Silencing of the transcriptional factor ZEB1 alters the steroidogenic pathway, and increases the concentration of testosterone and DHT in DU145 cells

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Abstract. Prostate cancer (PCa) is the second most common type of male malignancy worldwide. The transcription factor zinc finger E-box binding homeobox 1 (ZEB1) is associated with epithelial-mesenchymal transition and is also involved in regulation of androgen receptor (AR) expression, the main ligands of which are testosterone and dihydrotestosterone (DHT). These androgens are synthesized through the steroidogenic pathway within the prostate, and their synthesis is altered in PCa. The present study aimed to determine the ZEB1-induced alterations in androgen synthesis and AR expression in the DU145 PCa cell line. Reverse transcription-quantitative polymerase chain reaction, western blotting and immunocytochemistry were used to determine the mRNA and protein expression levels, and cellular localization of steroidogenic pathway enzymes in the DU145 cell line in response to ZEB1 silencing. Furthermore, the concentrations of testosterone and DHT were detected in cell culture medium using ELISA. ZEB1-silenced cells exhibited an increase in testosterone and DHT production, an increase in AR expression and an alteration in the steroidogenic pathway. In particular, steroidogenic acute regulatory protein and 5α-reductase 2 expression levels were decreased, whereas cytochrome P450 family 17 subfamily A member 1, 5α-reductase 1, aldo-keto reductase family 1 member D1 and aldo-keto reductase family 1 member C2 expression levels were increased. In conclusion, the present study provided novel information regarding the regulation of intratumoral androgen production in PCa, which is relevant for the progression of the disease to a castration-resistant form.

Introduction

Prostate cancer (PCa) is the second most common type of male malignancy worldwide and ~1.3 million new cases of PCa are predicted to occur in 2018. Furthermore, PCa is the fifth leading cause of cancer-associated mortality in men, and ~359,000 cases of PCa-associated mortality are predicted to occur worldwide in 2018 (1).

Androgens have an important role in the development and growth of the normal prostate gland, as well as in the proliferation of PCa cells (2,3). It has been reported that activation of the androgen receptor (AR) promotes the proliferation of PCa (3). Therefore, androgen deprivation therapy (ADT) is considered the gold standard for the treatment of PCa recurrence and metastasis. However, despite a good initial response to ADT, the majority of patients progress to aggressive castration-resistant PCa (CRPC) within 2-3 years (2,4). Various mechanisms underlying resistance to ADT have been identified, including AR hypersensitivity, mutations, amplification and splicing variants, in addition to intratumoral steroidogenesis (3,4). Regarding intratumoral steroidogenesis, distinct patterns of dysregulated expression of enzymes involved in androgen synthesis and metabolism have been reported, indicating that tumor cells from patients with CRPC exhibit increased expression of the steroidogenic enzymes hydroxysteroid dehydrogenase (HSD)3β1, HSD3β2, HSD17β1, HSD17β3, aldo-keto reductase family 1 member C3 (AKR1C3) and 5α-reductase 1 (5,6).

Notably, ADT may induce epithelial-mesenchymal transition (EMT); activation of this transdifferentiation program may increase tumor malignancy (7). A previous study demonstrated the importance of zinc finger E-box binding homeobox 1 (ZEB1), which is a canonical transcription factor of EMT, since it is not only a key EMT factor, but also an AR regulator (8). In the PC3 cell line, a negative loop of regulation has been identified between ZEB1 and the AR, resulting in decreased levels of AR in response to high ZEB1 expression and vice versa. Furthermore, the AR is a direct regulator of the ZEB1 gene, since it binds to two androgen response elements located 1,000 base pairs near the site of transcriptional initiation (8). However, to the best of our knowledge, the alterations...
occurring in androgen synthesis in relation to ZEB1 have not yet been established. Therefore, it may be hypothesized that ZEB1 alters androgen synthesis capacity in PCa.

The present study aimed to determine the alterations in androgen synthesis and AR expression induced by ZEB1 knockdown in the PCa cell line DU145. The results indicated that ZEB1 silencing significantly altered the expression of the cholesterol transporter (StAR), and enzymes involved in the synthesis and degradation of androgens, including cytochrome P450 family 17 subfamily A member 1 (CYP17A1), 5α-reductase 1, 5α-reductase 2, aldo-keto reductase family 1 member D1 (AKR1D1) and aldo-keto reductase family 1 member C2 (AKR1C2), thus resulting in an increase in testosterone and dihydrotestosterone (DHT) concentration in the cell culture medium. These findings may provide novel information regarding the regulation of androgen synthesis in PCa.

Materials and methods

Cell culture and transduction. The PCa cell line DU145 [cat. no. American Type Culture Collection (ATCC) HTB-81] was obtained from the ATCC (Manassas, VA, USA). ZEB1-knockdown cells (DU145 SH) and control cells (DU145 SCR) were produced in our laboratory according to protocols described in our previous study (9). Briefly, 5.5x10⁴ cells were transduced with 1 µg lentiviral vector containing a short hairpin (sh)RNA against ZEB1 [pLenti-6-shRNA (h ZEB1)-Rsv (RFP-Puro)] (GenTarget, Inc., San Diego, CA, USA). A shRNA against a random sequence was used as a control [pLenti-U6-shRNA (neg-control)-Rsv (RFP-Puro)] (GenTarget, Inc.). Subsequently, cells were selected with 1.5 µg/ml puromycin and effective silencing was verified using fluorescence microscopy, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting (9). These cell lines were cultured in 10% fetal bovine serum (Corning Incorporated, Corning, NY, USA) supplemented with RPMI-Phenol Red-free culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were treated with radioimmunoprecipitation assay lysis buffer mixed with protease inhibitor (Roche Diagnostics, Basel, Switzerland). The obtained homogenate was centrifuged at 26,500 x g (Beckman Coulter Allegra® 21R; Beckman Coulter, Inc., Brea, CA, USA) for 15 min at 4˚C. Finally, the supernatant was quantified using the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Subsequently, 50 µg protein under reducing conditions was separated by 10% acrylamide gel electrophoresis and the proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) at 50 mA and 4˚C overnight. Afterwards, the membranes were blocked with 5% milk solution in TBS-Tween 0.1% (TBST) for 2 h at room temperature, washed with TBST, and incubated overnight at 4˚C with the corresponding primary antibodies (Table II). The membranes were then washed and incubated with the horseradish peroxidase-conjugated respective secondary antibodies for 1 h at room temperature (Table II). Finally, the membranes were developed by chemiluminescence using an automatic system (Fusion FX5-XT; Vilber Lourmat Sté, Collégien, France). Semi-quantification of protein expression levels was conducted using ImageJ 1.52f software (National Institutes of Health, Bethesda, MD, USA), with β-actin used as a loading control.

Determination of testosterone and DHT levels in cell culture medium. A total of 4x10⁵ DU145 SH and DU145 SCR cells/plate were seeded into 100 mm plates containing 12 ml RPMI-Phenol red-free medium, and were cultured for 3 days. Subsequently, cells were counted using a Neubauer chamber. For steroid extraction, 300 µl culture media was removed and diethylether was added in a 5:1 ratio (solvent/sample). The samples were then vortexed for 2 min and incubated at 4˚C for 5 min, in order to allow correct separation of the phases. Subsequently, the organic phase was removed and evaporated in SpeedVac (Savant™ SC110; Thermo Fisher Scientific, Inc.). Finally, the samples were reconstituted in 500 µl 10% ethanol or methanol, in order to measure the levels of testosterone or DHT respectively, using an ELISA kit for testosterone serum detection (cat. no. 11-TESHU-E01; Alpco, Salem, MA, USA) and an ELISA kit for DHT serum detection (cat. no. 11-DHTHU-E01; Alpco), with a modification in the construction of the calibration curve. Briefly, since the samples were resuspended in solution with 10% ethanol or methanol, a novel calibration curve was constructed in these matrices using synthetic testosterone (Sigma-Aldrich; Merck KGaA) and synthetic DHT (Sigma-11-DHTHU-E01) at the same concentrations indicated in the kit calibration curves. To validate the method, a control sample of known concentration was measured and the minimum detectable dose was calculated. Afterwards, the measurement was performed according to the manufacturer protocols. Finally, the results were corrected to determine the concentration obtained for 10⁵ cells.
Immunocytochemistry. The DU145 SH and DU145 SCR cells were seeded on coverslips at 60% confluence and were fixed with PBS, 3% paraformaldehyde and 2% sucrose for 30 min at room temperature. Subsequently, cells were washed with PBS-Glycine for 15 min, permeabilized for 10 min at room temperature, with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) and washed with PBS-Glycine. Cells were then blocked in a humidified chamber using PBS-Glycine and 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 10 min and were incubated with the necessary primary antibodies diluted in blocking solution. The primary antibodies used are shown in Table III. Subsequently, cells were incubated in the dark with the corresponding Alexa Fluor® 488-conjugated secondary antibodies (Table III). Both incubations were performed for 1 h at 37°C. In addition, cells were incubated in the dark with DAPI (1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for nuclear staining. Finally, coverslips were washed and mounted with Fluorescence Mounting Medium (Dako; Agilent Technologies, Inc.) for subsequent observation with a Spinning Disk microscope (Olympus IX81; Olympus Corporation, Tokyo, Japan) at x40 magnification. Each of the markers was analyzed in triplicate for DU145 SH and DU145 SCR cells.
Statistical analysis. For statistical analysis of the data, the Mann-Whitney U test was applied using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Results were normalized to the results from DU145 SCR cells. The samples were processed in triplicate (n=3) and data are expressed as the means ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
Results

mRNA and protein expression, and cellular localization of the AR in a PCa cell line with ZEB1 silencing. Analysis of AR expression and subcellular localization demonstrated that ZEB1 silencing resulted in an increase in the mRNA and protein expression levels of AR (Fig. 1A and B), and AR was predominantly located in the perinuclear region (Fig. 1C).

mRNA and protein expression, and cellular localization of StAR, CYP17A1, 5α-reductase1, 5α-reductase2, AKR1D1 and AKR1C2 in a PCa cell line with ZEB1 silencing. The present study aimed to determine ZEB1 knockdown-induced alterations in the steroidogenic pathway (Figs. 2 and 3). Initially, the expression levels of the StAR transporter, which is involved in the first stage of the pathway (11,12), were evaluated by RT-qPCR, western blotting and immunocytochemistry. The results obtained indicated that the mRNA expression levels of the StAR were decreased in the DU145 SH cell line compared with in control DU145 SCR cells. Furthermore, the protein expression levels of StAR were also reduced in DU145 SH cells (Fig. 2A and G). The intracellular distribution of StAR was mainly cytoplasmic (Fig. 3A).

The enzyme CYP17A1, which participates in the third and fourth step of the steroidogenic pathway (11,12), was also analyzed by RT-qPCR, western blotting and immunocytochemistry. The results demonstrated that ZEB1 silencing resulted in an increase in the mRNA and protein expression levels of CYP17A1 compared with the non-silenced control (Fig. 2B and G). In addition, the intracellular distribution exhibited a cytoplasmic pattern (Fig. 3B).

Within the enzymes involved in androgen synthesis, two isoforms were analyzed, 5α-reductase 1 and 5α-reductase 2, which convert testosterone to DHT (12). The mRNA and protein expression levels of 5α-reductase 1 were increased in the ZEB1-silenced cells (Fig. 2C and G). With regards to its subcellular localization, 5α-reductase 1 was predominantly detected in the cytoplasm of DU145 SH and DU145 SCR.
Conversely, ZEB1 silencing induced a decrease in the mRNA and protein expression levels of 5α-reductase 2 compared with the corresponding control group (Fig. 2D and G). Furthermore, cytoplasmic intracellular distribution was detected in both cell lines (Fig. 3D).

The enzyme AKR1D1, which is responsible for inactivation of progesterone, 17OH-progesterone, androstenedione and testosterone, thus converting the substrates into 5β-metabolites (13), was analyzed. The results indicated that knockdown of ZEB1 resulted in an increase in the mRNA and protein expression levels of AKR1D1 compared with non-silenced control cells (Fig. 2E and G). In addition, the results of immunocytochemistry revealed that AKR1D1 exhibited nuclear location in DU145 SH cells (Fig. 3E).

Finally, the enzyme AKR1C2, which participates in the inactivation of DHT, was analyzed (14). The results revealed that ZEB1 silencing resulted in an increase in the mRNA and protein expression levels of AKR1C2 compared
Furthermore, AKR1C2 was strictly located in the nuclei of DU145 SH cells and was weakly detected in DU145 SCR cells (Fig. 3F).

Testosterone and DHT concentrations in the culture medium of ZEB1-silenced PCa cells. The results revealed that ZEB1 silencing resulted in an increase in testosterone and DHT concentrations in DU145 SH cells compared with in the DU145 SCR control cells (Fig. 4).

A summary of the steroidogenic pathway markers, including those associated with androgen synthesis and degradation, in DU145 SH and DU145 SCR cell lines is presented in Fig. 5.

Figure 4. Testosterone and DHT concentration detected in the cell culture media of the DU145 SH and DU145 SCR cell lines. (A) Testosterone concentration (pg/ml)/10^5 cells. (B) DHT concentration (pg/ml)/10^5 cells. Statistically significant differences were determined using the Mann-Whitney U test. n=3, *P<0.05. DHT, dihydrotestosterone DU145 SCR, control cells; DU145 SH, zinc finger E-box binding homeobox 1-knockdown cells.

Figure 5. Summary of the results obtained regarding markers of the steroidogenic pathway in control DU145 cells and ZEB1-knockdown DU145 cells. Androgen synthesis and degradation pathways are presented. Grey arrows indicate markers with increased expression in response to ZEB1 silencing; black arrows indicate markers with reduced expression in ZEB1-silenced cells; grey boxes indicate androgens with increased concentration in the cell culture media. ZEB1, zinc finger E-box binding homeobox 1.
Discussion

Our recent study revealed that DU145 cells express high levels of ZEB1; therefore, this cell line was selected to perform stable silencing in the present study. The results of our previous study demonstrated that cells with ZEB1 silencing undergo alterations in the expression levels of canonical markers of EMT, including E-cadherin and Vimentin; in particular, E-cadherin is increased and Vimentin is decreased in response to ZEB1 knockdown. In addition, it has been revealed that these cells exhibit a decrease in proliferation, migration and invasion (9). Taken together these results indicate that DU145 SH cells may acquire marked epithelial characteristics.

In the prostate, it has been widely described that testosterone synthesis begins with the transport of cholesterol to the inner mitochondrial membrane by StAR, followed by reactions catalyzed by the enzymes CYP11A1, CYP17A1, HSD3β, and AKR1C3 (11). In the present study, some of these markers were analyzed, and the results suggested that an increase in CYP17A1 may contribute to an increase in testosterone synthesis in cells with ZEB1 silencing. Therefore, CYP17A1 may be considered the key enzyme in the production of testosterone in these cells. This finding is in accordance with the findings of Montgomery et al (15), which revealed that, in samples from patients with CRPC, an increase in the expression of CYP17A1 and testosterone concentration was detected compared with in primary prostate tumors samples, thus suggesting intratumoral steroidogenesis in advanced stages and progression to CRPC. The production of DHT may be determined by detecting the enzymes 5α-reductase 1 and 5α-reductase 2, and testosterone concentration (16). The expression levels of the 5α-reductase isoforms were detected in the present study and suggested that the main factor affecting DHT production may be the initial concentration of testosterone, since the decrease in one isoform was compensated with an increase in the other.

Testosterone and DHT concentrations are also determined by their inactivation and subsequent degradation (13,14,16). Analysis of the enzyme AKR1D1, which is responsible for converting progesterone, 17OH-progesterone, androstenedione and testosterone into inactive metabolites (13), revealed that its expression was increased in ZEB1-silenced cells, which may result in a greater inactivation of these hormone metabolites; however, the concentration of testosterone was increased in ZEB1-silenced cells. These findings suggested that the inactivation performed by AKR1D1 was not sufficient to prevent the high levels of testosterone in these cells. Similarly, the AKR1C2 enzyme, which is responsible for the inactivation of DHT, was also increased in cells with ZEB1 knockdown.

The preferential nuclear localization of AKR1D1 and AKR1C2 suggested that after being translocated from the cytoplasm, androgen receptor-testosterone and androgen receptor-DHT complexes (17) may be inactivated in the nucleus. Taylor et al (18) analyzed the genomic profile of samples from patients with PCa, and revealed that AKR1C2 expression is increased in patients with advanced disease, thus suggesting that the increase in AKR1C2 expression determined in this study may be associated with more advanced stages of PCa. In addition, the expression levels of the enzyme AKR1C2 are correlated with the expression of AR (19). Huang et al (20) revealed that LNCaP PCa cells, which express AR, have a higher expression of AKR1C2 compared with in cell lines that do not express AR. It was also demonstrated that silencing AR in LNCaP cells produces a decrease in AKR1C2; however, the regulatory mechanism remains unknown. Furthermore, Ji et al (21) reported that DHT treatment of LNCaP and DU145 cells results in induction of AKR1C2 expression, indicating that this possibly occurs to counteract the increase in DHT concentration. These findings are in accordance with the present results, suggesting a possible regulation of androgen availability by the AR as a mechanism of control.

Notably, the relevance of increased concentration of androgens in cells with ZEB1 knockdown may be associated with activation of AR during EMT (6). Therefore, an increase in the expression of steroidogenic enzymes has been suggested as a mechanism underlying resistance to ADT (22,23). According to the present results, it may be hypothesized that an increase in androgen synthesis could lead to an increase in the activation of AR due to its increased availability.

The present results revealed that the isoform 5α-reductase 1 was increased whereas 5α-reductase 2 was decreased in response to ZEB1 knockdown. An expression switch between these isoforms has been suggested, since both enzymes act at different pH levels, and in the tumor environment, the activity of 5α-reductase 1 may be favored allowing an increase in the production of DHT (12). In addition, Audet-Walsh et al (24) revealed that the inverse regulation of these enzymes is conducted directly by the AR, which binds to the promoter sequences of the genes increasing the expression of 5α-reductase 1 and suppressing the expression of 5α-reductase 2.

Locke et al (25) reported an increase in StAR expression, and testosterone and DHT concentrations, in a castration-resistant xenotransplant model. Conversely, the present results suggested that an inverse relationship may exist between the concentration of androgens and StAR. It may be hypothesized that a reduction in StAR could act as a compensatory mechanism against the increase in androgens induced by ZEB1-independent mechanisms.

In conclusion, the silencing of transcription factor ZEB1 produced an alteration in the steroidogenic pathway and AR expression, specifically increasing the expression levels of CYP17A1, 5α-reductase 1, AKR1D1 and AKR1C2 enzymes, and decreasing the expression levels of StAR and 5α-reductase 2. In addition, ZEB1 knockdown increased testosterone and DHT concentrations. An increase in androgen concentration in tumor cells is a characteristic of patients with ADT resistance; therefore, alterations in ZEB1 expression may be associated with the mechanism underlying treatment failure in patients undergoing ADT and subsequent disease progression to CRPC.

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Results. All authors read and approved the final manuscript.

Authors' contributions
DH performed the gene expression studies, immunocytochemistry, ELISA and the statistical analyses. OOS transduced the cell line, and contributed to the gene expression and immunocytochemistry experiments. PV and MJT contributed to cell line, and contributed to the gene expression and immunocytochemistry, ELISA and the statistical analyses. OOS transduced the DH performed the gene expression studies, immunocytochemistry and EAC participated in the study design and interpreted the nocytochemistry experiments. PV and MJT contributed to

Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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