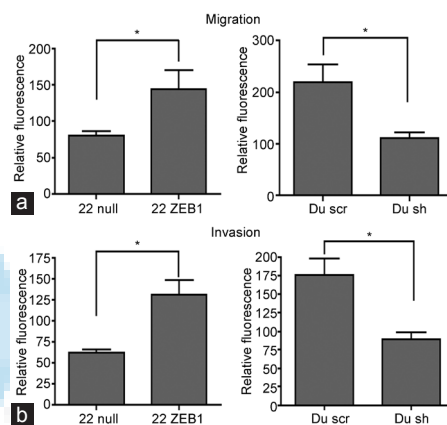


Figure 5: Invasion and migration assays. The figure shows the graphical comparison of (a) migration and (b) invasion of 22Rv1 cells with ZEB1 overexpression and DU145 cells with ZEB1 silencing compared to their respective controls using a modified Boyden chamber assay. $n = 5$, $*P < 0.05$ in all cases.

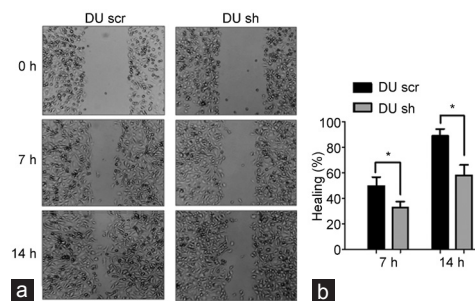


Figure 6: Wound-healing assay. (a) Photographs of artificially induced "wounds" in monolayers of DU145 cells with ZEB1 silencing at different time periods ($\times 10$, zoom). (b) Comparison of healing percentages of the studied cells at different times. $n = 5$, $*P < 0.05$ in all cases.

The metastasis process consists of many different steps such as migration of the tumoral cells with the posterior invasion to local or distant organs where the EMT program is a key event in many aspects of this phenomenon.²⁶ In our current research, we also report that ZEB1 expression by itself was also able to control tumoral capacities such as migration, invasion, and clonogenicity (as seen in the 22Rv1 cells with ZEB1 overexpression and the DU145 cells with ZEB1 silencing). In an interesting



work, Drake *et al.*²⁷ showed that a higher expression of ZEB1 conferred PC3 cells with a higher capacity to migrate through an endothelial barrier, proposing ZEB1 as a key element in the formation of distant metastasis. These evidences suggest that ZEB1 is a controller of multiple pathways during not only EMT induction, but also tumoral progression.

Moreno-Bueno *et al.*²⁸ reported, in breast cancer, that EMT inducers such as Snail and Slug were able to regulate common but also specific genetic programs during EMT, a fact that would support the idea of differential roles for these factors during tumoral progression. In summary, our research has shown that ZEB1 expression can regulate the EMT program in PCa cell lines as well as properties such as invasion and migration, suggesting that regulation of this factor may represent a therapeutic target to slow tumoral progression in PCa that could also avoid the side effects of current therapies.²⁹

AUTHOR CONTRIBUTIONS

OOS carried out the molecular studies, performed the statistical analysis, and drafted the manuscript. DH carried out the molecular studies. EAC and HRC participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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