The Retinoic Acid Synthesis Gene *ALDH1a2* Is a Candidate Tumor Suppressor in Prostate Cancer

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Abstract

Prostate cancer is the most common cancer among men in the United States, and aberrant DNA methylation is known to be an early molecular event in its development. Here, we have used expression profiling to identify novel hypermethylated genes whose expression is induced by treatment of prostate cancer cell lines with the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-aza-dC). Of the 271 genes that were induced by 5-aza-dC treatment, 25 also displayed reduced expression in primary prostate tumors compared with normal prostate tissue, and the decreased expression of only one gene, aldehyde dehydrogenase 1 family, member A2 (*ALDH1a2*), was also associated with shorter recurrence-free survival. *ALDH1a2* encodes an enzyme responsible for synthesis of retinoic acid (RA), a compound with prodifferentiation properties. By immunohistochemistry, we observed that ALDH1a2 was expressed in epithelia from normal prostate but not prostate cancer. Using bisulfite sequencing, we determined that the *ALDH1a2* promoter region was significantly hypermethylated in primary prostate tumors compared with normal prostate specimens ($P = 0.01$). Finally, transfection-mediated reexpression of wild-type ALDH1a2 (but not a presumptive catalytically dead mutant) in the prostate cancer cell line DU145 resulted in decreased colony growth ($P < 0.0001$), comparable with treatment with either 5-aza-dC or RA. Taken together, our findings implicate *ALDH1a2* as a candidate tumor suppressor gene in prostate cancer and further support a role of retinoids in the prevention or treatment of prostate cancer. (Cancer Res 2005; 65(18): 8118-24)

Introduction

Prostate cancer is the second leading cause of cancer death in men in the United States (1). One in six men will be diagnosed with prostate cancer in their lifetime and 1 in 33 will die from their disease. Localized prostate cancer can be treated by surgical resection or radiation but recurs in ∼25% of patients (2). Advanced disease is treated by androgen ablation. However, invariably, hormone-refractory prostate cancer recurs within 1 to 2 years (3). At that point, there are few treatment options, although recently taxane-based therapy has shown some promise in prolonging patient survival (4). An improved understanding of the molecular pathogenesis of prostate cancer is urgently needed to identify new targets and strategies for effective prevention or therapy.

Numerous molecular alternations have been implicated in prostate cancer development or progression. The *MYC* oncogene has been reported to be commonly amplified during prostate cancer progression (5). Of known tumor suppressor genes, mutation of *TP53* and loss of *PTEN* have been associated mainly with late-stage prostate cancer (6). Other molecular alternations include downregulation of *CDKN1B* (p27 and Kip1) and the homeobox gene *NKX3-1* (6). Altered expression of the androgen receptor has also been observed in hormone-refractory cancer (7).

Aberrant DNA methylation has also been frequently reported in prostate cancer (8). Hypermethylation of CpG islands within promoter regions of genes silences the expression of tumor suppressors, contributing to cancer (9). In prostate cancer, hypermethylation of glutathione *S*-transferase pi (*GSTP1*) is a frequent and early event, occurring in 90% of tumors and 70% of precancerous, prostatic intraepithelial neoplasia lesions (10, 11). *GSTP1* is a putative "caretaker" gene, metabolizing genotoxic compounds and protecting cells from DNA damage (12). Other genes identified as hypermethylated in prostate cancer include *CDKN2A*, *RASSF1A* [Ras association (RalGDS/AF-6) domain family 1], *CDH1* (E-cadherin), and *APC* (8).

The conventional approach to discovering aberrantly methylated genes has been to assay individual candidates, using methods such as bisulfite modification, where unmethylated (but not methylated) cytosines are converted to uracil followed by PCR amplification/DNA sequencing (13), or methylation-specific PCR (14). More recently, DNA microarray-based approaches have been used to systematically screen for hypermethylated genes in cancer. Two such methods that directly query DNA methylation are methylation-specific oligonucleotide microarray analysis (15), which uses arrayed oligonucleotide probes that distinguish bisulfite-modified PCR-amplified DNA products, and differential methylation hybridization (16), in which CpG island arrays are used to quantify PCR products generated following methylation-sensitive restriction enzyme digestion. While direct, both methods require specialized arrays representing CpG island sequences, and as such are not yet widely used.

An alternative microarray-based approach infers DNA methylation by the increased expression of genes following pharmacologic inhibition of DNMT1, the DNA methyltransferase responsible for maintaining established patterns of DNA methylation. While indirect, and practically restricted to cells in culture, this approach uses expression-profiling arrays in widespread use and has led to the identification of numerous genes silenced by DNA methylation in cancer (17–19).

Here, we have used expression profiling following pharmacologic inhibition of DNMT1 to identify novel hypermethylated genes in prostate cancer. Furthermore, by integrating findings with other

microarray data sets, we have identified the retinoic acid (RA) synthesis gene, aldehyde dehydrogenase 1 family, member A2 (*ALDH1a2*), and provide evidence that it functions as a tumor suppressor gene in prostate cancer.

Materials and Methods

Cell culture. LNCaP, DU145, and PC-3 cell lines were obtained from the American Tissue Type Collection (Manassas, VA), and MDA-PCa-2a was a kind gift of Dr. Nora Navone (M.D. Anderson Cancer Center). Cell lines were passaged in RPMI 1640 supplemented with 10% fetal bovine serum. For microarray experiments, cells were treated with 1 μmol/L 5-Aza-2′-deoxycytidine (5-aza-dC; Sigma, St. Louis, MO), or DMSO vehicle alone, for 4 to 6 days. Total RNA was prepared from cultured cells using the TRIZOL (Invitrogen, Carlsbad, CA) method.

Expression profiling. cDNA microarrays were obtained from the Stanford Functional Genomics Facility and contained 41,497 different human cDNAs, representing 19,599 human genes (UniGene clusters; ref. 20). We did expression profiling exactly as described (21). Briefly, for each cell line, 50 μg total RNA from 5-aza-dC-treated and control-treated cells were labeled with Cy5 and Cy3, respectively and then cohybridized to a cDNA microarray. Following overnight hybridization and washing, arrays were imaged using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Fluorescence ratios were extracted using GenePix Pro software, and the data uploaded into the Stanford Microarray Database (22) for storage, retrieval, and analysis. The complete microarray data sets are available at http://smd.stanford.edu, or from the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo (accession no. GSE2932). Fluorescence ratios were normalized for each array using a global normalization. We defined 5-aza-dC-induced genes as those with minimal expression in untreated samples (Cy3 intensity/background <3) and with substantial induction of expression in 5-aza-dC-treated samples (Cy5 intensity/background >3, and Cy5/Cy3 ratio >3). The microarray data set of primary prostate tumors was previously published (21).

Methylation analysis. Genomic DNA from cultured cells was isolated using the Qiagen Blood and Cell Culture DNA Maxi Kit and from previously reported (21) matching normal and tumor prostate specimens using the TRIZOL method. Bisulfite modification of *Hin*dIII-digested genomic DNAs was done exactly as described (14). For methylation-specific PCR analysis, we PCR amplified a 200-bp region within the CpG island upstream of the ALDH1a2 transcription start site, using primer pairs specific for either methylated or unmethylated bisulfite-modified sequences (Table S1). PCR was done on an Applied Biosystems GeneAmp 9700 instrument, using 50 ng bisulfite-modified DNA template, 1× AmpliTaq PCR buffer (Applied Biosystems, Foster City, CA), 500 μmol/L deoxynucleotide triphosphates (dNTP), 2 mmol/L MgCl$_2$, 200 ng each primer, and 2.5 units AmpliTaq DNA Polymerase (Applied Biosystems) in a 50-μL reaction for 30 cycles (94°C, 30 seconds; 65°C, 45 seconds; 72°C, 1 minute) followed by gel electrophoresis on a 2% TAE agarose gel and visualization using an Alpha Innotech imaging system. For bisulfite sequencing, we first PCR-amplified a 413-bp region within the CpG island of ALDH1a2, using primer pairs specific for bisulfite-modified sequences but not harboring CpGs (Table S1). PCR was done as above for 35 cycles (94°C, 30 seconds; 60°C, 50 seconds; 72°C, 1 minute). PCR products were cloned into pCR2.1 vector (Invitrogen), and four to eight clones per DNA specimen were dideoxy sequenced (Stanford PAN facility) using M13 primer.

Semiquantitative reverse transcription-PCR. For independent quantification of 5-aza-dC-induced ALDH1a2 expression in DU145 cells, we did semiquantitative reverse transcription-PCR (RT-PCR). Briefly, cDNA was synthesized by oligo(dT) priming using SuperScript First-Strand Synthesis System (Invitrogen), then PCR-amplified using 1/25th the RT reaction product for template, 1× AmpliTaq PCR buffer, 200 μmol/L dNTPs, 1.5 mmol/L MgCl$_2$, 10 pmol each gene-specific primer pair [ALDH1a2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control; Table S1], and 2 units AmpliTaq DNA Polymerase in a 50-μL reaction for either 25, 30, or 35 cycles (94°C, 60 seconds; 65°C, 60 seconds; 72°C, 1 minute) followed by gel electrophoresis on a 1.8% TAE agarose gel. The 318-bp ALDH1a2 and 152-bp GAPDH PCR products were quantified by Spot Densitometry (AlphaImager 5.5).

ALDH1a2 expression constructs. The full-length coding sequence of *ALDH1a2* (transcript variant 1) was PCR amplified from normal prostate cDNA using Platinum Pfx DNA Polymerase (Invitrogen) and gene-specific primers CGCGGATCCACATGACTTCCAGCAAGATAG (forward) and GCT-CTAGAGGAGTTCTTCTGGGGGATC (reverse), cloned in-frame into the *Bam*HI and *Xba*I sites of pcDNA6/V5-His (Invitrogen), and confirmed by DNA sequencing. A presumptive catalytically dead mutant ALDH1a2-441K (serine-to-lysine substitution at amino acid position 441), equivalent to the phenotypic-null *nof* allele of the zebrafish orthologue (23), was generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with mutagenic primers GTTATCGAAAGAGCCAATAAC**AAG**GACTTTG-GGACTCGTAGCAGC (forward) and GCTGCTACGAGTCCCAAAGTC**CTT**GT-TATTGGCTCTTTCGATAAC (reverse) and confirmed by DNA sequencing.

Cell transfection and colony assay. DU145 cells (8.5 × 10^5 cells per 6-well plate well) were transfected with 4 μg of empty vector, ALDH1a2, or ALDH1a2-441K expression construct, using LipofectAMINE 2000 reagent (Invitrogen). One day post-transfection, cells were seeded at 2.0 × 10^3 cells per well in 6-well plates, and the following day, 8 μg/mL blasticidin (Invitrogen) were added to culture medium. After 10 days in selection medium, cells were fixed with 10% acetic acid/10% methanol, stained with 0.4% crystal violet and visible colonies counted. Cells were also collected 24 hours post-transfection for Western blot analysis. In separate experiments, DU145 cells were seeded at 2.0 × 10^2 cells per well in 6-well plates and treated with either 1 μmol/L all-*trans*-RA (ATRA, Sigma), 1 μmol/L 5-aza-dC, or DMSO vehicle alone, for 10 days, changing the medium every 4 days. After 10 days, cell morphology was evaluated by phase-contrast microscopy and visible colonies counted.

Western blot analysis. Total protein was extracted from empty vector, ALDH1a2, or ALDH1a2-441K-transfected DU145 cells using 1× radio-immunoprecipitation assay lysis buffer (Sigma) following the manufacturer's instructions. For Western blot, 20-μg protein lysate was electrophoresed and transferred overnight to Optitran nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). After blocking in 5% dried milk for 1 hour, the blot was incubated sequentially with anti-V5 antibody (1:5,000, Invitrogen) and horseradish peroxidase (HRP)–conjugated anti-mouse IgG antibody (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour each. α-Tubulin was evaluated as a protein loading control (1:1,000 anti-α-tubulin, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:10,000 HRP-conjugated anti-mouse IgM, Jackson ImmunoResearch Laboratories). Detection was carried out using the ECL kit (Amersham Biosciences).

Immunohistochemistry. A tissue microarray was constructed comprising 21 primary prostate tumors and matched normal prostate specimens, each represented by four 2-mm cores. A 4-μm section was cut from the tissue microarray block, deparaffinized in Citrisolv (Fisher Scientific, Hampton, NH) and hydrated in a graded series of alcohol solutions. Aldh1a2 polyclonal affinity-purified antibody (23) was used at 1:100 dilution and incubated overnight at 4°C. After washing, a goat anti-rabbit IgG antibody conjugated to biotin (Jackson Immunoresearch Laboratories) was applied followed by an anti-biotin antibody coupled to alkaline phosphatase (Jackson Immunoresearch Laboratories), each for 1 hour. Chromogenic detection was carried out with the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/INT (DAKO, Carpinteria, CA).

Results

Identification of *ALDH1a2* as a 5-aza-dC–induced gene in prostate cancer. To identify candidate genes whose expression was silenced by aberrant DNA methylation in prostate cancer, we treated four different prostate cancer cell lines (LNCaP, DU145, PC-3, and MDA-PCa-2a) with the DNA methyltransferase inhibitor 5-aza-dC. For each of the cell lines, RNA isolated from treated and untreated cells was directly compared by microarray hybridization to cDNA microarrays representing ∼19,600 different genes (Fig. 1*A*). In total, we identified 271 genes (represented by 356 different cDNAs; Table S2) whose expression was low in untreated cells and induced at least 3-fold by 5-aza-dC in at least one cell line (see Materials and Methods).

Because we could not distinguish direct and indirect effects of 5-aza-dC on gene expression, we sought to prioritize the list of candidate hypermethylated genes by cross-referencing our gene list with other microarray data sets (Fig. 1*A*). Of the 271 genes induced by 5-aza-dC, 25 genes (49 cDNAs) were also found among the 862 genes (1,235 cDNAs) we previously identified to display significantly decreased expression in prostate tumors compared with normal prostate tissue (21), as would be expected for genes aberrantly methylated in prostate cancer. Furthermore, only one of the 25 genes was found among the 19 genes (20 cDNAs) we previously identified whose expression was significantly negatively correlated with recurrence-free survival, suggesting clinical relevance. That gene, *ALDH1a2*, was induced 6-fold by 5-aza-dC in DU145 cells; a result we independently validated by semiquantitative RT-PCR (Fig. 1*B*), displayed significantly reduced expression in prostate tumors ($P < 0.0001$; Fig. 1*C*), and its decreased expression was associated with significantly shorter recurrence-free survival time ($P = 0.03$; Fig. 1*D*).

ALDH1a2 is expressed in normal but not cancerous prostate epithelium. That ALDH1a2 was reexpressed in 5-aza-dC-treated DU145 prostate cancer epithelial cells, and that its expression was reduced in primary prostate tumors compared with normal prostate tissue suggested that it was normally expressed in the epithelial compartment of prostate tissue. However, we could not rule out the possibility that its decreased expression in prostate tumors merely reflected a decreased stromal content of tumor specimens. To characterize the expression of ALDH1a2 in prostate tissue, we did immunohistochemistry on normal prostate and prostate tumor specimens in tissue microarray format using an antibody specific for ALDH1a2. Strong staining was apparent in a minority of normal prostate specimens. Nonetheless, our results (Fig. 2) indicate that ALDH1a2 is expressed in normal prostate
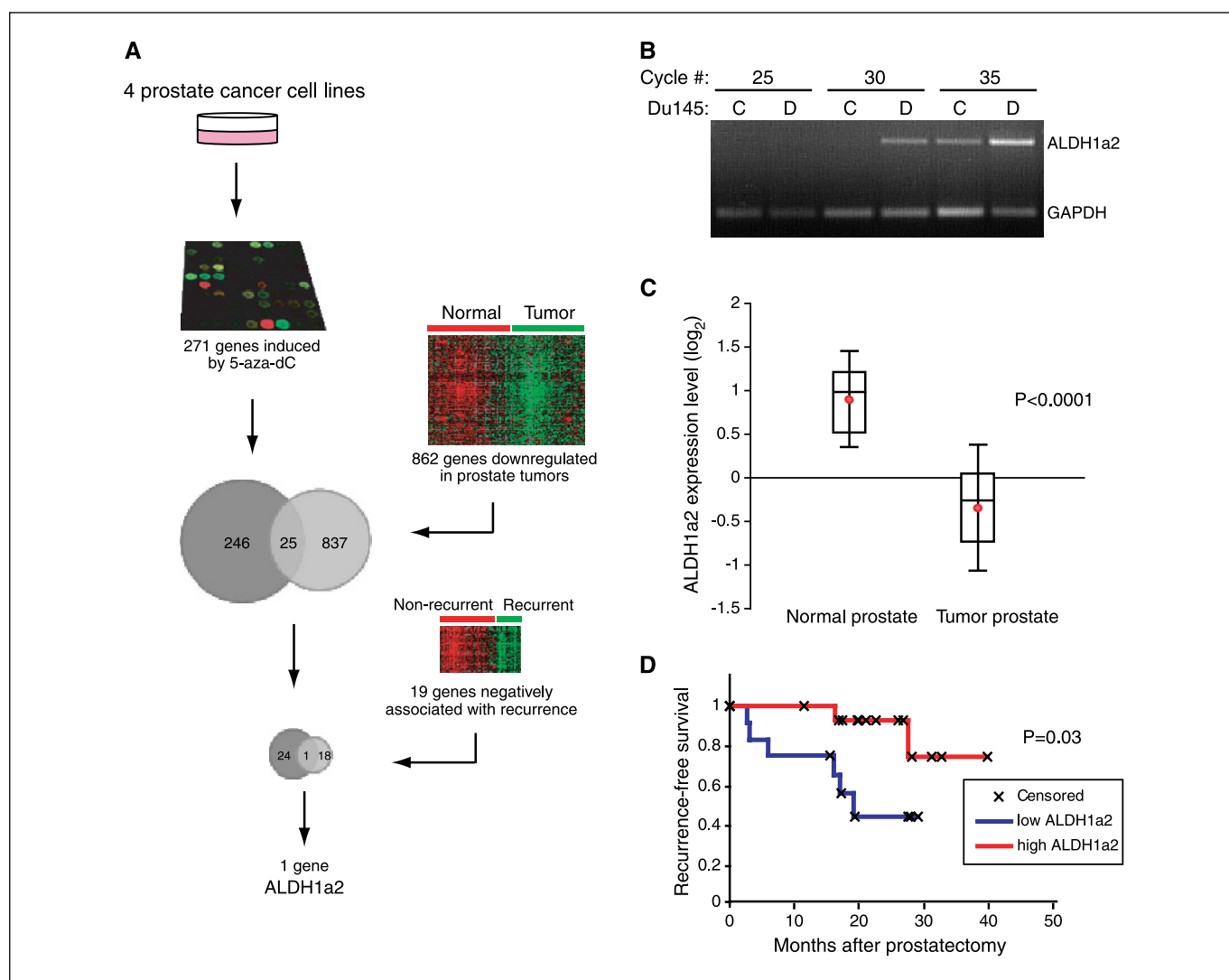

Figure 1. Identification of ALDH1a2. *A,* flow diagram depicting the cross-referencing of 5-aza-dC-induced genes with other microarray data sets to identify *ALDH1a2* as a candidate tumor-suppressing gene silenced by hypermethylation in prostate cancer (see text for details). *B,* semiquantitative RT-PCR validating induction of ALDH1a2 by 5-aza-dC treatment in DU145 cells. C, control treated (DMSO-vehicle alone). D, drug treated (5-aza-dC). At 35 cycles, ALDH1a2 displayed 3.6-fold induction relative to internal GAPDH control. *C,* reduced expression of ALDH1a2, measured by DNA microarray (21), in prostate tumors compared with normal prostates. Mean expression (red dot) and median expression with 25th and 75th quartile values (box plots); *bars,* ±SD. *P* (Student's *t* test). *D,* Kaplan-Meier analysis depicts reduced (i.e., below median) ALDH1a2 expression, measured by DNA microarray, associated with shorter prostate-specific antigen recurrence-free survival (*P* indicated).

epithelial and that its expression is lost in prostate tumor epithelium, consistent with DNA methylation-mediated transcriptional silencing.

ALDH1a2 promoter is hypermethylated in prostate cancer. *ALDH1a2* is associated with a ~1.5-kb CpG island at its 5′ end, overlapping the transcription start site (Fig. 3*A*). To determine whether *ALDH1a2* was indeed hypermethylated in prostate cancer, we directly characterized the methylation status of CpG dinucleotides within the CpG-island both in DU145 cells and in primary prostate tumors. Methylation-specific PCR analysis showed hypermethylation of *ALDH1a2* in DU145 cells, which was partially reversed following 5-aza-dC treatment (Fig. 3*B*). Furthermore, direct sequencing of bisulfite-modified DNA from clinical specimens revealed significantly higher levels of *ALDH1a2* DNA methylation in primary prostate tumors compared with normal prostates ($P = 0.01$; paired Student's t test; Fig. 3*C*). The highest level of DNA methylation was observed for the single prostate cancer lymph node metastasis analyzed (Fig. 3*C*). Consistent with DNA methylation–mediating transcriptional silencing, ALDH1a2

transcript levels in prostate tumors were inversely correlated with DNA methylation levels ($R = 0.77$; Fig. 3*C*).

Reexpression of ALDH1a2 suppresses colony formation. ALDH1a2 carries out the second step in RA synthesis from vitamin A (retinol), catalyzing the conversion of retinal to RA. RA is known to promote cell growth arrest, differentiation, and apoptosis (24), immediately suggesting a plausible pathogenetic connection between loss of ALDH1a2 expression and tumor development.

To explore the effect of RA on prostate cancer cell growth, we cultured DU145 cells (which express retinoid receptors RARα, RARγ, and RXRα; ref. 25) in the presence of either ATRA or 5-aza-dC, the latter which induces expression of *ALDH1a2* (among other genes). When seeded at low density, both ATRA- and 5-aza-dC-treated DU145 cells formed smaller and fewer colonies compared with untreated cells (Fig. 4*A-B*) and displayed flatter, less-refractile morphology (Fig. 4*A*).

To more directly characterize the effect of ALDH1a2 expression on prostate cancer cell growth, we transfected DU145 cells with a plasmid construct directing its expression. Three alternatively spliced transcript variants (sharing the same promoter) have been annotated for the *ALDH1a2* locus; for our experiments, we selected to express transcript variant 1, which we determined by RT-PCR to be the most highly expressed in normal prostate tissue (data not shown). Following transfection, reexpression of ALDH1a2 was confirmed by Western blot (Fig. 4*D*). When seeded at low density, significantly fewer colonies formed in ALDH1a2-transfected cells compared with the empty-vector control ($P < 0.0001$; Fig. 4*E-F*); the reduction in colony formation (1.6-fold) was comparable with ATRA treatment (1.4-fold).

To further clarify the tumor-suppressive function of ALDH1a2, we constructed a presumptive catalytically dead mutant equivalent to the phenotypic null allele *no-fin* (*nof*) of the zebrafish orthologue (23), in which a single nonpolar amino acid (position 441) was replaced by a charged lysine residue within a highly conserved region of the catalytic domain (Fig. 4*C*). In contrast to the wild-type allele, expression of the mutant allele (confirmed by Western blot; Fig. 4*D*) resulted in no significant reduction in colony formation (Fig. 4*E-F*), implicating ALDH1a2 enzymatic activity in the suppression of tumor cell growth.

Discussion

Here we have combined pharmacologic inhibition of DNA methyltransferase with microarray analysis to identify candidate genes whose expression was silenced by aberrant DNA methylation in prostate cancer. By cross-referencing these genes with other microarray data sets, we identified *ALDH1a2* as a gene whose expression was induced by DNA demethylation, reduced in prostate tumors compared with normal prostate, and negatively-correlated with tumor-free survival. We have further shown that ALDH1a2 is expressed in normal but not cancerous epithelium, that the *ALDH1a2* promoter is hypermethylated in prostate tumors, consistent with methylation-mediated gene silencing, and that reexpression of wild-type ALDH1a2 (but not a presumptive catalytically-dead mutant) in prostate cancer inhibits cell growth, consistent with a tumor suppressor function.

Interestingly, we also observed lower-level *ALDH1a2* methylation in normal prostate specimens. As "normal" specimens were derived from morphologically normal regions adjacent to tumors, it is possible that *ALDH1a2* methylation is an early event in prostate cancer development and that our findings reflect a "field effect"
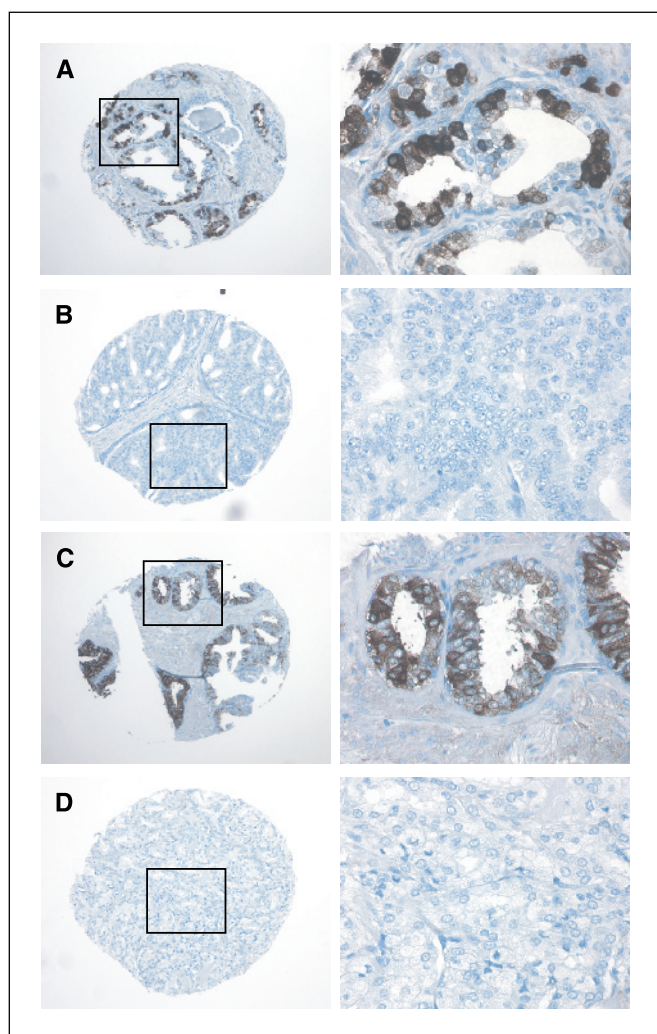

Figure 2. ALDH1a2 expression in prostate tissue. Immunostaining using tissue microarray format shows ALDH1a2 expression in epithelium from normal prostate but not from matching tumor specimens. Images from tissue cores of two different normal prostates (*A* and *C*) and matched prostate tumors from the same individuals (*B* and *D*) at 100× (*left*) and 400× (*right*) original magnification. Chromogenic detection (brown precipitate); counterstaining done with hematoxylin.
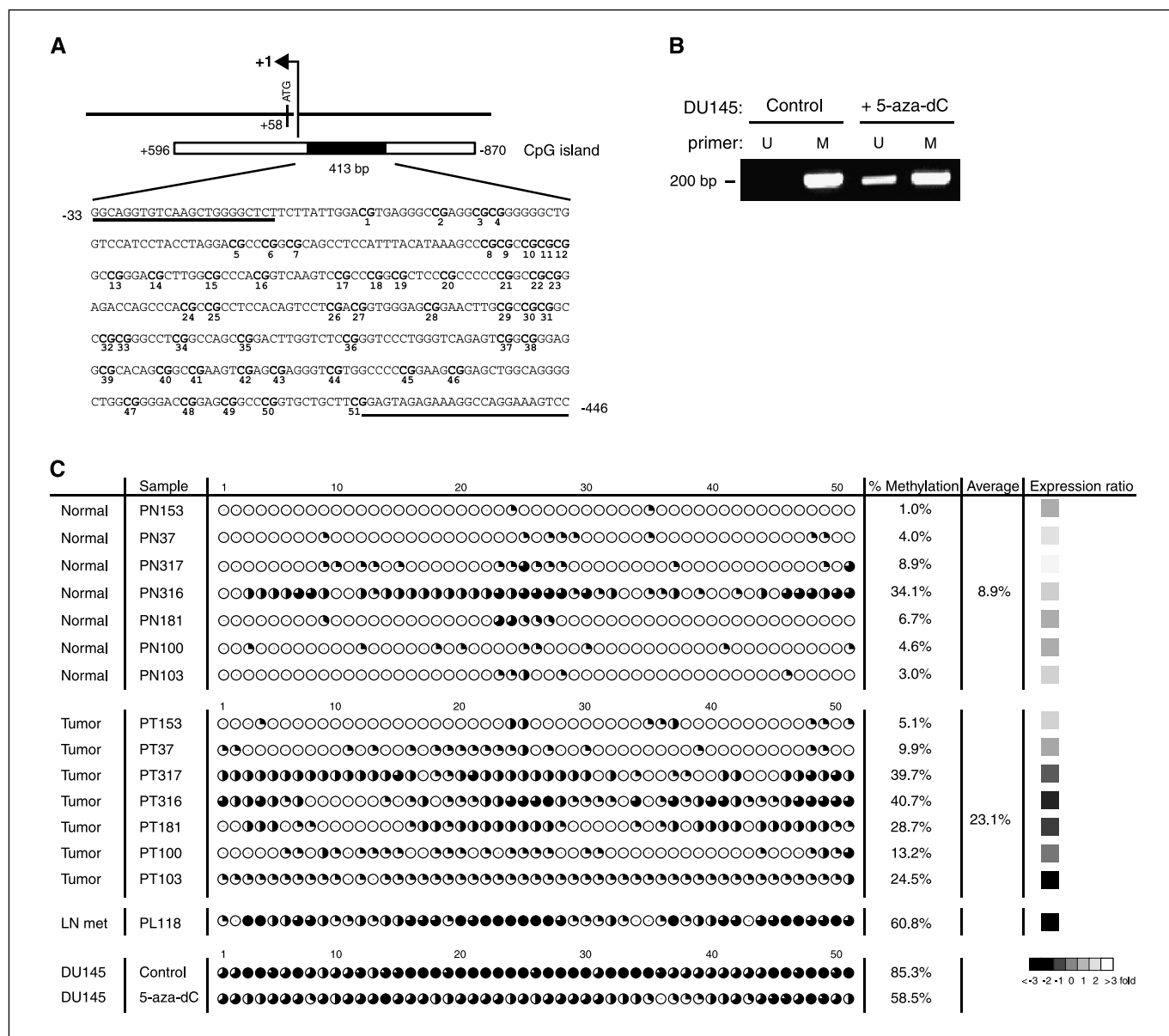
Figure 3. Hypermethylation of ALDH1a2 promoter in prostate cancer. *A,* schematic depiction of *ALDH1a2* promoter-associated CpG island. DNA sequence is numbered from the transcriptional start site. The 51 CpG dinucleotides within a 413-bp central region of the CpG island, assayed by bisulfite sequencing (PCR primers indicated), are numbered. *B,* methylation-specific PCR analysis of *ALDH1a2* promoter methylation in 5-aza-dC- or control-treated DU145 cells. U, unmethylated primer pair; M, methylated primer pair. *C,* bisulfite sequencing analysis of *ALDH1a2* promoter methylation in normal and prostate tumor samples. Filled pies indicate the fraction of sequenced clones exhibiting methylation at each CpG dinucleotide; numbering of CpG sites refers to schematic above. Fraction of CpG sites methylated is significantly higher in prostate tumors compared to normal prostate samples (*P* = 0.01, paired Student's *t* test). Corresponding mean-centered mRNA levels, measured by cDNA microarray, are indicated by gray scale.

(26, 27). The timing of *ALDH1a2* methylation during tumor development and progression remains to be investigated.

ALDH1a2 is a member of the human aldehyde dehydrogenase family that includes 17 genes encoding different substrate specificities, of which *ALDH1a1, ALDH1a2*, and *ALDH6* catalyze the oxidation of retinal to RA (28, 29). Oxidation to RA occurs locally at the tissue sites where RA affects biological processes. ALDH1a2 itself has been studied mainly in the context of normal prenatal development (30, 31).

RA has been shown to promote cell growth arrest, differentiation and apoptosis in a variety of cell types (24). RA affects these cellular processes through binding to RA receptors (RARα,

RARβ, and RARγ) and retinoid X receptors (RXRα, RXRβ, and RXRγ; ref. 32). Activated receptors, most typically heterodimers of RAR and RXR, directly regulate gene transcription through binding to the RA response element in the promoters of target genes. The transcriptional targets that mediate the actions of RA on growth arrest, differentiation, and apoptosis are not fully understood, but evidence suggests downstream effects on activator protein 1 (AP-1), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/Akt pathways, as well as cyclins and CDK inhibitors (33).

Retinoids are required for normal prostate development (34). Several lines of evidence also support a link between altered RA signaling and

the development of prostate cancer. In animal models, diets deficient in vitamin A lead to an increased number of spontaneous and chemically induced tumors (ref. 33 and references therein). In humans, epidemiologic studies have linked low serum vitamin A levels to a higher incidence of subsequent prostate cancer (35, 36).

At the cellular level, several studies have identified molecular defects in RA signaling in prostate cancer. The receptor *RARβ2* is frequently hypermethylated in prostate cancer (37, 38), and RXRβ exhibits reduced expression in prostate cancer compared with normal prostate tissue (39). It has also been reported that breast cancer cells, unlike their nontransformed counterparts, are unable to synthesize RA from retinol (40), due in MCF7 cells to loss of expression of ALDH6 (41). Similarly, prostate tumors, compared with normal prostate tissue, contain comparable levels of retinol
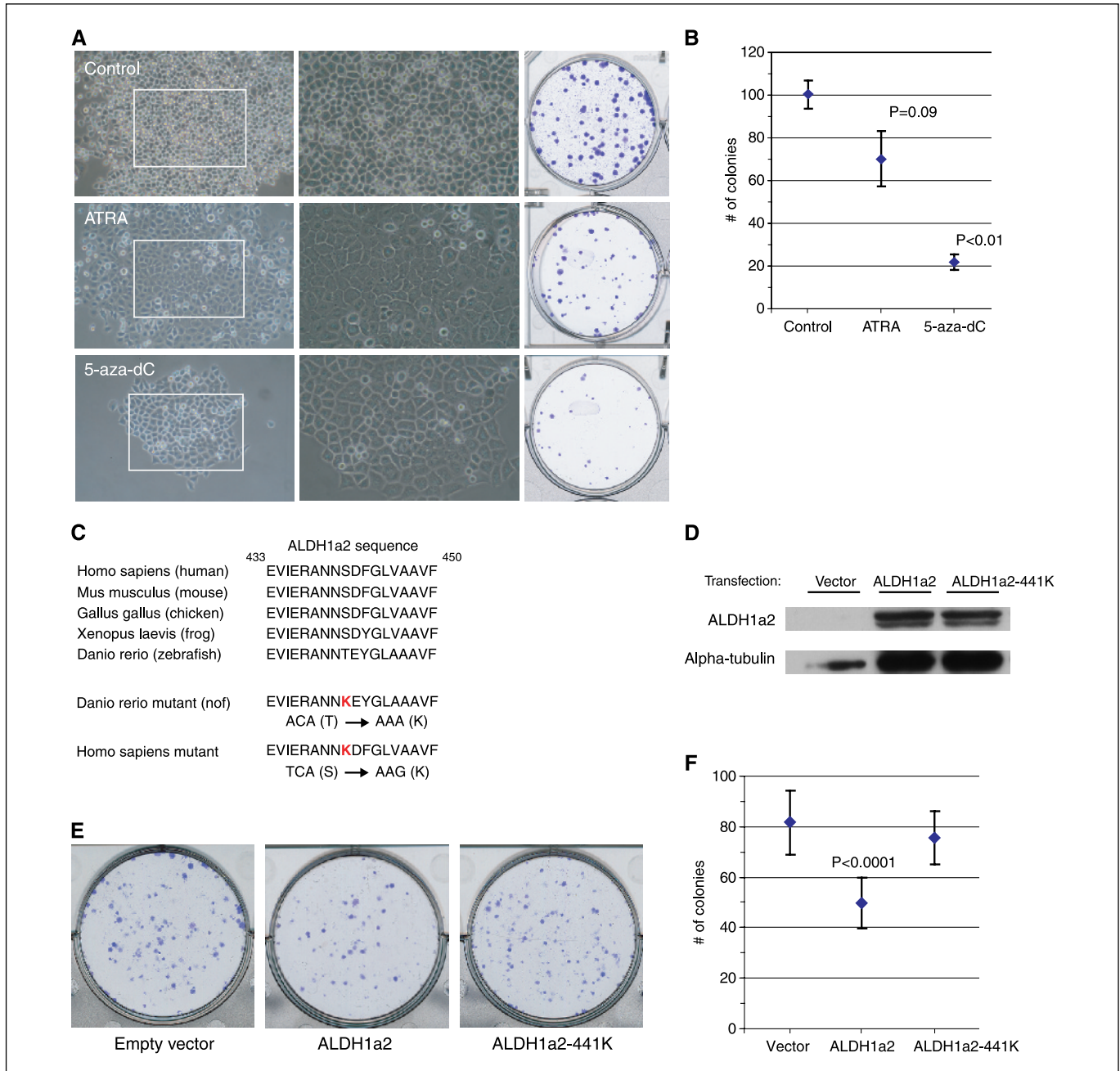

Figure 4. Reexpression of ALDH1a2 suppresses colony formation. *A,* representative colony morphology (10× and 20× original magnification, *left*), and crystal violet stained colony assay (*right*), of DU145 cells treated with DMSO vehicle (*top*), 1 μmol/L ATRA (*middle*) or 1 μmol/L 5-aza-dC (*bottom*). *B,* quantification of colonies/well from three 6-well plate wells, for control, ATRA- and 5-aza-dC-treated DU145 cells. *Points,* mean; *bars,* ±SD. *P* (Student's *t* test) indicated for comparisons of treated versus control. *C,* amino acid sequence homology of tetrapod and zebrafish *ALDH1a2* orthologues shown for a conserved region within the catalytic domain (residues numbered). Also shown are the mutant residues defining the zebrafish *no-fin* (*nof*) null allele, and the ALDH1a2-441T human analogue. *D,* Western blot (anti-V5) analysis of ALDH1a2 (57 kDa) expression in empty-vector-, ALDH1a2-, and ALDH1a2-441T-transfected DU145 cells 24 hours post-transfection; with α-tubulin loading control. *E,* representative colony formation assay in empty-vector-, ALDH1a2-, and ALDH1a2-441T-transfected DU145 cells. *F,* quantification of colonies per well from twelve 6-well plate wells, for empty-vector-, ALDH1a2-, and ALDH1a2-441T-transfected DU145 cells. *Points,* mean; *bars,* ±SD. *P* (Student's *t* test) indicated for significant comparison of ALDH1a2 versus vector control.

but 5- to 8-fold lower levels of RA (42). Our findings suggest the methylation-silenced expression of ALDH1a2 may be responsible for lower RA levels and altered RA signaling, contributing to the development of prostate cancer.

Retinoids have attracted much interest in the prevention or treatment of prostate cancer. Retinoids have been shown to inhibit prostate cancer growth both in cell culture (43–46) and in animal models (43, 47–49). Clinical trials using retinoids in the treatment of prostate cancer have thus far shown only modest efficacy (50–52). Our findings implicate *ALDH1a2* as a tumor suppressor gene in prostate cancer and provide rationale for further investigating retinoids for the prevention or treatment of prostate cancer.

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