

Androgen receptor as a regulator of ZEB2 expression and its implications in epithelial-to-mesenchymal transition in prostate cancer

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Abstract

Zinc finger E-box-binding protein 2 (ZEB2) is known to help mediate the epithelial-to-mesenchymal transition, and thereby it facilitates cancer metastasis. This study was initiated to explore whether ZEB2 expression differs in prostate cancer (PCa, $n=7$) and benign prostatic hyperplasia (BPH, $n=7$) tissues. In PCa tissues, the levels of both immunoreactive ZEB2 and androgen receptor (AR) were found to be significantly higher ($P<0.05$) when compared with BPH tissues. Co-regulation of AR and ZEB2 prompted us to investigate the role of androgenic stimuli in ZEB2 expression. ZEB2 expression was found to be significantly ($P<0.05$) upregulated after androgen stimulation and downregulated following AR silencing in LNCaP cells, an androgen-dependent PCa cell line. This finding suggested AR as a positive regulator of ZEB2 expression in androgen-dependent cells. Paradoxically, androgen-independent (AI) cell lines PC3 and DU145, known to possess low AR levels, showed significantly ($P<0.05$) higher expression of ZEB2 compared with LNCaP cells. Furthermore, forced expression of AR in PC3 (PC3-AR) and DU145 (DU-AR) cells led to reductions in ZEB2 expression, invasiveness, and migration. These cells also exhibited an increase in the levels of E-cadherin (a transcriptional target of ZEB2). Co-transfection of AR and ZEB2 cDNA constructs prevented the decline in invasiveness and migration to a significant extent. Additionally, ZEB2 downregulation was associated with an increase in miR200a/miR200b levels in PC3-AR cells and with a decrease in miR200a/miR200b levels in AR-silenced LNCaP cells. Thus, AR acts as a positive regulator of ZEB2 expression in androgen-dependent cells and as a negative regulator in AI PCa cells.

Key Words

- ▶ androgen receptor
- ▶ EMT
- ▶ ZEB2
- ▶ prostate cancer

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Introduction

It is well recognized that androgens play a vital role in development, growth, and progression of prostate cancer (PCa), the most common nondermatological malignancy in

men (Mohler 2008). Surgical ablation of androgens and anti-androgen therapies are the major treatment modalities for locally advanced or metastatic PCa (Miyamoto et al. 2004).

Although these treatment strategies are initially effective, the disease eventually progresses to an androgen-independent (AI) state (Feldman & Feldman 2001). Nonetheless, AI PCa cells continue to rely on androgen receptor (AR) for their growth and survival (Balk 2002). Hence, AR-mediated signaling and its effectors are considered to be attractive targets for the treatment of AI PCa, which is often associated with metastasis.

Sufficient evidence has shown that the epithelial-to-mesenchymal transition (EMT) facilitates metastasis of cancerous cells (Klymkowsky & Savagner 2009). EMT is characterized by the gain of mesenchymal characteristics, such as mobility, invasiveness, and loss of epithelial features, that is, cell–cell adhesion, cell polarity, etc. Several strides have been carried out to identify the factors contributing to EMT in PCa. Transforming growth factor beta (Zhu & Kyprianou 2010), nicotinamide adenine dinucleotide-dependent histone deacetylase or SIRT1 (Byles *et al.* 2012), platelet-derived growth factor (Kong *et al.* 2009), TMPRSS2/ERG (Leshem *et al.* 2011), SNAI2 (SLUG) (Emadi Baygi *et al.* 2010), DAB2IP (Xie *et al.* 2010), Hsp27 (HSPB1) (Shiota *et al.* 2013), and miRNAs (Ru *et al.* 2012) have all been identified as EMT regulators in PCa. Interestingly, the majority of these factors mediate EMT via zinc finger E-box-binding protein (ZEB) family members, such as ZEB1 (δ EF1 or AREB6) and ZEB2 (Smad-interacting protein 1 (SIP1)) (Gregory *et al.* 2011).

The role of the androgen axis in PCa pathogenesis has been described previously (Nieto *et al.* 2007). However, data available on the role of androgens in EMT regulation are contradictory. While some investigators have reported androgen-induced activation of EMT and its effectors (Zhu & Kyprianou 2010, Anose & Sanders 2011), others have demonstrated EMT activation in response to androgen deprivation (Sun *et al.* 2012). A recent study by Izumi *et al.* (2013) has implicated AR as an inhibitor of EMT activation in PCa cells. These conflicting data warrant more studies directed toward deciphering the role of androgenic stimuli in EMT and PCa metastasis.

In this study, ZEB2 expression was examined in i) primary PCa and benign prostatic hyperplasia (BPH) tissues, ii) an androgen-dependent (AR-positive) PCa cell line (LNCaP), before and after the attenuation of AR expression, and iii) AI PC3 and DU145 cells (expressing low levels of endogenous AR), before and after the forced expression of AR. We also investigated whether the modulation in ZEB2 expression is accompanied by any changes in downstream functions (e.g. invasion and migration).

Materials and methods

Antibodies

Human AR MABs and secondary antibodies conjugated to HRP or FITC were procured from Dako (Glostrup, Denmark). E-cadherin, p63, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from BD Biosciences (San Jose, CA, USA), Sigma–Aldrich, and Calbiochem (Darmstadt, Germany) respectively. Human ZEB2 and α -methylacyl-CoA racemase (AMACR) antibodies were purchased from Sigma–Aldrich, mouse and rabbit IgGs from Millipore (Billerica, MA, USA), Alexa Fluor 488-conjugated secondary antibodies from Life Technologies, and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA, USA).

Tissue samples

Use of prostate tissue samples was approved by the NIRRH Ethics Committee for Clinical Studies. BPH tissues ($n=7$) were obtained using transurethral resection and PCa biopsies ($n=7$), by core needle biopsy.

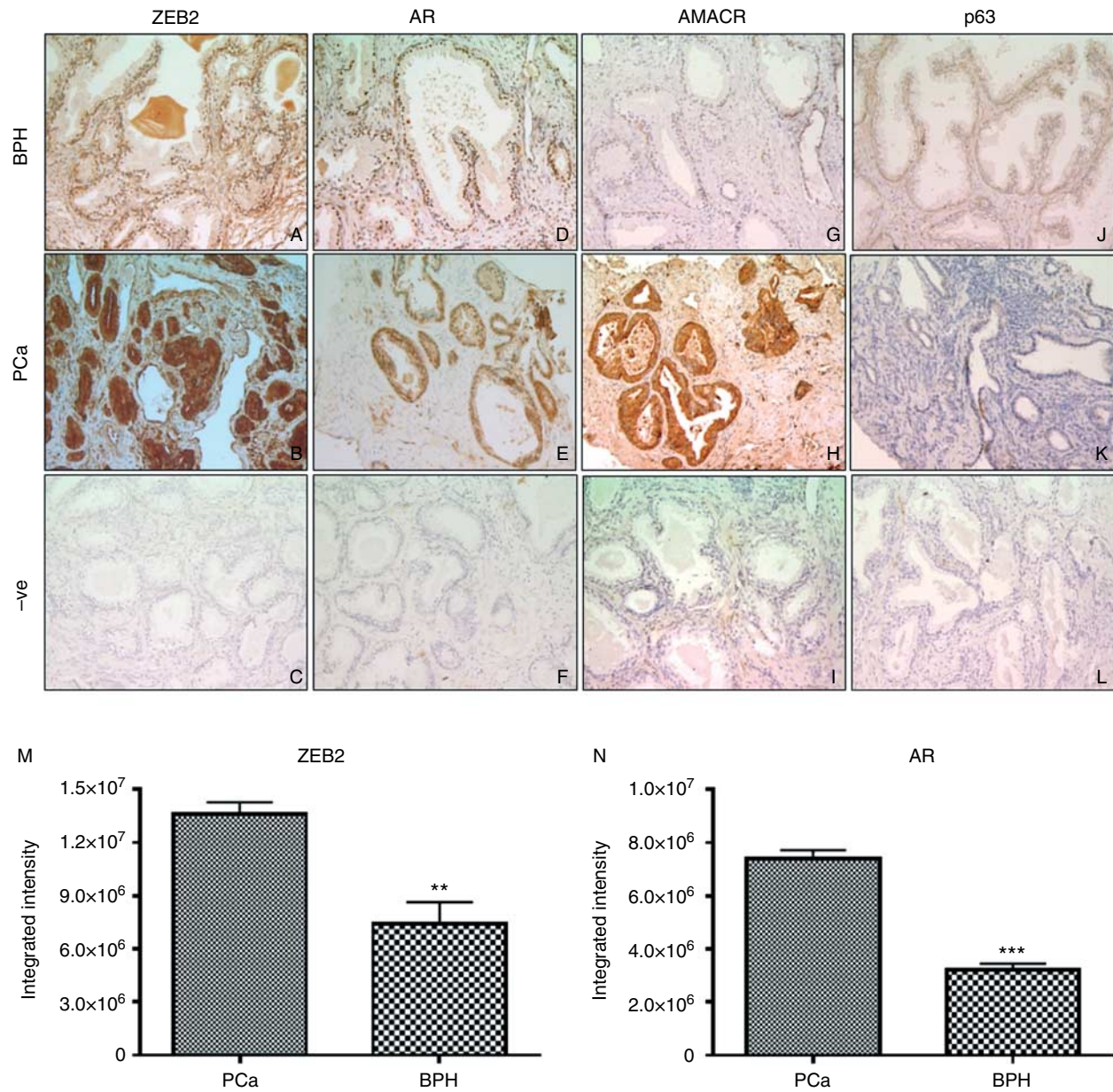
Immunohistochemistry

Prostate tissue sections of 5 μ m thickness were deparaffinized and rehydrated in descending grades of methanol. After quenching endogenous peroxidase activity, sections were microwaved for 10 min in antigen unmasking solution (Vector Laboratories). For detection of nuclear proteins, sections were permeabilized in 0.1% Triton X-100 in PBS (pH 7.4) for 10 min. After blocking, sections were incubated with either the respective primary antibody or rabbit/mouse IgGs and then with biotinylated secondary antibodies. Reaction products were detected using avidin–biotin–HRP complex and diaminobenzidine. Immunostained sections were counterstained with hematoxylin.

Staining intensities of the immunoprecipitates were determined using an image analysis software – Aperio Image Scope, version v11.2.0.780 (Aperio, Vista, CA, USA). A minimum of ten areas from each section were randomly selected for measuring integrated optical density.

Cell lines and their maintenance

Androgen-dependent prostate carcinoma LNCaP-FGC cell line (CRL-1740) was procured from the American

**Figure 1**

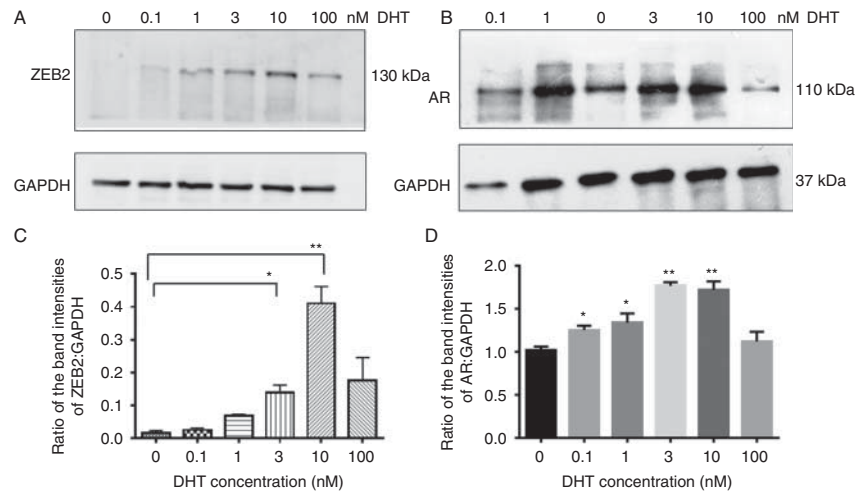
Immunolocalization of ZEB2 (A and B), AR (D and E), AMACR (G and H), and p63 (J and K) proteins to the prostate tissues of patients with benign prostatic hyperplasia (BPH) (A, D, G and J) and prostate cancer (PC) (B, E, H and K). (C, F, I and L) sections where primary antibodies were

replaced with IgGs. Magnification 20 \times . (M and N) semiquantitative analysis to compare the intensities of immunoreactive proteins ((M) ZEB2 and (N) AR) in BPH and PCa tissues. ***P* value <0.001 and ****P* value <0.0001.

Type Culture Collection (Manassas, VA, USA), whereas AI PCa cell lines, PC3 (CRL-1435) and DU145 (ATCC-HTB-81), were obtained from National Centre for Cell Sciences (Pune, India). The cell lines were grown in phenol red-free RPMI-1640 media (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Invitrogen).

5 α -Dihydrotestosterone stimulation experiments

LNCaP cells (1 \times 10⁵/well), seeded in RPMI-1640 supplemented with 10% charcoal-stripped FBS, were stimulated with different concentrations of 5 α -dihydrotestosterone (DHT). After 72 h, cell pellets were resuspended in protein lysis buffer (9 M urea, 4% CHAPS (3-[(3'-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 40 mM Tris) to obtain total protein extracts.

**Figure 2**

Detection of ZEB2 (A) and AR (B) proteins in androgen-stimulated LNCaP cells. Blots were reprobed to detect GAPDH for normalization of total protein load. (C and D) Densitometric analyses to depict the ratios of

the intensities of ZEB2 or AR:GAPDH respectively. **P* value <0.05 and ***P* value <0.005.

DNA transfection

The WT *AR* cDNA construct was kindly gifted by Dr Donald Tindall (Mayo Clinic, Rochester, MN, USA). The cDNA construct for *ZEB2* (pFlagstrep hSIP1–3) was a generous gift from Dr Danny Huylebroeck (University of Leuven, Belgium). Cells (1×10^5 /well) were transfected with the cDNA constructs using Lipofectamine Plus (Invitrogen) as per the manufacturer's instructions. After 48 h, cells were suspended in either RNA extraction buffer or protein lysis buffer. For optimization of co-transfections, cells were first transfected with different concentrations of *AR* or *ZEB2* cDNA constructs (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

siRNA transfection

LNCaP (1×10^5 /well) cells were transfected with siRNAs (Qiagen) targeting two different regions of the human *AR* gene or scrambled oligo (SO) siRNA, as per the manufacturer's instructions (Qiagen) for 72 h.

Quantitative real-time PCR

Total RNA was isolated using RNeasy Microkit (Qiagen) and converted to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturers' instructions. Transcripts encoded by the target and endogenous control 18S rRNA genes were amplified using specific TaqMan primer/probes.

Relative quantity ($RQ = 2^{\Delta\Delta Ct}$) of transcripts was calculated by the $\Delta\Delta Ct$ method, in which expression of the target gene is normalized against 18S rRNA expression. ΔCt was calculated by subtracting *Ct* of the endogenous control from that of the target gene and $\Delta\Delta Ct$ was calculated by subtracting ΔCt of control from that of test. The values were expressed as $RQ \pm S.E.M.$

miRNA isolation

miRNAs were extracted using the mirVana miRNA isolation kit (Life Technologies) according to the manufacturer's instructions. RNA samples were converted to cDNA using miRNA gene-specific primers and a TaqMan miRNA RT Kit (ABI). TaqMan primer/probes (Life Technologies) were used for amplification of the *MIR200A* and *MIR200B* genes. A conserved region of *U6* (*RNU6-50P*) snRNA was amplified in each sample to normalize the RNA input among different samples.

Immunoblotting

Nuclear proteins were extracted using a ProteoJET cytoplasmic and nuclear protein extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. Extracts of total protein were obtained by suspending cells in protein lysis buffer. Nuclear or total proteins (10 μ g), resolved in 10% SDS-PAGE gels, were transferred to PVDF membranes (Millipore). After blocking,

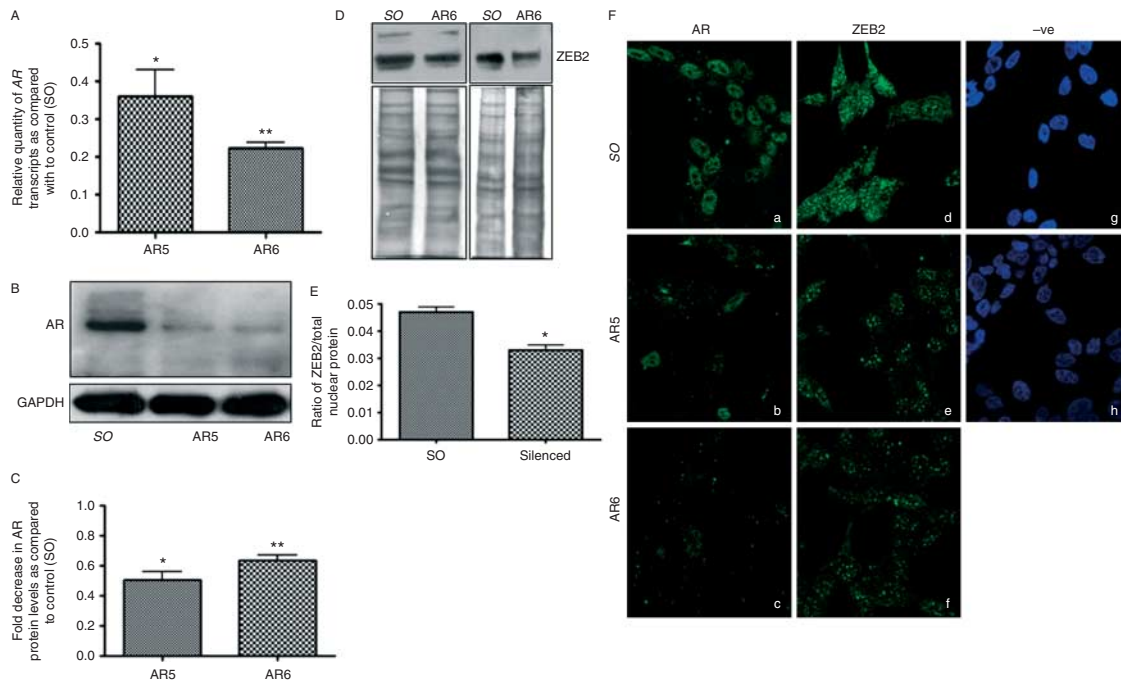


Figure 3

Relative levels of AR transcript (A) and AR protein (B) in LNCaP cells transfected with AR5/AR6 siRNAs, compared with those transfected with scrambled siRNA (SO). (C) Fold change obtained by densitometric analysis of the ratios of the intensities of AR to that of GAPDH in AR- and SO-transfected cells. (D) ZEB2 protein levels in the nuclear extracts of AR- and SO-transfected cells. The blot was stained with Coomassie blue after immunodetection, to normalize

nuclear protein load. (E) Densitometric analysis to depict the ratios of ZEB2:total nuclear protein load. **P* value < 0.05 and ***P* value < 0.005. (F) Immunofluorescent detection of AR (a, b and c) and ZEB2 (d, e and f) proteins in LNCaP cells, transfected with either SO siRNA (a and d) or AR siRNAs (AR5 (b and e) and AR6 (c and f)). In negative controls (g and h), primary antibodies were replaced with mouse or rabbit IgGs. Magnification, 63 \times .

the blots were sequentially incubated with the respective primary and secondary antibodies. Immunoreactive bands were detected using an Advanced ECL detection kit (Amersham) and their densities were measured using the IQTL Software (GE Biosciences, Pittsburgh, PA, USA).

Immunofluorescence and phalloidin staining

Cells, fixed in 3.7% paraformaldehyde in PBS, were permeabilized in 0.1% Triton X-100. Blocking (1% BSA for 1 h) was followed by incubation with appropriate primary antibodies and then with FITC or Alexa Fluor-conjugated secondary antibody. For F-actin staining, fixed and permeabilized cells were incubated with Phalloidin stain (Life Technologies) for 30 min and analyzed using confocal laser microscopy (Carl Zeiss, Jenna, Germany).

In vitro invasion assay

Cells (2×10^5) suspended in 250 μ l plain media were placed in the top chamber of matrigel-coated transwells (Nunc, Roskilde, Denmark). Transwells without matrigel were

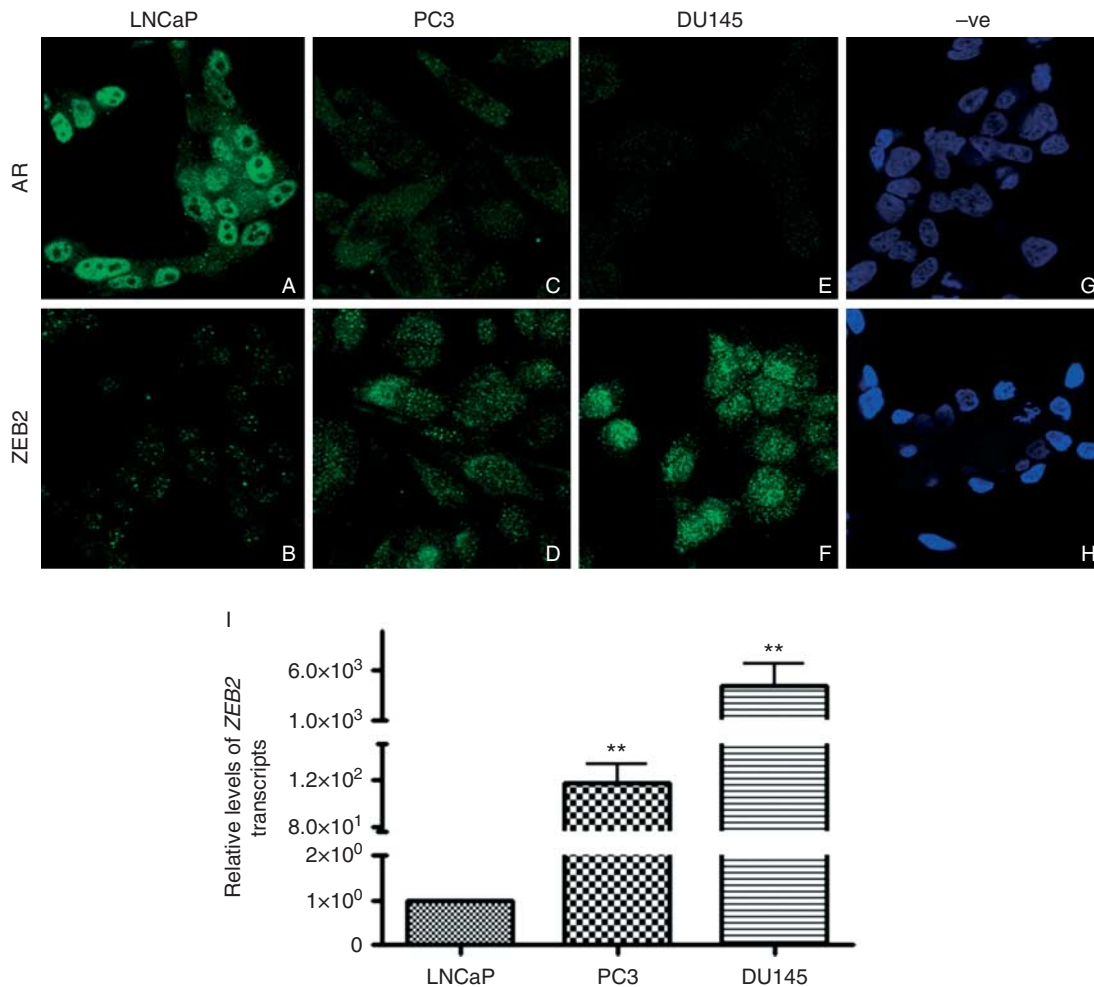
used as controls. Medium with 10% sera was added to the lower chamber. After 48 h, transwells were permeabilized and hematoxylin stained. Non-invading cells were removed from the top of transwells. The percentage of invasion was calculated using the following formula. Number of cells in the control transwell – number of cells in the transwell containing matrigel/100.

Wound-healing assays

Cells (5×10^5) were treated with 5 μ g/ml Mitomycin C (Sigma–Aldrich) for 2 h. This was followed by creation of a wound using a sterile 200 μ l micropipette tip. Images of different regions of the wound at 0 and 24 h were taken. The percentage of the wound covered was calculated using the following formula: distance of the wound after 24 h/distance of the wound at 0 h \times 100.

Statistical analysis

Data are expressed as mean \pm s.d. Student's unpaired *t*-test was used to determine the significance of difference

**Figure 4**

Immunofluorescent detection of AR (A, C and E) and ZEB2 (B, D and F) proteins in androgen-dependent LNCaP (A and B), and androgen-independent PC3 (C and D) and DU145 (E and F) cells. In negative controls,

primary antibodies were replaced with mouse IgGs (G) or rabbit IgGs (H). Magnification 63×. (I) Relative levels of ZEB2 transcripts in PC3 and DU145 cells, compared with LNCaP cells. ***P* value <0.005.

between control and experimental samples. A *P* value of <0.05 was considered to be significant.

Results

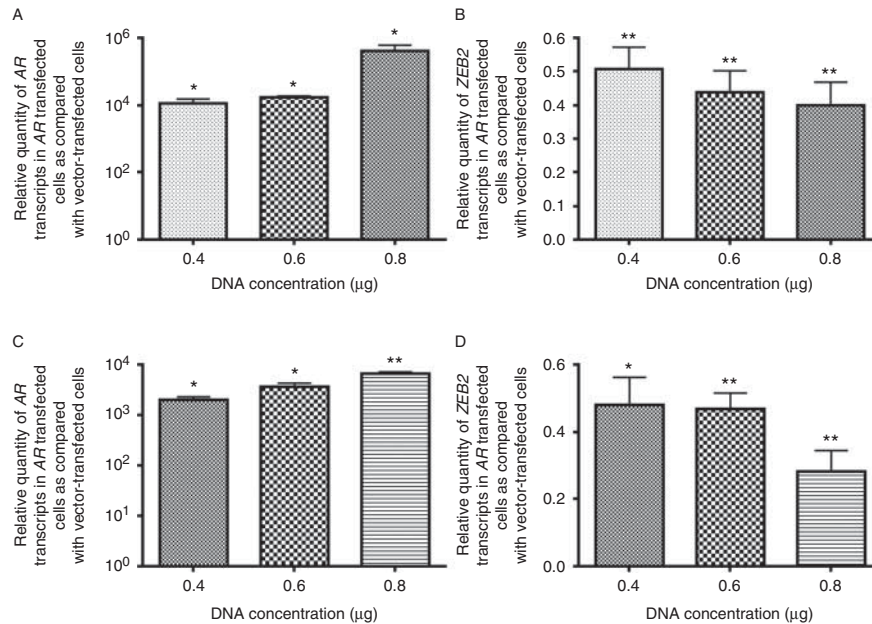
Immunoreactive ZEB2 and AR levels are elevated in PCa tissues

BPH and primary PCa tissues were immunostained for AMACR (Fig. 1G and H) and p63 (TP63) (Fig. 1J and K), to distinguish the glands representing PCa and BPH phenotypes respectively.

In BPH tissues, AR was immunolocalized to the nuclei of the secretory epithelial cells. AR immunostaining was weak or negligible in the basal epithelial cells of BPH

tissues (Fig. 1D and Supplementary Fig. 2, see section on supplementary data given at the end of this article). On the other hand, immunoreactive AR was predominantly localized to the cytoplasm of the secretory epithelial cells in PCa tissues.

ZEB2 protein was detected in the nuclei of the secretory and basal epithelial cells in the BPH tissues (Fig. 1A and Supplementary Fig. 2). In PCa tissues, it was localized to nuclear as well as cytoplasmic compartments of the secretory epithelial cells (Fig. 1B and Supplementary Fig. 2). Semiquantitative analyses indicated significantly higher expression of ZEB2 (*P*<0.001) and AR proteins (*P*<0.0001) in the secretory epithelial cells of PCa, compared with BPH tissues (Fig. 1M and N).

**Figure 5**

Relative levels of AR (A and C) and ZEB2 (B and D) transcripts in AR overexpressing PC3 (A and B) or DU145 (C and D) cells, compared with the respective empty vector-transfected parental cells. **P* value < 0.05 and ***P* value < 0.005.

ZEB2 expression is stimulated by androgen in LNCaP cells

DHT stimulation led to a dose-dependent (0.1–10 nM) increase in the levels of ZEB2 (Fig. 2A and C) and AR (Fig. 2B and D) in androgen-dependent LNCaP cells. Interestingly, both AR and ZEB2 levels were found to be lesser in 100 nM treated cells, compared with 10 nM treated cells. Thus, ZEB2 and AR proteins appeared to be co-regulated in response to androgen stimulation. Results suggested a positive regulation of ZEB2 expression by androgens in androgen-dependent cells.

ZEB2 expression is regulated by AR in LNCaP cells

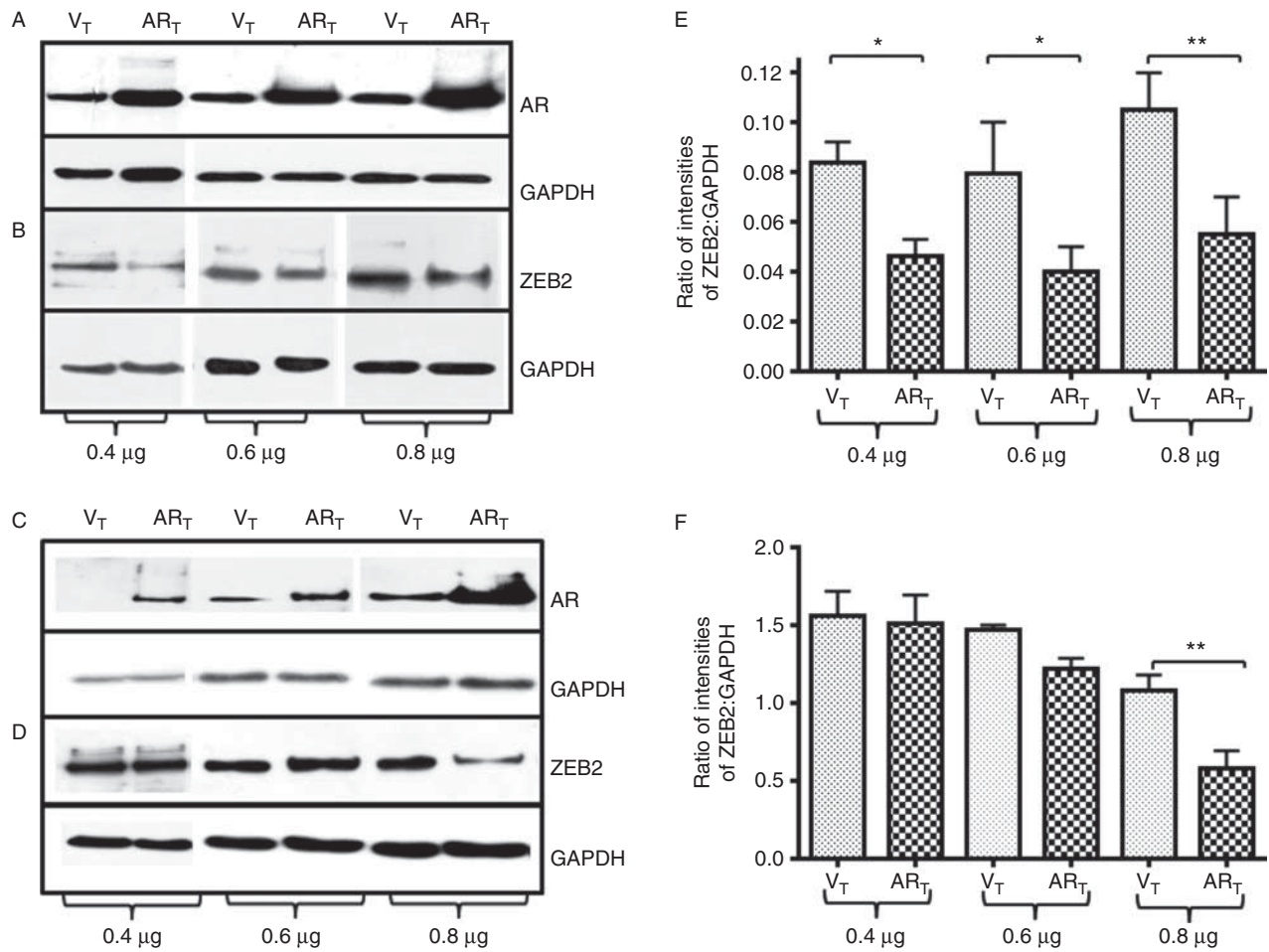
LNCaP cells were transfected with different concentrations of AR5 and AR6 siRNAs (data not shown). Maximal inhibition of AR expression was observed in cells transfected with 15 nM siRNAs (AR5 and AR6). The levels of AR transcript (Fig. 3A) and AR protein (Fig. 3B and C) were significantly lesser (*P* < 0.05) in the AR-silenced cells, compared with the SO-transfected cells.

The levels of nuclear ZEB2 were found to be significantly (*P* < 0.05) lesser in the AR-silenced cells, compared with the SO-transfected cells (Fig. 3D and E). The decrease in nuclear ZEB2 levels hinted at the possibility of a decline in the transcriptional activity of ZEB2.

Immunocytochemical localization corroborated the results obtained by q-PCR and immunoblotting. As expected, immunoreactive AR levels were lesser in AR siRNA-transfected cells (Fig. 3Fb and c), compared with SO-transfected cells (Fig. 3Fa). Immunoreactive ZEB2 protein levels were also found to be lower, especially in the nuclei of AR-silenced cells (Fig. 3Fe and f). This reaffirmed that AR is a positive regulator of ZEB2 expression in androgen-dependent LNCaP cells.

Basal levels of AR and ZEB2 in androgen-dependent and AI cells

Immunoreactive AR levels were higher in androgen-dependent LNCaP cells (Fig. 4A), than in AI PC3 and DU145 cells (Fig. 4C and E). By contrast, basal levels of ZEB2 protein as well as transcripts were found to be higher in PC3 and DU145, than in LNCaP cells (Fig. 4A, B, C, D, E, F, G, H and I). Thus, ZEB2 expression appeared to be correlated with metastasis. It is well established that PC3 and DU145 cells are more aggressive, compared with LNCaP cells. This was further corroborated by higher expression of vimentin (i.e. mesenchymal marker) and lesser expression of E-cadherin (i.e. epithelial marker) in PC3 and DU145 cells, compared with LNCaP cells

**Figure 6**

AR (A and C) and ZEB2 (B and D) protein levels in AR overexpressing PC3 (A, B and E) and DU145 (C, D and F) cells (AR_T), and the respective empty vector-transfected parental cells (V_T). Blots were probed for GAPDH to

normalize the total protein load. (E and F) Densitometric analyses to depict the ratios of the intensities of ZEB2 to that of GAPDH in V_T and AR_T cells. **P* value < 0.05 and ***P* value < 0.005.

(Supplementary Fig. 3, see section on supplementary data given at the end of this article).

AR as a regulator of ZEB2 expression in PC3 and DU145 cells

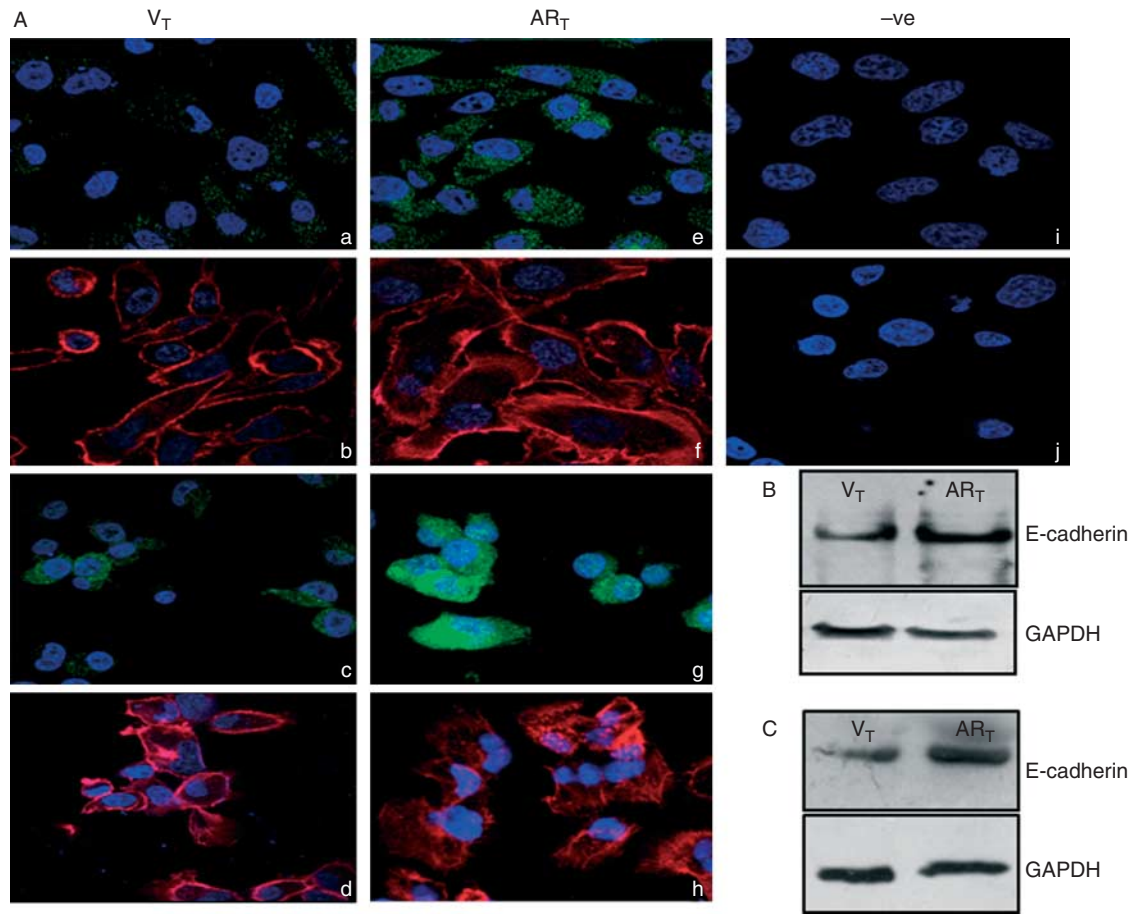
Then, we investigated whether an increase in the levels of AR modulates the ZEB2 expression in AI cells. Transfection of PC3 (Figs 5A and 6A) and DU145 cells (Figs 5C and 6C) with the AR cDNA construct led to a significant ($P < 0.05$) increase in the levels of AR transcript (Fig. 5A and C) and also in the levels of AR protein (Fig. 6A and C). However, ZEB2 was found to be significantly ($P < 0.05$) down-regulated at the transcript (Fig. 5B and D) and protein levels (Fig. 6B and D) in AR-transfected cells. Extent of the decrease in ZEB2 levels was proportional to the increase in

AR levels (Fig. 6E and F). Thus, in AI cells, AR acts as a negative regulator of ZEB2 expression.

E-cadherin expression is upregulated in AR overexpressing PC3/DU145 cells

Immunofluorescent localization and immunoblotting revealed higher levels of E-cadherin in AR overexpressing PC3 cells (PC3-AR), compared with the parental PC3 cells (Fig. 7Aa, e and B). This pattern was also evident in DU-AR cells (Fig. 7Ac, g and C). It is likely that the decline in ZEB2 levels contributes to higher expression of E-cadherin in PC3-AR and DU-AR cells.

PC3-AR and DU145-AR cells, displaying modulation in the levels of ZEB2 and E-cadherin, appeared to be morphologically distinct (Fig. 7Af and h) from their respective

**Figure 7**

(A) Detection of E-cadherin by immunofluorescence (a, e, c and g) and detection of F-actin by phalloidin staining (b, f, d and h) in PC3 (a, b, e and f) and DU145 (c, d, g and h) cells, transfected with either the empty vector

(V_T) (a, b, c and d) or AR cDNA constructs (AR_T) (e, f, g and h). (i and j) Mouse IgGs stained PC3 and DU145 cells respectively. (B) (PC3) and (C) (DU145) Immunodetection of E-cadherin in the protein lysates of V_T and AR_T cells.

parental cells (Fig. 7Ab and d). They looked more epithelioid and had more intense and diffused F-actin localization, as indicated by phalloidin staining (Fig. 7f and h).

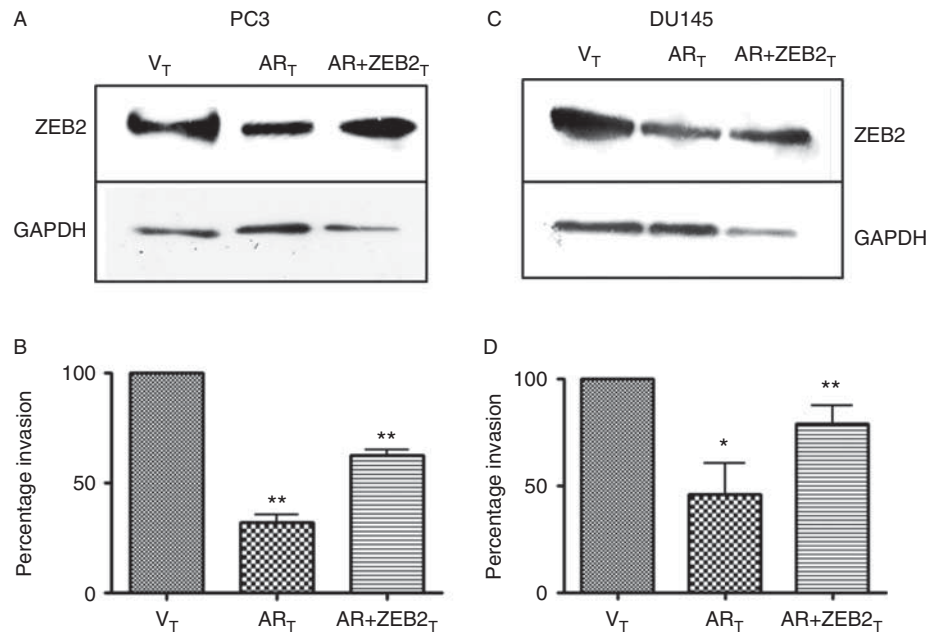
A decrease in ZEB2 expression is associated with reduced invasiveness and migration in AR overexpressing PC3/DU145 cells

AR overexpression led to a significant ($P < 0.05$) reduction in the invasiveness (Fig. 8B and D) and migration (Fig. 9) of PC3 and DU145 cells. To validate the assumption that AR-induced reduction in ZEB2 levels contributes to a decrease in invasion and migration, PC3 and DU145 cells were co-transfected with the cDNA constructs encoding AR and ZEB2 proteins (Figs 8 and 9). An increase in the levels of ZEB2 restored invasion (Fig. 8B and D) and migration (Fig. 9), to a significant ($P < 0.05$) extent, in the co-transfected cells.

miR200a and miR200b levels are altered in PC3-AR cells

miR200a and miR200b levels were found to be significantly ($P < 0.005$) higher in LNCaP, compared with PC3 and DU145 cells (Fig. 10A). A similar pattern was observed for the AR protein in these cell lines (Fig. 4A, B, C, D, E, F, G and H). On the other hand, ZEB2 protein demonstrated a reverse pattern, i.e. highest levels in DU145 and lowest levels in LNCaP cells (Fig. 4A, B, C, D, E, F, G and H). This prompted us to *in silico* explore the presence of androgen response elements (AREs) and ZEB2-binding sites in *MIR200A* and *MIR200B* genes.

Using an in-house Perl-based tool, regions spanning 1–1000 bp upstream of the transcription initiation sites in *MIR200A* and *MIR200B* genes were scanned for the presence of ZEB2-binding sites – CACCTG (E-BOX2), CAGGTG (Z-BOX1), and CAGGTA (Z-BOX2). *MIR200A*

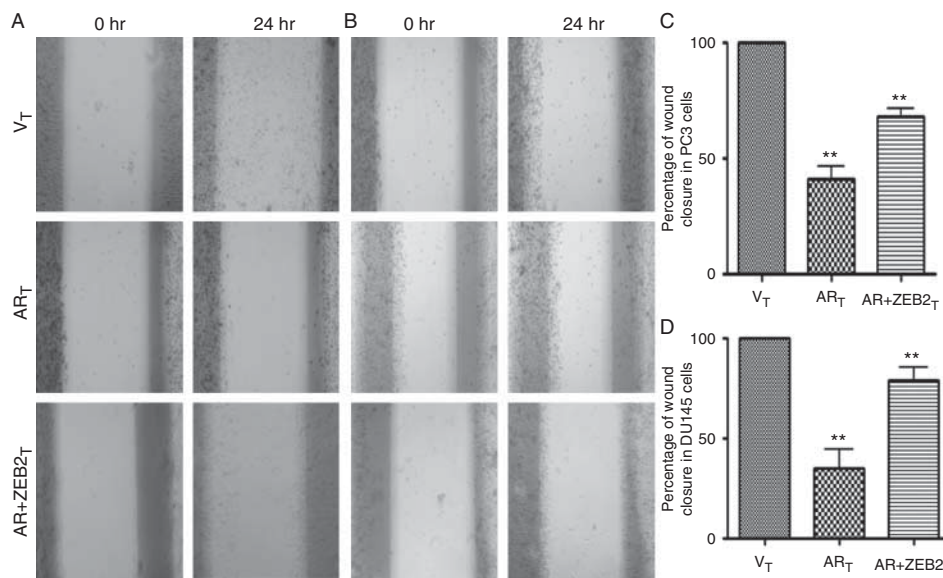
**Figure 8**

ZEB2 levels in the protein lysates of PC3 (A) and DU145 (C) cells transfected with either empty vector (V_T) or AR (AR_T) or AR + ZEB2 (AR + ZEB2_T). Percentage of invasion in PC3 (B) and DU145 (D) cells of V_T, AR_T and AR + ZEB2_T cells. **P* value < 0.05 and ***P* value < 0.005.

and *MIR200B* genes were found to have ZEB2-binding sites (Table 1). AREs were not present in the scanned regions.

AR silencing in LNCaP cells led to a significant decrease in miR200a and miR200b levels (Supplementary

Fig. 4, see section on supplementary data given at the end of this article). This indicated the possibility of AR being a positive regulator of MIR200A/MIR200B expression in androgen-dependent LNCaP cells. In AI PC3 cells also, an

**Figure 9**

Wound healing in PC3 (A and C) and DU145 (B and D) cells transfected with either the empty vector (V_T), or AR (AR_T) or AR + ZEB2 constructs (AR + ZEB2_T). Magnification, 4×. (C and D) The percentage of wound closures. ***P* value < 0.005.

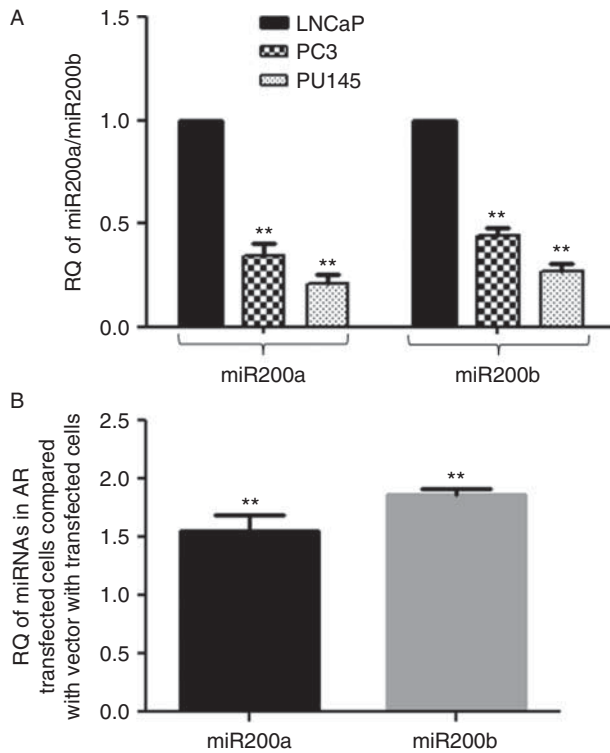


Figure 10 (A) miR200a and miR200b levels in androgen-independent PC3 and DU145 cells, compared with androgen-dependent LNCaP cells. (B) miR200a and miR200b levels in AR overexpressing PC3 cells. **P* value < 0.05 and ***P* value < 0.005.

increase was observed in the miR200a/miR200b levels, after AR overexpression (Fig. 10B). The increase in miR200a/miR200b levels could be either a cause or a result of the decrease in ZEB2 levels in PC3-AR cells.

Discussion

EMT is a major event that enables tumor cells to become metastatic (Thiery & Sleeman 2006). Cells undergoing EMT demonstrate a decrease in the expression levels of epithelial proteins such as E-cadherin and junction plakoglobin, and an increase in the expression of mesenchymal markers, such as vimentin, fibronectin, and α -smooth muscle actin (Acloque et al. 2009). These

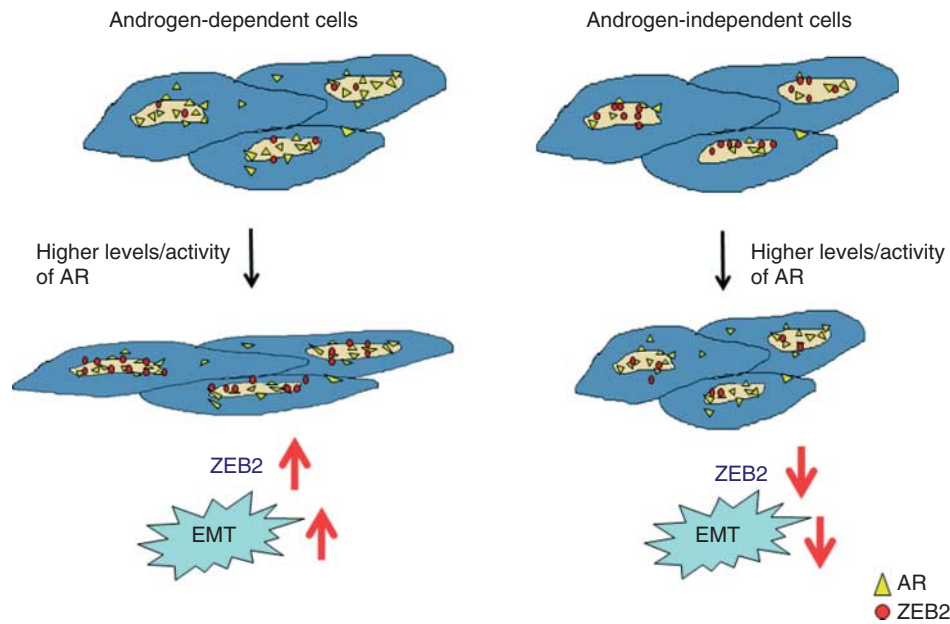
alterations lead to the loss of cell–cell adhesion and a gain of mobility. EMT is also known to be associated with the expression of ZEB2 during development (Vandewalle et al. 2005). ZEB2 expression has also been reported in tumors of the breast, stomach, liver, and ovary (Katoh & Katoh 2009). This study showed significantly higher levels of immunoreactive ZEB2 in PCa compared with BPH tissues. PCa tissues also showed significantly higher levels of AR. These findings raise the possibility of the co-regulation of AR and ZEB2 and also the existence of a functional nexus between these two transcription factors.

Our data demonstrate the positive regulation of ZEB2 expression by androgens in androgen-dependent LNCaP cells. The entry of the mouse *Zeb2* gene into the database of androgen-responsive genes (<http://argdb.fudan.edu.cn/>) is in agreement with our observations. Our microarray data also demonstrate a twofold decrease in the levels of *ZEB2* transcripts, following the silencing of AR expression in LNCaP cells (data not shown). By contrast, an inverse relationship was observed between AR and ZEB2 in AI PC3 and DU145 cells, known for their low levels of AR expression (Alimirah et al. 2006). These cells, when overexpressing AR, show a significant decline in ZEB2 levels. This in turn leads to a reduction in the invasiveness and migration of PC3-AR/DU-AR cells, which could have occurred due to a modulation in the levels of E-cadherin, a transcriptional target of ZEB2 (Postigo & Dean 2000). Indeed, an increase in the levels of E-cadherin was observed in PC3-AR and DU145-AR cells. Co-transfection with a *ZEB2* cDNA construct prevented the decline in invasiveness and migration. An increase in E-cadherin expression and a decline in invasiveness and migration were probably downstream effects of reduced levels/activity of ZEB2 in AR overexpressing AI cells.

Attempts were also made to investigate whether the AR-mediated modulation in ZEB2 levels involves post-transcriptional regulation by miRNAs. There is evidence to suggest that miR200 family members regulate EMT by targeting ZEB proteins (Gregory et al. 2008). This finding encouraged us to investigate the possibility of a relationship between miR200a/miR200b and AR. Basal levels of miR200a/miR200b were found to be significantly higher

Table 1 *In silico* scanning for the presence of ZEB2-binding sites in the regulatory regions (–1 to –1000 bp) of *MIR200A* and *MIR200B* genes

Gene names	Accession number	Regions scanned	E-box2 CACCTG	Z-box1 CAGGTG	Z-box2 CAGGTA
<i>MIR200A</i>	Gi:224589800	1 102 243–1 103 242	–	–941, –418, –364	–
<i>MIR200B</i>	Gi:224589800	1 101 484–1 102 483	–	–938, –494, –182	–

**Figure 11**

EMT regulation by androgen receptor (AR) in prostate cancer is a cell context-dependent phenomenon. AR acts as a positive regulator of ZEB2 expression and EMT in androgen-dependent cells and as a negative regulator in androgen-independent prostate cancer cells.

in LNCaP cells compared with PC3 and DU145 cells. This observation partly explains the lower abundance of ZEB2 in LNCaP cells. Furthermore, AR silencing in LNCaP cells led to a decrease in the levels of miR200a/miR200b. This finding lends credence to the possibility that AR is a positive regulator of miR200 expression in androgen-dependent LNCaP cells (Supplementary Fig. 3). However, the AR silencing-mediated decrease in miR200a/miR200b levels was not accompanied by an increase in ZEB2 expression in LNCaP cells. It is likely that the AR-mediated upregulation of ZEB2 expression in LNCaP cells does not rely on the participation of miR200a/miR200b.

In AI PC3 cells, AR overexpression leads to an increase in miR200a/miR200b levels. However, it is not clear whether an increase in MIR200A/MIR200B expression precedes or succeeds the decline in ZEB2 levels. It has been postulated that there exists a double-negative feedback loop between ZEB and miR200s, which regulates the stability and interchangeability of the EMT phenotype (Gregory *et al.* 2008, Brabletz & Brabletz 2010). It is likely that the AR-induced decrease in ZEB2 expression leads to an increase in miR200a/miR200b levels. This presumption is supported by *in silico* studies, which revealed the presence of ZEB2-binding sites in the regulatory regions of the *MIR200A* and *MIR200B* genes (Table 1).

This study provides conclusive evidence for the AR-mediated regulation of ZEB2 expression in PCa cells.

However, this regulation appears to be a context-dependent event (Fig. 11). AR binds to more than 200 proteins that either repress or activate the transcriptional activity of AR. These co-repressors or co-activators may compete for a common, or at least partially overlapping, binding site on AR (Nagy *et al.* 1999). Different levels or the differential recruitment of binding proteins may modulate the ability of AR to act as a repressor in one context and as an activator in another context (van de Wijngaert *et al.* 2012). In addition, AR binding to chromatin is known to be dependent on certain co-regulatory proteins, such as forkhead protein FOXA1. A modulation in the levels of these co-regulatory proteins is also known to alter the AR cistromes (Sahu *et al.* 2011). Divergent observations with regard to ZEB2 expression, as observed in this study, could also result from distinct AR transcriptional programs in androgen-dependent and -independent cell lines.

This study supports the inferences drawn by Zhu & Kyprianou (2010), which suggest that AR maintenance is necessary for EMT regulation. Long-term androgen deprivation downregulates AR expression and induces EMT, which often lead to recurrent prostate tumor growth (Liu *et al.* 2012). Clinical evidence also suggests that intermittent, rather than continuous, androgen deprivation therapy is more beneficial to patients with locally advanced, metastatic prostate tumors (Boccon-Gibod *et al.* 2007, Suzuki *et al.* 2008). Our study also hints that

the loss of AR expression favors EMT in AI PCa cells. Thus, the regulation of the expression of AR as well as its co-regulators and downstream effectors may have remarkable implications for metastasis in PCa cells.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0514>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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