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The Presence of Androgen Receptor Elements Regulates ZEB1 Expression in the Absence of Androgen Receptor

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ABSTRACT

Zinc finger E-box binding homeobox 1 (ZEB1) is a transcription factor that plays a central role in the epithelial to mesenchymal transition (EMT) of cancer cell lines. Studies on its regulation have mostly focused on the negative 3'UTR binding of miR200c. Interestingly, it has been previously reported that androgen receptor (AR) regulates ZEB1 expression in breast and prostate cancers. In order to validate this, various ZEB1 promoter deletions were cloned into a luciferase reporter system to elucidate the contribution of two putative androgen response elements (AREs). The in vivo contribution of AR was also assessed in cell lines after R1881 treatment using qPCR with prostate specific antigen (PSA) as the positive control. We discovered that AR upregulates the levels of expression of ZEB1 10-fold on a luciferase promoter that only contains the distal ARE. However, when the proximal ARE is included, no additional activation is apparent with AR or its hormone independent variant, AR-V7. Furthermore, we demonstrate here that a promoter construct containing both AREs activates transcription of ZEB1 even in the AR-null cell lines DU145 and PC3. Incubation of the AR-positive cell line, LNCaP with R1881, failed to substantially increase the expression levels of ZEB1. Despite the presence of AREs in the promoter region, it appears that ZEB1 expression can be induced even without AR. In addition, the region around the distal ARE is a potent repressor in AR-null cell lines. J. Cell. Biochem. 116: 115–123, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: ANDROGEN RECEPTOR (AR); ZEB1/TCF8; PROSTATE CANCER; OVOL1; OVOL2; EPITHELIAL TO MESENCHYMAL TRANSITION (EMT); LUCIFERASE ASSAYS

ZEB1 is a zinc finger transcription factor that can be overexpressed in various cell lines to induce EMT by suppressing E-cadherin (CDH1) and MYB [Moes et al., 2012; Hugo et al., 2013]. ZEB1 is also indirectly able to induce stem cell factors through repression of miR200 family members which themselves repress stemness factors including Sox2 and Klf4 [Wellner et al., 2009].

Abbreviation: AR, androgen receptor; ARE, androgen receptor element; EMT, epithelial to mesenchymal transition; GFP, green fluorescent protein; ZEB1, zinc finger E-box-binding homeobox 1.
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Induction of ZEB1 protein has been shown in response to long-term exposure to chemotherapy [Kim et al., 2013], incubation with M2 macrophages [Roca et al., 2013] and hypoxia [Salnikov et al., 2012] highlighting the importance of the tumor micro-environment [Yang et al., 2014]. The exact sequence of events that leads environmental stress to increase ZEB1 expression remains unknown. Interestingly, downregulation of ZEB1 is generally thought to be mediated at the mRNA level by miR200c, thus causing a negative feedback in which miR200c represses ZEB1 and vice-versa [Moes et al., 2012]. However, little has been done to determine which factors positively regulate ZEB1 expression.

One of the most common treatments for PCa is chemical castration which inhibits the positive feedback of androgens within the cancer cells. Although androgen deprivation is the "gold standard" of therapy, it has been suggested that therapy induces or causes a positive selection for aggressive, androgen-independent cancer cells [Sun et al., 2012]. Castration also reduces the levels of circulating androgens, which is the ligand that binds to AR and allows it to localize to the nucleus [Ni et al., 2013]. While prostate cancer is initially dependent on androgen in the early stages of the disease, it rapidly becomes androgen-insensitive if the patient is treated with anti-androgens [Hu et al., 2009]. Several known mechanisms in which this malady can become androgen-insensitive are: (1) AR can be overexpressed to levels that allow it to become even more sensitive to even low levels of circulating androgen or androgen-like hormones; (2) splice variants that lack the ligand binding domain can be produced which localize to the nucleus in the absence of hormones; (3) phosphorylation of the ligand binding domain; and (4) prostate cancer can simply down-regulate AR in favor of other methods of growth [Pienta and Bradley, 2006]. The use of anti-androgens may even lead prostate cancer to become more aggressive through EMT [Sun et al., 2012]. This report investigates how AR may potentially contribute to EMT by modulating ZEB1 expression, a master regulator that can independently lead to an EMT in prostate cancer models.

MATERIALS AND METHODS

DNA EXPRESSION PLASMID CONSTRUCTS

AR-V7 and AR-FL were cloned into the pcDNA3.1 vector. GFP-AR and GFP-ARV7 were cloned into GFP-C1 [Hu et al., 2012]. All ZEB1-luciferase constructs used were in pGL4 (Promega) and pGL4-ZEB1$_{-867/-66}$ was specifically obtained from Dr. Carole Mendelson of UT Southwestern [Renthal et al., 2010].

CELL CULTURE

DU145-TxR and PC3-TxR cell lines were described previously [Li et al., 2011] and all cell lines are described in (Table I). A GFP-ARV7 variant of DU145-TxR was constructed and designated DU145-TxR-GFP-ARV7. PC3-Epi is a single cell E-cadherin/CDH1 positive PC3 clone and its M2 macrophage induced EMT derivative, PC3-EMT were described previously [Roca et al., 2013]. LNCaP-GFP and LNCaP-ARV7 were obtained from Dr. Charles Sawyers [Watson et al., 2010]. All cells were incubated at 37 °C in RPMI media (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) in >95% humidity and 5% carbon dioxide [Zeng et al., 2011].

TABLE I. Description of the Cell Lines Used

Cell line	Description
PC3-EPI	CDH1+ subpopulation of PC3
PC3-EMT	M2 induced mesenchymal clone of PC3-Epi
PC3-TxR	PC3 cells treated with long term Paclitaxel
DU145	Derived from brain metastasis
DU145-TxR	DU145 cells treated with long term Paclitaxel
DU145-TxR-GFP-ARV7	DU145-TxR stable transfected with GFP-ARV7
LNCaP-GFP	LNCaP stably transfected with GFP
LNCaP-ARV7	LNCaP stable transfected with untagged ARV7

QUANTITATIVE POLYMERASE REACTION (qPCR)

Cells were disrupted with lysis buffer containing 1% 2-mercaptoethanol and shredded with a QIAshredder column (Qiagen) [Li et al., 2011]. Total RNA from lysed cells was extracted and purified using an RNeasy kit (Qiagen). The concentration and purity of RNA was assessed using a NanoDrop (ThermoScientific). Synthesis of cDNA was performed using an iScript cDNA synthesis kit (Bio-Rad). The cDNA products were diluted 20-fold with DEPC treated water before use in the Taq-man assays. Primer/probe sets were premixed (Applied Biosystems).

IMMUNOBLOTTING

Cells were lysed with protease inhibitors and centrifuged to separate the debris. Each sample was quantified by BCA analysis (Thermo) and diluted to 0.5 μg/μL. 20 μL was separated on a 4–15% SDS-PAGE and transferred to a nitrocellulose membrane using a TransBlot (Bio-Rad). The membranes were incubated with 1× blocking buffer (Sigma) for 1 h. The membranes were then incubated with a primary antibody at 4 °C overnight followed by a secondary (1:20,000) IRDye IgG antibody (Li-Cor Biosciences) for 1 h. All antibodies (Supplemental Table S1) were purchased from a vendor and diluted with 1× blocking buffer. The Odyssey Infrared Imaging System (Li-Cor) was used to detect protein expression while the SeeBlue ladder (Invitrogen) was used to assess molecular weight.

LUCIFERASE ASSAY

Cell lines were seeded into 24-well plates at 50,000 cells per well and transiently transfected the next day using X-tremeGene HP (Roche). The indicated ZEB1 reporter was transfected in the same ratio as the CMV-renilla luciferase control vector in order to be about 20% of the total amount of DNA transfected [Mooney et al., 2014; Rajagopalan et al., 2014]. Data reported were normalized by dividing the firefly values by the CMV-renilla luciferase values in each well then normalized again by dividing all values by the indicated reference. The error bars indicate the standard errors which was calculated using the normalized data. The total amount of DNA for each well was always 0.5 μg and so the amounts were kept consistent using pcDNA3 as the empty vector control.

RESULTS

ZEB1 EXPRESSION IN CELL LINES

The expression of various epithelial and mesenchymal cell markers was assessed in the eight prostate cancer cell lines (Table I) used in these studies. PC3-TxR and DU145-TxR came from cell lines treated

with the chemotherapy paclitaxel which causes EMT [Kim et al., 2013]. Likewise, PC3-EMT was induced to a mesenchymal phenotype by incubation with M2 macrophages [Roca et al., 2013]. ZEB1 expression was used to categorize the cell lines into two groups, one that had extremely low expression of ZEB1: (PC3-Epi, LNCaP-GFP, LNCaP-ARV7) and another group with high expression: (PC3-TxR, PC3-EMT, DU145, DU145-TxR, DU145-TxR-ARV7) (Fig. 1A and B). Other markers including E-cadherin/CDH1 and OVOL1/2 supported these categories (Fig. 1C).

ZEB1 PROMOTER DISSECTION

ZEB1 expression was modulated at the promoter level in seven out of eight cell lines tested (Fig. 2). Interestingly, LNCaP-GFP and LNCaP-ARV7 had almost identical patterns to each other and very similar patterns to their AR-null counterparts. The presence of the region from ZEB1$_{-65/+49}$ elicited a dramatically higher ZEB1 expression in seven out of eight cell lines tested. The increased expression in the constructs with ZEB1$_{-65/+49}$ may be due to the presence of a transcriptional start site; however, it is interesting that the scale of activation is very similar across cell lines.

AR REGULATES THE DISTAL ARE IN ZEB1

To elucidate whether AR could positively regulate ZEB1 levels via distal AREs, luciferase assays were conducted. These luciferase assays were performed in the AR-null cell line, DU145, and revealed that increasing amounts of AR were able to increase activity on luciferase promoters in an R1881 dependent manner (Fig. 3). As expected, increasing amounts of ARV7 had a similar effect on luciferase activity independent of synthetic androgen, R1881. It should be noted that in both DU145 and DU145-TxR cells AR translocated to the nucleus in an R1881 dependent manner while GFP-AR/ARV7 did not require R1881 for AR translocation (immunofluorescence not shown). In order to confirm binding of AR to the proposed sequence, a mutant containing four nucleotides in the proposed ARE was developed (schematic is in Supplemental Fig. S1). Interestingly, it had no effect on ZEB1 promoter activity, possibly because the mutant was close enough to a consensus sequence that the binding kinetics were not altered.

BOTH THE DISTAL AND PROXIMAL ARE TOGETHER ARE NOT AR DEPENDENT

Inclusion of both AREs (Androgen Receptor Elements) were postulated to be synergistic [Anose and Sanders, 2011]. Inexplicably however, AR failed to further activate AR transcription on any of the promoters (Fig. 4). Additionally, mutation of the proximal ARE had either no effect or a positive effect on some promoters. In order to determine the relationship between the two AREs, cell lines were transfected with two ZEB1 constructs, pGL4-ZEB1$_{-867/+50}$ and pGL4-ZEB1$_{-740/+50}$, surprisingly, removing the entire distal ARE had a positive effect on ZEB1 transcription in six out of eight cell lines (Fig. 5A); however, further addition of AR failed to increase transcription in DU145 luciferase promoter assays (Fig. 5B) on the pGL4-ZEB1$_{-740/+50}$. Interestingly, this figure actually confirms that both AREs synergize in AR positive LNCaP, however, it also indicates that both AREs together are inhibitory to all AR negative cell lines. Since the transcriptional start site is present in all constructs, this figure indicates that there is an inhibitory region within ZEB1$_{-867/-741}$ that is not regulated by AR.

R1881 FAILED TO INDUCE ZEB1 EXPRESSION WITH ENDOGENOUS AR

In order to determine whether a positive effect on ZEB1 transcription would be seen in a more physiological system, the AR positive LNCaP-GFP or LNCaP-ARV7 were transiently transfected with either ZEB1$_{-867/-66|+51/+291}$ or ZEB1$_{-867/+291}$ luciferase promoters and incubated with various concentrations of R1881 (Fig. 6A and B). These promoters were used since they had the greatest difference in expression (41/32-fold for LNCaP-GFP and LNCaP-ARV7) compared to ZEB1$_{-867/-66|+51/+367}$ or ZEB1$_{-867/+367}$ (9/7.5-fold for LNCaP-GFP and LNCaP-ARV7) as shown in (Fig. 2). In this experiment, phenol-red free RPMI, supplemented with charcoal stripped FBS was used to prevent the possibility of AR activation and its nuclear

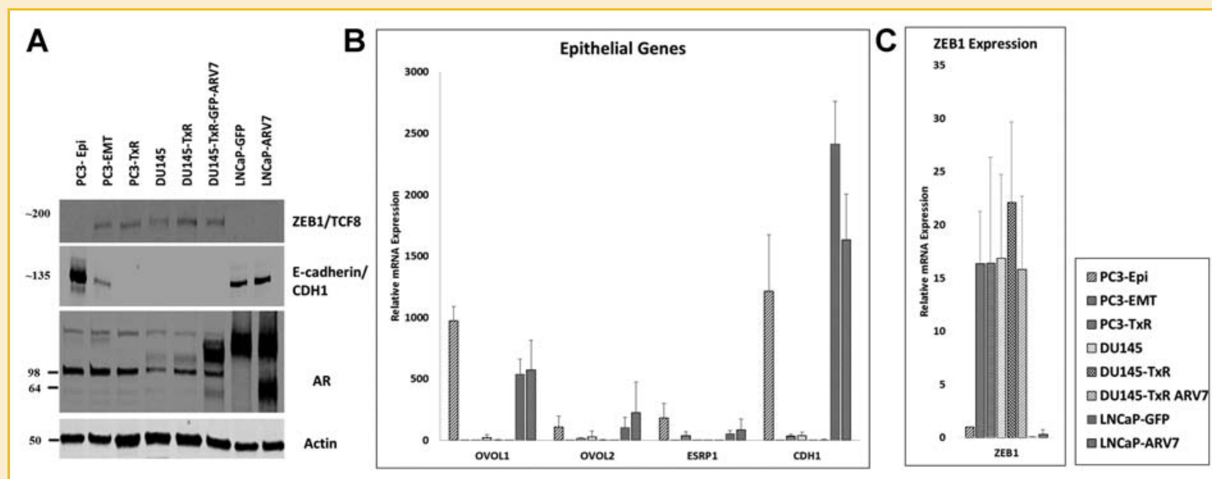

Fig. 1. Various cell lines were assayed for mesenchymal and epithelial markers. (A) Immunoblotting for ZEB1, E-cadherin, AR, and β-Actin. (B) qPCR for the epithelial markers OVOL1/2, ESRP1, and E-cadherin with PC3-TxR normalized to 1. (C) qPCR for the mesenchymal ZEB1 and ZEB2 with PC3-Epi normalized to 1.

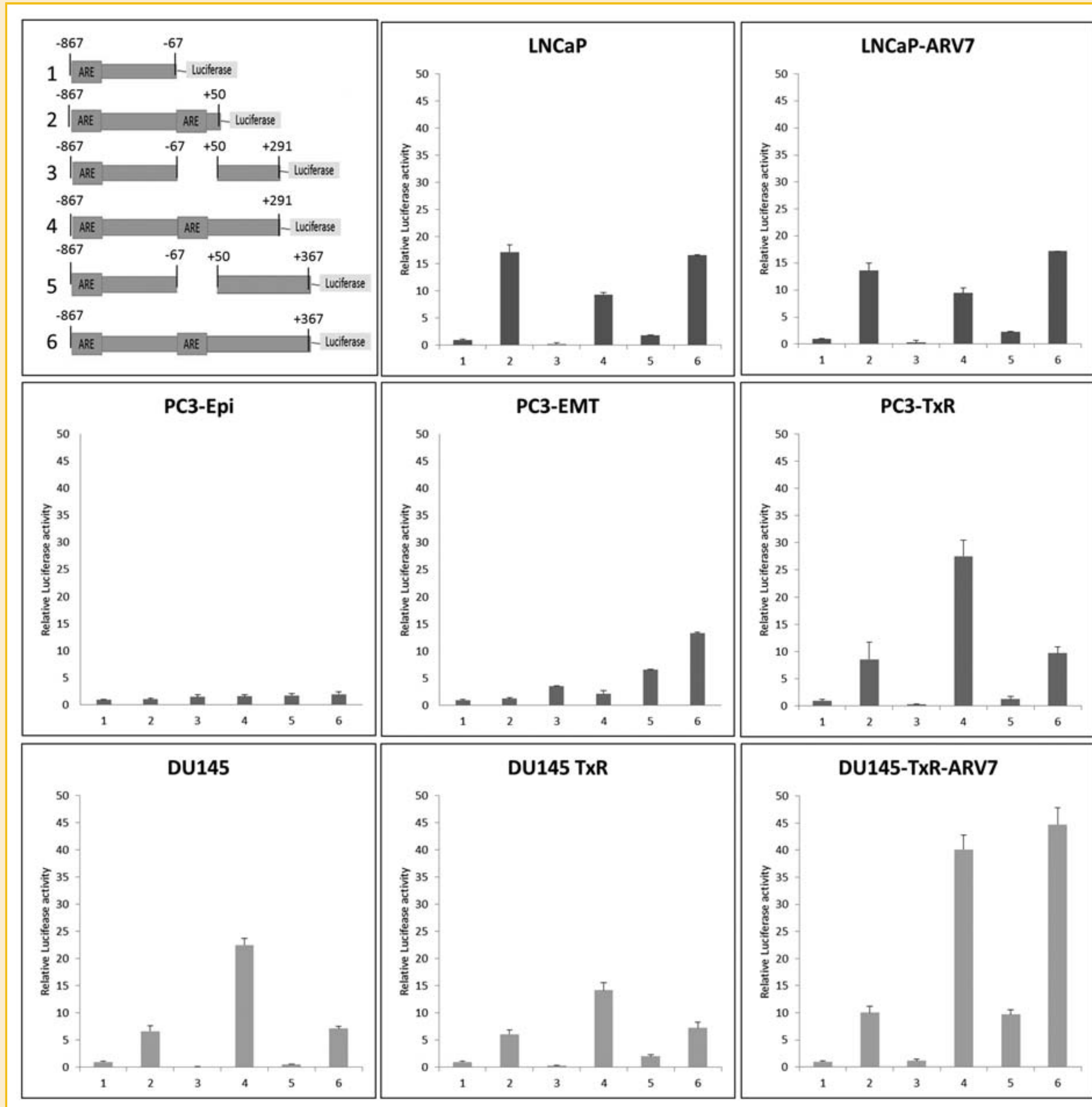

Fig. 2. The region around the proximal ARE regulates the ZEB1 promoter in the absence of AR. Each well of a 24 plate was transfected with 100 ng of the specified ZEB1 promoter, 100 ng CMV-renilla, and 400 ng pcDNA3. Experiments were done a minimum of three times in quadruplicate and normalized to pGL4-ZEB1$_{-866/-67}$.

translocation. Interestingly, R1881 had no effect on the luciferase promoters. Additionally, results from qPCR revealed that endogenous ZEB1 was not affected while PSA had a characteristic upregulation due to AR signaling. These results (Fig. 6C and D) coincide with a previous report indicating that R1881 failed to induce endogenous ZEB1 expression [Anose and Sanders, 2011].

DISCUSSION

It has been reported that AR is positively associated with ZEB1 expression in triple negative breast cancer cells [Graham et al., 2010]. However, AR is only expressed in the latest stages of breast cancer while it is expressed in the early stages of prostate cancer and even in normal prostate tissue where it is vital for prostate development and differentiation. Another study did demonstrate that constitutively active AR, but not full length AR caused upregulation of ZEB1 in LNCaP cells [Cottard et al., 2013]. Interestingly, the immunoblotting and qPCR results from the previous study were done for a maximum of 9 days after transient transfections by sorting out GFP positive cells. This data is actually not contradictory to the work described here since we used stably transfected cell lines for our qPCR and immunoblotting studies. Using transient transfection may produce different results since AR may be able to upregulate ZEB1 in the short-term but does not lead to a stable EMT.

Another report demonstrates that the presence of both AREs are essential to ZEB1 transcription in PC3-AR cells which have been

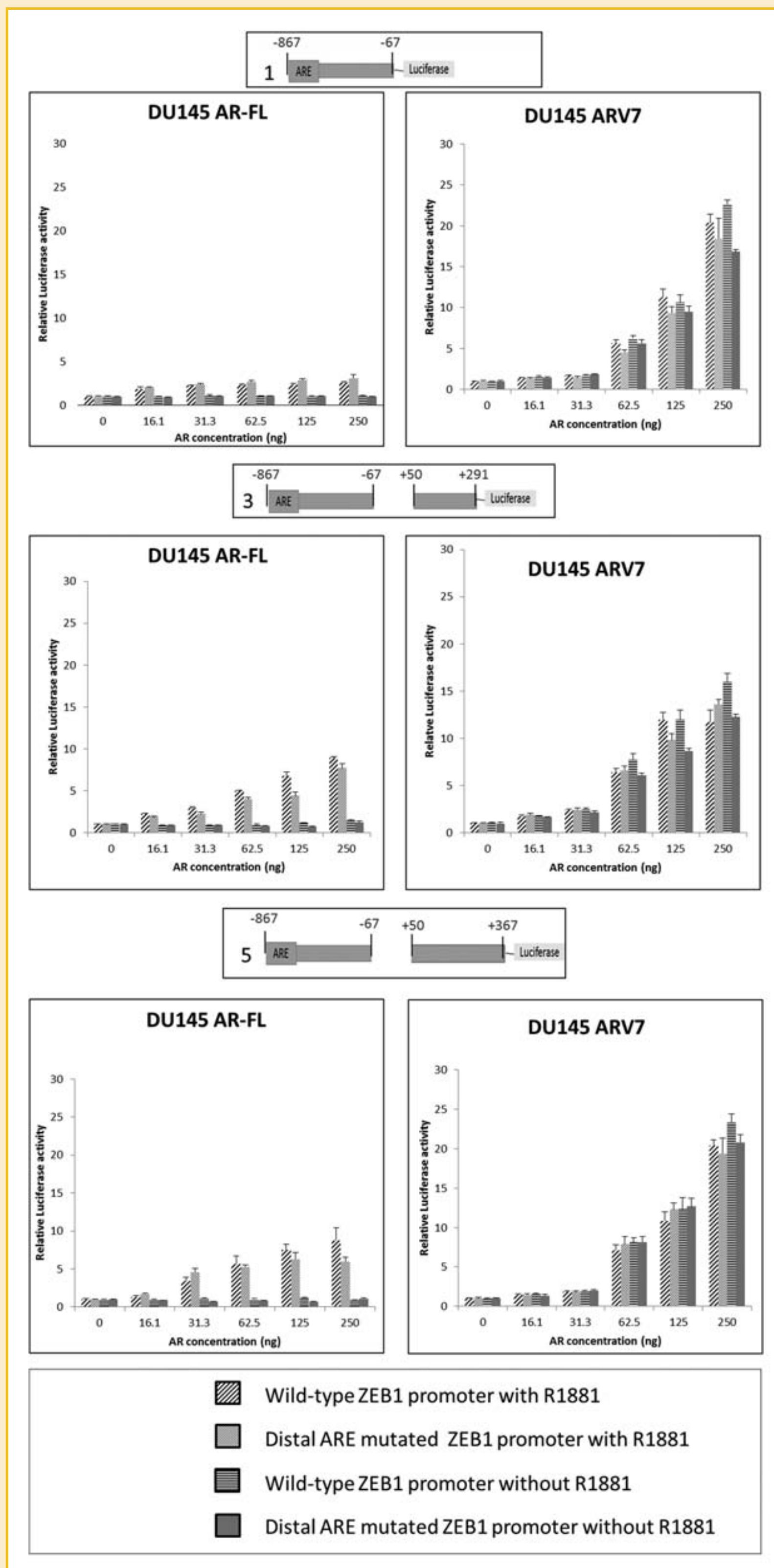

Fig. 3. The region around the distal ARE is regulated by AR. A schematic is shown on the left, which depicts the distal ARE promoter nucleotides underlined and the mutated nucleotides as lower case letters. The luciferase assays were conducted with various promoter lengths in DU145 cells. Firefly luciferase activity was divide by CMV-renilla activity and the resulting ratio was normalized to 1 in each graph for no AR.

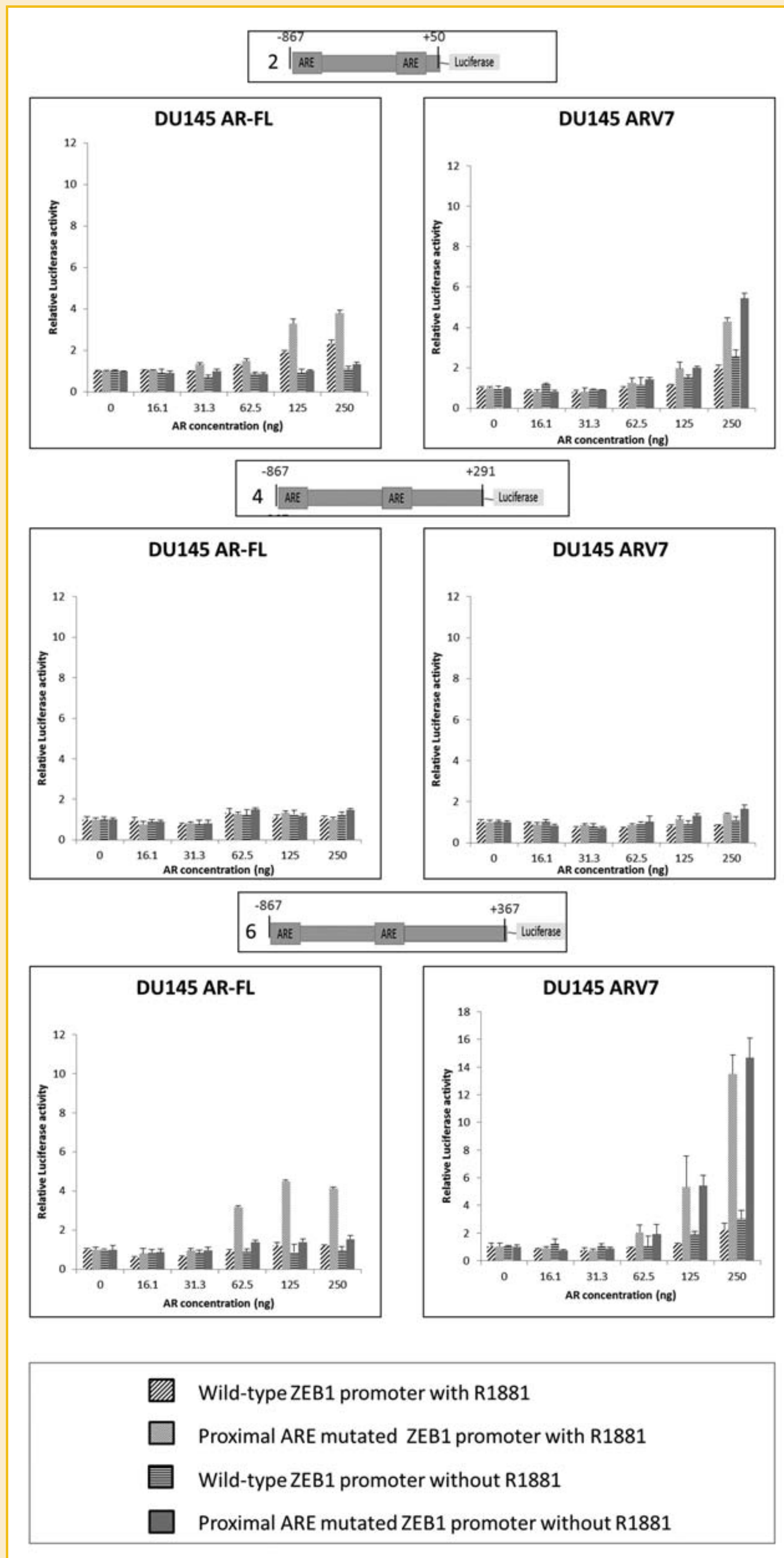

Fig. 4. The region around the distal but not the proximal ARE is regulated by AR. A schematic is shown on the left, which depicts the proximal ARE promoter nucleotides underlined and the mutated nucleotides as lower case letters. The luciferase assays were conducted with various promoter lengths in DU145 cells. Firefly luciferase activity was divide by CMV-renilla activity and the resulting ratio was normalized to 1 in each graph for no AR.

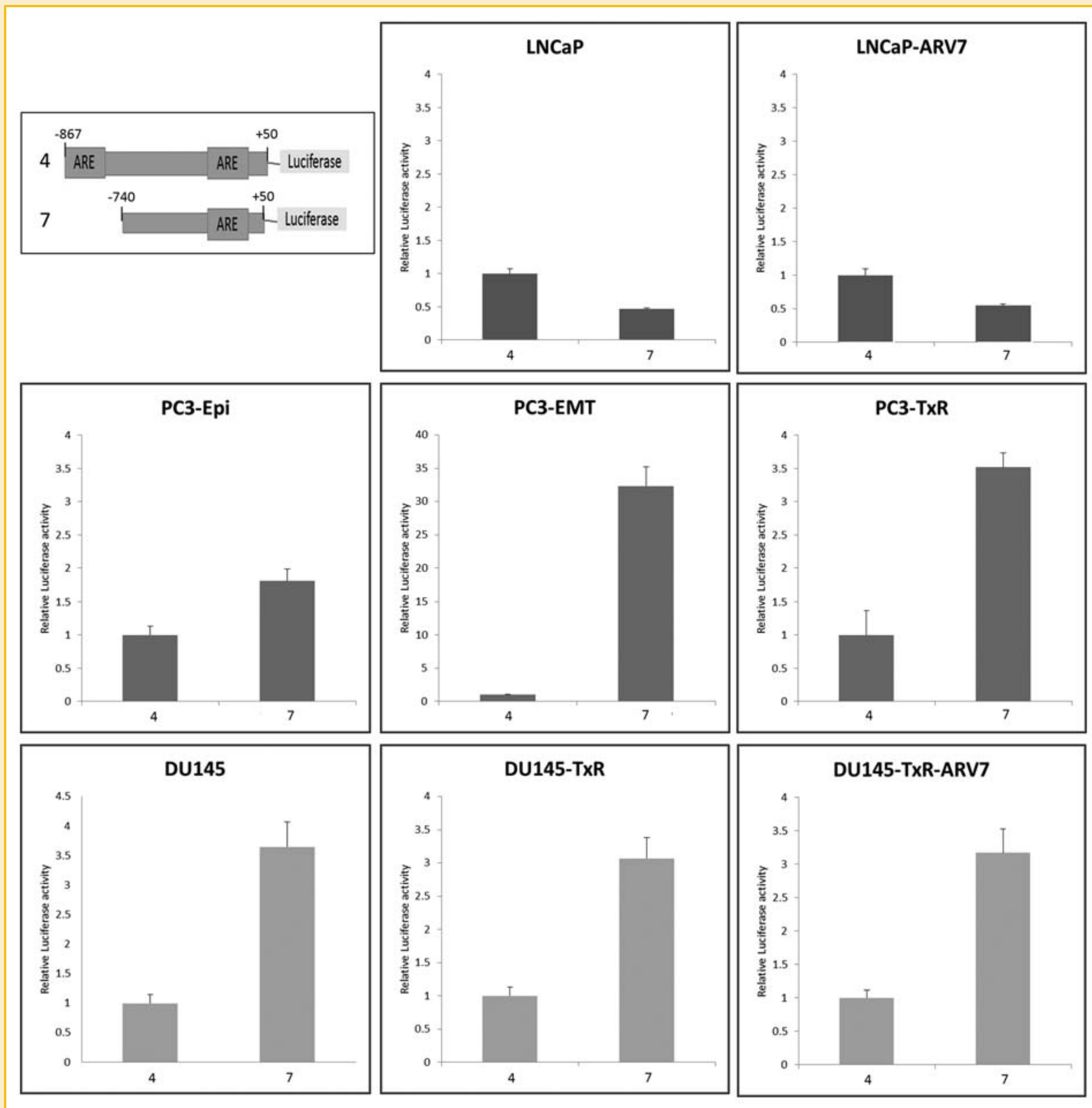

Fig. 5. The region around the distal ARE is repressive in AR-negative cell lines. (A) Each well of a 24 plate was transfected with 100 ng of the either pGL4-ZEB1$_{-866/+50}$ or pGL4-ZEB1$_{-740/+50}$ promoter, 100 ng CMV-renilla and 400 ng pcDNA3. Experiments were done a minimum of three times in quadruplicate and normalized to pGL4-ZEB1$_{-866/+50}$. (B) DU145 cells were transfected with pGL4-ZEB1$_{-740/+50}$ with various amounts of AR. The case with no AR was normalized to 1. Mutations were done to the 3′ ARE as shown in Figure 4.

stably transfected with full length AR [Anose and Sanders, 2011]. The authors further demonstrate that dihydrotestosterone (DHT) was able to upregulate ZEB1 on an artificial β-galactosidase reporter in LNCaP and LNCaP derivatives in a dose dependent manner. However, the authors did not demonstrate the upregulation of endogenous ZEB1 expression in these cell lines, which is consistent with our results [Anose and Sanders, 2011].

Although our data would suggest that AR does not regulate ZEB1 under physiological conditions, this may not be compulsory. Cancer is a heterogeneous cell population that can have differing DNA methylation patterns, histone marks, and transcription factor expression [Mooney et al., 2010a,b; Rajagopalan et al., 2011; Kulkarni et al., 2012]. In this way, there may be situations in which the truncated ZEB1 promoters used in this study actually mimic the in vivo situation better than the longer constructs containing both distal and proximal AREs.

In the systems used in this report the distal ARE is AR sensitive (Fig. 3) whereas the proximal ARE is not (Fig. 4). Inclusion of the proximal ARE is more strongly activating than the distal ARE (Fig. 2). In fact, the inclusion of both AREs was actually somewhat repressive in six out of eight cell lines even in the absence of AR (Fig. 5). It is most likely that there is a transcription initiation

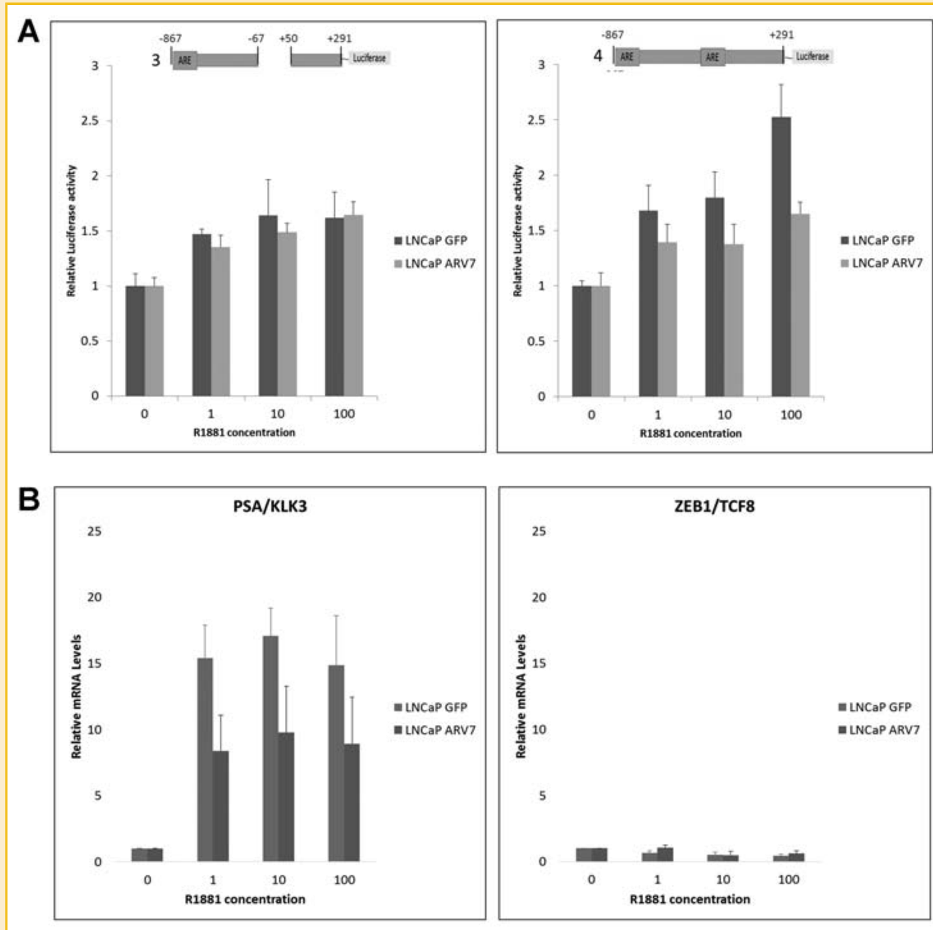

Fig. 6. Endogenous AR or stably expressed ARV7 had little effect on ZEB1 expression. (A) Either pGL4-ZEB1$_{-866/-66|+51/+291}$ or pGL4-ZEB1$_{-866/+291}$ were transfected into the indicated LNCaP cell line with various amounts of R1881. Normalization was done to the no R1881 control. (B) The indicated LNCaP cells were grown for 36 h in media supplemented with various amounts of R1881. Triplicate qPCR for ZEB1/PSA is shown normalized to β-Actin. Experiments in (A, B) were done in charcoal stripped FBS with colorless RPMI.

Fig. 7. Models of AR-independent and -dependent ZEB1 modulation. AR binds in the vicinity of the proposed distal ARE as a dimer whereas in our model, the proximal ARE is not bound by AR at all instead some unknown factors which enhance ZEB1 promoter activity to a larger degree than AR.

complex that binds in the vicinity of the proximal ARE (Fig. 7). This unknown complex may not include AR and is actually activated in AR positive and negative cell lines. ChIP-seq and ChIP-onChIP studies also suggest that these sites are not bound by AR in AR positive cell lines: abl, LNCaP, and VCaP cell lines [Wang et al., 2009; Massie et al., 2011]. AR itself can slightly upregulate the expression of ZEB1 however, its effect is only on the distal ARE which is normally bound by repressors in both AR negative and positive cell lines. Future work should focus on the region of the proximal ARE in order to determine the identities of actual transcription factors that regulate the ZEB1 promoter and on the distal ARE to determine repressors of ZEB1 expression.

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