Androgen Deprivation Causes Epithelial–Mesenchymal Transition in the Prostate: Implications for Androgen-Deprivation Therapy

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Abstract
Androgen deprivation is currently a standard-of-care, first-line therapy for prostate cancer in the United States. Although this regimen effectively regresses androgen-dependent disease, relapse often occurs in an androgen-independent manner and is associated with poor prognosis. Such castration-resistant prostate cancer represents a major clinical challenge, and the mechanisms underlying castration resistance are not fully understood. Epithelial–mesenchymal transition (EMT) is a key developmental process and has also been implicated in cancer metastasis and therapeutic resistance in recent years. However, the factors contributing to EMT in human cancers remain unclear. Here, we show that both normal mouse prostate tissue and human LuCaP35 prostate tumor explants display an EMT as well as increased stem cell-like features following androgen deprivation. Importantly, we observed similar changes in mesenchymal features in prostate tumors from patients treated with androgen-deprivation therapy. In addition, we have delineated a feedback loop involving the androgen receptor and the Zeb1 transcription factor that seems to mediate this transition. In summary, we show for the first time that androgen deprivation induces EMT in both normal prostate and prostate cancer, revealing a potentially important consequence of a standard-of-care treatment for prostate cancer. This finding could have significant implications for second-line treatment strategies in this clinical setting.

Introduction
Prostate cancer is the most frequently diagnosed cancer in men in the United States, accounting for 10% of all cancer-related deaths (1). Androgen-deprivation therapy is a standard-of-care treatment for prostate cancer and efficiently controls the growth of androgen-dependent tumors. Unfortunately, the majority of these cancers ultimately become refractory to hormone deprivation and emerge as castration resistant (2–4). Such castration-resistant prostate cancer represents a significant clinical challenge, and a better understanding of the biologic mechanisms that contribute to tumor regrowth is of critical importance.

Epithelial–mesenchymal transition (EMT) was initially identified as a developmental process during which epithelial cells acquire a migratory and invasive mesenchymal phenotype. It is typically characterized by the coordinated loss and gain of various epithelial (e.g., E-cadherin) and mesenchymal (e.g., N-cadherin and vimentin) markers, respectively. A number of distinct molecular processes that mediate EMT can also serve as diagnostic readouts, including expression of the transcriptional repressors of E-cadherin, such as Twist, Snail, Slug, and Zeb1/2 (5, 6). In recent years, the significance of EMT in tumor biology has become more widely appreciated (7). In particular, EMT has been shown to promote tumor metastasis, confer cancer stem cell properties, and mediate resistance to traditional therapeutics in diverse preclinical model systems of mammary, lung, pancreatic, and bladder cancer (8–13).

Recently, a few studies have highlighted a role for EMT in facilitating prostate cancer progression and metastasis (14–16). The underlying drivers of EMT in prostate are poorly understood, and the impact of androgen-deprivation therapy on EMT has not yet been carefully examined. In this study, we present preclinical and clinical data supporting a shared link between androgen deprivation and EMT in both normal prostate tissue and prostate cancer.
Materials and Methods

Microarray and data analysis

Gene expression profiling for LuCaP35 xenografts was carried out by GeneChip human genome U133 Plus 2.0 array (Affymetrix). Samples were prepared with the RNeasy Mini Kit (QIAGEN), and microarray studies were carried out following standard protocols as previously described (17). Data were analyzed by the bioconductor packages with R software. Using a fold change cutoff as 2 and a P\textsubscript{value} as 0.05, we identified a set of genes that were differentially expressed (DE) between castration and control samples. Pathway analysis of these DE genes by Ingenuity Pathway Analysis 8.0 (IPA 8.0) identified an embryonic stem cell–like gene signature in castration samples. Human secreted genes were also manually selected from the DE list to create an additional signature. The heat maps of the above-mentioned 2 signatures were then produced by R function heat map, in which Euclidean distance and complete linkage were used.

Gene expression profiling with prostate stem cells was carried out on whole mouse genome 4 × 44K array (Agilent). The Lin\textsuperscript{−}CD44\textsuperscript{−}CD133\textsuperscript{−}Sca-1\textsuperscript{−}CD117\textsuperscript{+} and Lin\textsuperscript{−}CD44\textsuperscript{+}CD133\textsuperscript{−}Sca-1\textsuperscript{+}CD117\textsuperscript{−} cells were isolated through fluorescence-activated cell sorting (FACS) as previously described (18). Following FACS, cells were spun down, and RNA was isolated with the Arcturus PicoPure RNA isolation Kit (Applied Biosystems). Typically, 1,000 to 5,000 cells were used for each sample, and at least 30 mice were used to generate one set of samples. Quantity and quality of total RNA samples were determined by a ND-8000 spectrophotometer (Thermo Scientific) and a Bioanalyzer 2100 (Agilent Technologies), respectively. The first round of amplification and the second round of cDNA synthesis were carried out by the MessageAmp II aRNA Amplification Kit (Applied Biosystems). Cynarine-5 dye was then incorporated through an in vitro transcription reaction using the Quick Amp Labeling Kit (Agilent Technologies). A total of 750 ng of each Cynarine-5 labeled test sample was pooled with Cynarine-3 labeled Universal Mouse Reference (Stratagene) and hybridized onto Agilent’s Whole Mouse Genome 4 × 44K arrays as described in the manufacturer’s protocol. The arrays were washed, dried, and scanned on the Agilent scanner. Agilent’s Feature Extraction Software (version 10.7) was used to analyze acquired array images. Bioconductor R package "limma" was used for data analysis. Moderated t-statistics and P values were obtained by empirical Bayes statistics for differential expression, and moderated SEs determined by this approach were reported. Limma reports log; fold change (logFC) and the moderated t-statistic (t), and the SE was inferred as (logFC/t).

The processed microarray data set GSE17862 was downloaded from the National Center for Biotechnology Information (NCBI) website. Bioconductor R package "limma" was used to carry out a paired t test to compare the gene expression values between days 14 and 0 upon androgen removal.

The gene expression levels for individual probes in GDS1390 and GDS2562 were obtained from the NCBI Gene Expression Omnibus (GEO) website. All plots and statistical analyses, unless specified, were conducted using Prism GraphPad5.

Quantitative real-time PCR

RNA was isolated with the RNeasy RNA isolation Kit (QIAGEN), and cDNA was synthesized by the High Capacity Reverse Transcription Kit (Applied Biosystems). The gene expression levels were quantified using Taqman assays (Applied Biosystems) and the \(\Delta\Delta CT\) method. The genes and the Taqman assay identification numbers are listed as following: Zeb1 (Hs00232783_m1), Zeb2 (Hs00207691_m1), E-cadherin (Hs00170423_m1), N-cadherin (Hs00169953_m1), Vimentin (Hs00185584_m1), Twist1 (Hs01675818_s1), AR (Hs00171172_m1), and GUSB (4333767F). GUSB was used as the internal control for normalization.

Animal studies

All animal studies were conducted following the guidelines of the Genentech Institutional Animal Care and Use Committee. Nonobese diabetic/severe combined immunodeficient mice (Charles River Laboratory) were used to maintain LuCaP35 xenografts. Subcutaneous xenograft tumor volumes were calculated by multiplying the 3 dimensions and \(\pi/6\) (volume = \(\pi/6 \times L \times W \times H\); ref. 19). Surgical castration was achieved by removing both testicles through a microincision between the 2 testicles. Castrated and control athymic nude mice were obtained from Charles River Laboratory.

Cell culture

Unless specified, LNCaP, DU145, PC3, and CWR22Rv1 cell lines were cultured in RPMI 1640 containing 10% FBS. Cell lines were maintained by a core facility at Genentech that routinely uses short tandem repeat fingerprinting to verify cell line identity.

Antibodies and other reagents

Antibodies used are listed as follows: E-cadherin (Cell Signaling, 24E10); N-cadherin (Cell Signaling, #4061); Vimentin (Cell Signaling, 14C10); Zeb1 (Cell Signaling, D80D3); Twist1 (Scbt, Twist2C1a); AR (Epitomics, EP670Y). All antibodies for FACS were previously described (18). Gene expression plasmid pCMV-XL5-AR is from OriGene. Other reagents are Trichostatin A (TSA; Sigma); 5-Aza-2-deoxycytidine (5-Aza; Sigma); puromycin (Clontech); PBS (Sigma); charcoal stripped serum (Gibco); PhenoRedFree RPMI 1640 (Gibco); dihydrotestosterone (Innovative Research of America).

RNA interference

Androgen receptor (AR) knockdown was achieved by transient transfection of LNCaP cells with siGENOME SMARTpool siRNAs targeting human AR (Dharmacon), using Lipofectamine 2000 (Invitrogen). A pool of nontargeting siRNAs (siGENOME SMARTpool control, non-targeting #1) was used as the control. ZEB1 knockdown was conducted by lentivirus-mediated short hairpin RNA (shRNA) in pLKO.1-puro vectors. Lentiviruses were packaged following standard protocols as previously described (20). A vector containing a scrambled shRNA sequence was used as nontargeting control (NTC; Sigma; SHC002). The clone identification numbers for the 2 shRNA sequences for ZEB1 are #1, NM_030751.2-572s1c1 and #2, NM_030751.2-5706s1c1 (Sigma).
Drug sensitivity assay
A total of 7,500 LNCaP cells in the volume of 100 μL were plated in each well of a 96-well plate. The next day, docetaxel (Taxotere; Sanofi-Aventis) was added directly to the wells to reach concentrations over a range of 0.02 to 50 nmol/L. Three days after drug treatment, cell viability was quantified by the CellTiter-Glo assay (Promega), following the manufacturer’s protocols. Each assay was conducted in quadruplicate.

Cell proliferation assay
LNCaP cells were plated in poly-α-lysine coated 12-well plates (BD Bioscience) in triplicate, and cell densities were measured with Incucyte (Essen Bioscience) imaging every 2 hours.

Results
Androgen deprivation induces an EMT in normal mouse prostate tissue, associated with stem cell-like features
A notable feature of the mouse prostate is its strong regenerative capacity: It can undergo many rounds of regression and regrowth following physical and chemical castration and androgen supplementation, respectively. It is widely believed, therefore, that these castrated and regressed prostates are enriched for stem cells (21, 22). Considering the conceptual association between stem cells and EMT, we hypothesized that the castrated prostate tissue may undergo an EMT. To test this hypothesis, we evaluated the gene expression profiles of intact and castrated mouse prostate tissue using microarray analysis (GDS2562; ref. 23). Consistent with this hypothesis, we found that the expression of N-cadherin, Zeb1, Twist1, and Slug were upregulated 3 days after castration and continued to increase over time (Fig. 1A). Furthermore, these increases were either partially or completely reversed after 3 days of testosterone replenishment (Fig. 1A). Next, we examined prostates from mice that had been castrated long term (4 months). As expected, these prostates had shrunk significantly compared with age-matched controls (Fig. 1B). In addition, we observed a dramatic decrease in E-cadherin protein levels, accompanied by notable increases in N-cadherin and vimentin.
Androgen deprivation causes an EMT in prostate cancer in vivo

To examine whether androgen deprivation could affect EMT and ‘stemness’ in prostate cancer, we used the well-characterized LuCaP35 prostate cancer model. This tumor explant model was initially derived from a lymph node metastasis from a prostate cancer patient and is maintained through in vivo passing. Importantly, this tumor model recapitulates many characteristics associated with androgen-dependent human prostate cancer, including (i) AR positivity, (ii) responsiveness to androgen deprivation, and (iii) elevated prostate-specific antigen production (24). We castrated male mice with established LuCaP35 xenografts and isolated regressed tumors 4 weeks later prior to relapse (Fig. 2A and B). As expected, LuCaP35 tumor growth depended on androgen, and the tumor sizes decreased following androgen deprivation (Fig. 2B). We then carried out microarray expression analysis to compare the gene expression profiles between regressed and control tumors. Using \( P < 0.05 \) and absolute fold change more than 2 as the cutoff, we observed approximately 1,600 upregulated probes and approximately 1,000 downregulated probes. Global expression analysis using Ingenuity Pathway Analysis revealed the “human embryonic stem cell pluripotency” signature as one of the top listed DE canonical pathways (Fig. 2C). A closer examination of this pathway revealed that the regressed tumors had increased expression of 2 stemness markers, WNT5a and WNT5b (25). In addition, the regressed tumors also exhibited activated TGF-β signaling, as suggested by decreased expression of LEFTT1, an antagonist of TGF-β signaling (26), and increased expression of SMAD3, a downstream effector of TGF-β signaling (Fig. 2D).

In addition to increased ‘stemness’ and activated TGF-β signaling, castration affected the expression of many secreted factors (Fig. 2E), and resulted in significantly increased expression of a few ligands or their receptors that have been reported to induce EMT, including insulin-like growth factor (IGF1), JAG1, fibroblast growth factor receptor 2 (FGFR2), and platelet-derived growth factor D (PDGF-D) (Fig. 2E, Supplementary Table S1 and data not shown; ref. 6). Therefore, we hypothesized that an EMT was induced in the androgen-deprived tumors. Indeed, the castrated tumors had decreased levels of E-CADHERIN and augmented levels of N-CADHERIN and VIMENTIN compared with control tumors (Fig. 3A). In addition, the transcriptional suppressors of E-CADHERIN—ZEB1, ZEB2, and TWIST1—were also enriched after castration (Fig. 3A), although no changes in either SNAI1 or SNAI2 mRNA were observed (data not shown). These microarray findings were further validated by quantitative real-time PCR (qRT-PCR; Fig. 3B).

Collectively, our in vivo data have shown that androgen deprivation promotes EMT in both normal prostate tissue and prostate cancer. We also wanted to determine whether similar observations could be recapitulated in cell line models. ‘In vitro castration’ was carried out by culturing LNCaP cells in phenol-red–free RPMI 1640 supplemented with charcoal-stripped serum (CSS) that was low in hormone levels; control cells were cultured in the same media plus 10 nmol/L dihydrotestosterone (DHT) CSS + DHT, an AR agonist. Cells cultured in CSS grew slowly, tended to form spheres, and were severely
impaired in their ability to attach to tissue culture plates (Fig. 5A and B). This is consistent with the notion that cells that have undergone EMT exhibit reduced adherence to the basement membrane (5). Importantly, testosterone rescued cell growth and promoted cell attachment (Fig. 5A and B). In addition, both N-cadherin protein (Fig. 5C) and \( \text{ZEB1} \) mRNA (Fig. 5D) levels were markedly increased in cells cultured in CSS, whereas both increases were more modest and marginal in cells cultured in the presence of testosterone (CSS + DHT). This suggests that these effects were largely due to testosterone and not due to other factors that may have been depleted from the CSS. A functional feature of cells that have undergone EMT in many cancer models is increased resistance to therapeutics (7). To determine whether androgen deprivation promoted resistance to chemotherapeutic agents in this model, we treated cells with docetaxel, the most commonly used chemotherapeutic reagent for prostate cancer in the clinic. As shown in Fig. 5E, compared with cells cultured in FBS, LNCaP cells cultured in CSS exhibited increases in both IC\(_{50}\) and IC\(_{90}\) and contained a relatively larger population of cells that survived docetaxel treatment, suggesting increased chemotolerance in these cells. Furthermore, docetaxel sensitivity was fully rescued by the presence of testosterone (CSS + DHT). Together, these data indicate that the EMT caused by androgen deprivation could also be partially reconstituted in a cell-line model.

\( \text{Zeb1 mediates androgen deprivation–induced EMT via a bidirectional, negative feedback loop with AR} \)

Three families of transcription factors have been identified as direct regulators of EMT: (i) Snail and Slug, (ii) Twist, and
(iii) Zeb1 and Zeb2. ZEB1 expression was consistently increased following physical or chemical castration in normal prostate (Fig. 1), the LuCaP35 xenograft model (Fig. 3), human prostate specimens (Fig. 4), and the cultured LNCaP cell line (Fig. 5). This suggests that Zeb1 may be an important mediator of EMT in the prostate following androgen deprivation. To interrogate the mechanism by which androgen regulates Zeb1, we examined various markers of EMT across 4 different prostate cancer cell lines that are either sensitive (LNCaP) or resistant (CWR22Rv1, PC3, and DU145) to chemical castration (29). In the castration-resistant cell lines, the expression of at least one mesenchymal marker (N-cadherin, vimentin, Zeb1, or Twist1) was detected (Fig. 6A), supporting the association between castration resistance and EMT. Strikingly, AR and Zeb1 expression was mutually exclusive of each other, indicating a possible negative feedback between AR and Zeb1.

To test whether a negative feedback loop exists between AR and Zeb1, we examined the effect of AR or ZEB1 knockdown on the reciprocal gene. To this end, we carried out AR RNA interference in AR-expressing LNCaP cells and ZEB1 RNA interference in Zeb1-expressing CWR22Rv1 cells. The results showed that AR knockdown reduced Zeb1 expression, while ZEB1 knockdown increased AR expression, confirming the feedback loop between AR and Zeb1.

Figure 3. Androgen deprivation induces an EMT in LuCaP35 xenografts. A, relative mRNA levels of E-CADHERIN, N-CADHERIN, VIMENTIN, ZEB1, ZEB2, and TWIST1 in the control and castrated LuCaP35 tumors, based on the microarray described in Fig. 2. B, qRT-PCR validation of the microarray data examining the same genes as in A. GUSB was used as the endogenous normalization control. C, E-cadherin, N-cadherin, and vimentin protein levels of 3 matched LuCaP35 xenografts with or without 5-week castration.
in Zeb1-expressing PC3 cells (Fig. 6B–E). As shown in Fig. 6B and D, we were able to achieve efficient knockdown of both AR and Zeb1 protein; however, neither AR RNA interference nor ZEB1 RNA interference alone was sufficient to cause significant changes in ZEB1 or AR mRNA levels, respectively (Fig. 6C and E). We hypothesized that differentiation status may be critical in regulating AR and ZEB1 levels and that the addition of chromatin remodeling agents (e.g., the class I and II histone deacetylase inhibitor TSA and the DNA methylation inhibitor 5-Aza) could facilitate the modulation of both genes. The combined treatment of TSA and 5-Aza effectively increased ZEB1 mRNA levels in LNCaP cells (Fig. 6C), consistent with the predicted existence of CpG methylation sites in the ZEB1 promoter (ENCODE database; ref. 30). Importantly, siRNA-mediated knockdown of AR further enhanced ZEB1 mRNA levels in the presence of TSA and 5-Aza treatments, suggesting that AR negatively regulates ZEB1 expression (Fig. 6C). Similarly, in PC3 cells, the knockdown of ZEB1 by 2 independent shRNAs in the presence of 5-Aza and TSA induced a dramatic increase in AR mRNA levels (Fig. 6E). This effect correlated well with the effectiveness of ZEB1 knockdown and suggests that Zeb1 similarly represses AR expression. We also examined whether overexpression of AR or Zeb1 could inhibit the expression of the reciprocal gene. As shown in Fig. 6F, we carried out transient transfection of AR in PC3 cells and observed a significant decrease in Zeb1 protein expression. In addition, due to the technical challenges associated with expressing recombinant Zeb1 (molecular weight, 200 kDa), we took a different approach and achieved increased ZEB1 expression by treating the LNCaP cells with 5-Aza and TSA (Fig. 6C), and observed dramatically decreased expression of AR (Fig. 6G). Collectively, these data strongly suggest a bidirectional negative feedback loop between AR and Zeb1 (Fig. 6H).

Discussion

In this study, we have examined the effect of androgen deprivation on a wide variety of both normal prostate and prostate cancer model systems. Together, these data have revealed for the first time that androgen deprivation induces an EMT and have uncovered a potentially important yet overlooked consequence of the standard of care treatment for prostate cancer. Moreover, we have uncovered a bidirectional, negative feedback loop between AR and Zeb1 that is important for mediating EMT following androgen deprivation.

The impact of androgen deprivation on E-cadherin, N-cadherin, or vimentin individually has been suggested by several studies both in vitro and in vivo. Consistent with our observations, Tanaka and colleagues recently showed an association between increased N-cadherin and androgen deprivation as well as metastasis in both LNCaP xenografts and human clinical samples (31). In addition, LAPC9 xenografts that had been passaged in castrated mice showed increased N-cadherin expression (32). Furthermore, an LNCaP clone that had been selected in CSS had low E-cadherin expression (33).
Interestingly, this same clone was shown in an independent study to express high vimentin levels as well (34). Finally, consistent with our observations that \textit{ZEB1} expression increases under castrated conditions, Xu and colleagues observed an 80% reduction in \textit{miR-200b} (inhibitor of Zeb1) levels in chemically castrated LNCaP cells via RNA sequencing (35). In contrast to these studies, one study showed that androgen supplementation rather than deprivation promoted EMT in both LNCaP and the AR protein–negative PC3 cells (36). This discrepancy is perplexing, and we speculate that one possible factor could be differences in the culture history of LNCaP cell lines. Indeed, according to the same study, the effect of androgen on EMT was most significant in LNCaP TGF\textbetaRII cells, an LNCaP-derived, TGF-\textbeta –responsive clone that stably expresses recombinant TGF\textbetaRII (36).

We have uncovered a novel and bidirectional negative feedback loop between AR and Zeb1. Interestingly, the androgen—AR—Zeb1 axis seems to be ubiquitous and not restricted to the adult male prostate. Androgen injection into pregnant female mice inhibited Zeb1 expression in the urogenital system of female embryos (37). In addition, estrogen supplementation, which was previously used clinically to block androgen function, has been shown to stimulate Zeb1 expression in the uterus (38) as well as in human foreskin cells (39). These studies support the inhibitory effect of AR (or AR signaling) on Zeb1. Reciprocally, we searched the ENCODE database and identified 3 Zeb1-binding sites in \textit{AR} genomic sequences (30), suggesting that Zeb1 could potentially function as a transcriptional suppressor of AR. Taken together, these studies strongly implicate a conserved link between AR and Zeb1, although the detailed mechanism(s) by which AR and Zeb1 inhibit each other is yet to be determined.

Clinically, our study could have implications for the design of second-line therapy for prostate cancer. EMT has been identified as a common mechanism underlying therapeutic resistance and has been linked to poor prognosis in many types of cancer, including prostate cancer (40). Here, we show that whereas androgen deprivation can effectively control prostate tumor size initially, it simultaneously promotes EMT, an unintended consequence that may ultimately lead to castration resistance. Hence, a rational alternative approach may be to inhibit EMT in combination with androgen deprivation therapy to prevent disease progression. Indeed, an antagonist antibody targeting N-cadherin was recently shown to effectively delay the establishment of castration resistance (32). Furthermore, blocking EMT at an even later stage when castration resistance is already established could still be effective because \textit{ZEB1} RNA interference could reverse EMT and inhibited the migration/invasion of androgen-independent DU145 cells \textit{in vitro} (Supplementary Fig. S1). Taken together, our

Figure 5. Androgen deprivation increases \textit{ZEB1} and N-cadherin expression in LNCaP cells, associated with chemoresistance. LNCaP cells were cultured in RPMI1640 plus 10% FBS, RPMI1640 plus 10% charcoal stripped serum in the absence (CSS) and presence of supplemented 10 nmol/L DHT (CSS + DHT) for at least 4 weeks. The following aspects of these cells are shown: representative microscopy images (A), cell growth rates (B), N-cadherin and GAPDH Western blots (C), and \textit{ZEB1} mRNA levels, quantified by qRT-PCR \((n = 3, 2\text{-tail grouped } t\text{ test was carried out between CSS and (CSS} + \text{DHT}); ^* , P < 0.05; D), and survival curves of these cells treated with the indicated docetaxel concentrations. The dashed line denotes 50% survival (E).
findings provide a rationale for clinical trials combining EMT blockade and androgen-deprivation therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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