ATDC/TRIM29 Drives Invasive Bladder Cancer Formation through miRNA-Mediated and Epigenetic Mechanisms

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Abstract

Bladder cancer is a common and deadly malignancy but its treatment has advanced little due to poor understanding of the factors and pathways that promote disease. ATDC/TRIM29 is a highly expressed gene in several lethal tumor types, including bladder tumors, but its role as a pathogenic driver has not been established. Here we show that overexpression of ATDC in vivo is sufficient to drive both noninvasive and invasive bladder carcinoma development in transgenic mice. ATDC-driven bladder tumors were indistinguishable from human bladder cancers, which displayed similar gene expression signatures.

Introduction

Bladder cancer is the fifth most common malignancy and the second most common cause of death among genitourinary tumors in the United States (1). Risk factors for bladder cancer development include smoking, male sex, and inflammation (2). Ninety percent of bladder cancers are urothelial carcinomas, which are divided into two types: noninvasive and invasive tumors. Noninvasive bladder cancer accounts for 80% of bladder tumors, is low-grade, multifocal, and frequently recurs after resection (3). Only 15% of these tumors will progress to lethal, muscle-invasive bladder cancers, and identification of those at risk for progression has been challenging. Muscle-invasive high-grade bladder cancer accounts for 20% of all bladder tumors and approximately 50% of patients develop lethal metastases despite aggressive therapy (4). Therefore, understanding the pathways that govern tumor behavior is essential to develop better treatment strategies.

Clinically, ATDC was highly expressed in bladder tumors in a manner associated with invasive growth behaviors. Mechanistically, ATDC exerted its oncogenic effects by suppressing miR-29 and subsequent upregulation of DNMT3A, leading to DNA methylation and silencing of the tumor suppressor PTEN. Taken together, our findings established a role for ATDC as a robust pathogenic driver of bladder cancer development, identified downstream effector pathways, and implicated ATDC as a candidate biomarker and therapeutic target. Cancer Res; 75(23): 5155–66. ©2015 AACR.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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uroplakin-expressing urothelial cells cooperates with Hras mutation or Pten inactivation to drive noninvasive bladder cancer in mice (13, 14). Inactivation of p53, Rb, or Pten individually in tg mice results in urothelial hyperplasia, but is insufficient to induce invasive bladder cancer (15, 16). p53−/− and Rb−/− double knockout mice develop urothelial hyperplasia, but only develop muscle-invasive bladder cancers when treated with the carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine (BNB; ref.15). In contrast, combined Pten and p53 deficiency or conditional inactivation of Notch signaling alone promoted formation of muscle-invasive bladder cancers in mice without addition of BNB (16, 17).

The ataxia-telangiectasia group D complementing (ATDC) gene (also known as TRIM29) belongs to the tripartite motif protein family. ATDC has been reported to be expressed in bladder cancers (18), but its functions are unclear. ATDC promotes pancreatic cancer cell proliferation in vitro and tumor growth and metastasis in vivo by upregulation of β-catenin signaling (19) and has also been shown to bind p53 and antagonize p53-mediated functions (20). To determine the function of ATDC in tumor formation, we constitutively overexpressed ATDC in a genetically engineered mouse model. ATDC overexpression induced nonmuscle-invasive and muscle-invasive urothelial carcinomas that were phenotypically indistinguishable from their human counterparts. Furthermore, ATDC expression was common in human bladder cancer and molecular alterations present in human bladder cancers were similar to those present in the invasive bladder cancers that developed in ATDC transgenic mice. Here we characterize the role of ATDC in bladder cancer and identify molecular mechanisms by which ATDC promotes bladder tumorigenesis.

Materials and Methods

Generation of transgenic mice

The CAG-ATDC transgene (construction described in Supplementary Materials and Methods) was linearized and microinjected into the pronuclei of fertilized eggs of FVB/NJ mice (Jackson Laboratory) by the University of Michigan Transgenic Animal Model Core. CAG-ATDC founder lines were identified by PCR analysis using FLAG-ATDC specific primers (Fig. 1A; Supplementary Materials and Methods). Three founder lines were identified carrying 6 (line A), 4 (line B), and 2 (line C) copies of the transgene. The mice were inbred with FVB/NJ mice and studies were performed using the second to fourth generations. Animal experiments were approved by the University of Michigan Animal Care and Use Committee and were performed in accordance with established guidelines.

Quantitative RT-PCR

Quantitative RT-PCR was conducted similar to previously described methods (19). Detailed information about probes and methods available in Supplemental Materials and Methods.

Immunohistochemical staining

Bladder cancer or normal bladder samples were fixed, stained and examined as previously described (19). All human samples were obtained following approval by the Institutional Review Board of the University of Michigan Medical Center. A bladder cancer tissue microarray (TMA) was constructed by the University of Michigan Medical Center’s Tissue Procurement Facility and a bladder cancer TMA was provided by Dr. Lotan (University of Texas Southwestern Medical Center, Dallas, TX). The primary antibodies for immunohistochemical staining were ATDC (Sigma), Pten (Cell Signaling Technology), DNMT3A (Abcam). Quantitation and scoring of immunohistochemistry was done as previously described (21, 22).

Cell culture and transfection

Cell lines (UC9, UC14, UC10, T24, SV-HUC1) and primary cells (w81, pa19) were purchased from ATCC or derived as previously described (23, 24). All cell lines were analyzed and authenticated by targeted genomic and RNA sequencing. Cells were grown in DMEM or F12K media with 10% serum (Gibco) under standard culture conditions. ATDC and Pten lentiviral expression constructs were created by cloning relevant restriction-digested (NheI/XbaI) cDNA into a pLentiLoxRSV vector (University of Michigan Vector Core) using restriction digestion and ligation. Viral particles were packaged by University of Michigan Vector Core. ATDC, PTEN, and DNMT3A shRNA and control shRNA constructs were purchased from Sigma Aldrich. Transfection and generation of stable cell lines was done as previously described (19).

Immunoblot analysis

Immunoblot analysis was done as previously described (19) using antibodies directed against ATDC (Santa Cruz Biotechnology), PTEN and RELA (Cell Signaling Technology), DNMT3A and DNMT3B (Santa Cruz Biotechnology), at a 1:1,000 dilution. β-Actin antibody (Sigma) served as a loading control.

Proliferation assays

Cell proliferation was measured using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS Kit (Promega) as described (19).

 Invasion assays

In vitro invasion assay were done using Cell Invasion Assay Kit (Chemicon/Milipore International) and performed as previously described (19).

Orthotopic bladder xenograft assays

Luciferase was transduced into UC9 or UC14 cell lines stably transfected with ATDC-targeting shRNA or control shRNA as noted above. The protocol for generation of orthotopic bladder xenograft tumors has been previously described (25). A 24 g catheter was inserted into the bladders of 6-week-old female NOD/SCID mice and 2.5% trypsin was instilled and then washed. A total of 1 x 10^5 cells were then instilled into each bladder. Tumor formation and growth was followed using bioluminescence as previously described (19) using an IVIS 200 imaging system (Xenogen Biosciences). Five weeks after cancer cell injection, mice were euthanized with carbon dioxide inhalation and extent of primary tumor growth assessed.

Methylation-specific PCR

Specific methods used for methylation specific PCR, quantitative methylation–specific PCR, primers, and conditions are available in Supplementary Materials and Methods.
Affymetrix gene expression profiling analysis

Gene expression profiles of normal control bladders from wt littermates and invasive bladder carcinoma samples from CAG-ATDC mice were obtained using the Affymetrix 3000 7G Gene Chip platform, as we have described previously and as outlined in Supplementary Materials and Methods (26).

Oncomine molecular concepts analysis

A CAG-ATDC gene expression signature was generated by taking all genes upregulated 2-fold or greater in our invasive CAG-ATDC bladder tumors compared with littermate control bladder tissue and then identifying the subset of these genes upregulated in all three tumors and with human homologs. This produced a 77 gene-expression signature unique to our CAG-ATDC bladder cancer tumors (Supplementary Table S3). Automated enrichment analysis using Oncomine, where concepts are automatically evaluated for enrichment by disproportional overlap with over 10,000 molecular concepts, including other gene expression signatures (27, 28) was performed using our 77 gene CAG-ATDC bladder tumor signature (1 in CAG-ATDC BlCa tumors- uploaded into Oncomine as a custom concept). Enriched concepts were identified by automated analysis in Oncomine and selected interaction nodes and edges were exported into Cytoscape for visualization using force directed layout.
miRNA transfection and detection

miR-29A, B, and C specific primers and TaqMan probes and mirVana qRT-PCR miRNA Detection Kits were obtained from Life Technologies. miR-29A, B, and C mimics, antisense oligos, and scrambled control miR were also obtained from Life Technologies. miR-29A, B, and C mimics, antisense oligos, and mirVana qRT-PCR miRNA Detection Kits were obtained from Life Technologies. mimics and antisense miRNAs were transfected in six-well plates by using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer protocol. miRNA from UC9 or UC14 cells with or without ATDC shRNA expression were isolated and purified using mirVana miRNA Isolation Kit (Life Technologies). qRT-PCR analysis for miRNAs was performed in triplicate with TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II (Applied Biosystems) according to the instructions of the manufacturer. Real-time RT-PCR was performed on ViiA 7 system (Applied Biosystems) using the following conditions: after 10 minutes at 95 °C, 40 cycles were performed at 95 °C for 15 seconds, and 60 °C for 1 minute. The data were normalized to small nucleolar RNA U6 (housekeeping control) using the C_{\text{t}} method.

siRNA transfection

Nontargeting control sequences (D-001810-10-05) or SMARTpool MYC (L-003282-02) or RELA (L-003533-00) targeting siRNA were purchased from Dharmacon. Transfections were carried out using Lipofectamine RNAiMAX (Life Technologies) as above.

Statistical analysis

Proliferation and invasion data are represented as mean ± SEM from at least three independent experiments. Significance of differences between groups was evaluated by Student t test or ANOVA. P < 0.05 was considered significant. Pairwise association of expression between ATDC and DNMT3A and PTEN was tested using the Jonckheere-Terpstra test to compare immunohistochemical intensity scores (1–4). The association between bladder cancer stage and ATDC expression classified as medium or high compared with absent or low was also tested with the Jonckheere–Terpstra test.

Results

ATDC induces bladder tumor formation in a transgene dosage-dependent manner

To determine the contribution of ATDC to bladder cancer formation, we generated mice globally overexpressing ATDC from a constitutive promoter (CAG-ATDC; Fig. 1A). Using quantitative PCR and immunoblotting we identified three founder transgenic lines (A, B, and C) harboring 6, 4, and 2 copies of the CAG-ATDC transgene, respectively (Fig. 1A–C). We then examined ATDC transgene expression in founder lines by qRT-PCR, Western blot analysis, and IHC and found transgene dosage-dependent overexpression in all tissue types (Fig. 1B–G and Supplementary Fig. S1).

CAG-ATDC transgenic mice were observed closely for phenotypic abnormalities. Starting at 8 months of age, the predominant phenotype was the development of bladder outlet obstruction secondary to tumor formation (Fig. 1H). While control mice lacking the ATDC transgene displayed normal urothelial histology (Fig. 11 and J), analysis of bladders from line A (6 tg copies) demonstrated a spectrum of urothelial neoplasia ranging from dysplasia to muscle-invasive urothelial carcinoma (Fig. 1K–M and Supplementary Fig. S2; Supplementary Table S1). Fourteen percent of line A mice (6 tg copies; 3/21) developed only low-grade noninvasive tumors and 29% (6/21) developed either invasive carcinoma or CIS. Nine percent (2/22) of line B mice (4 tg copies) developed noninvasive and 5% (1/22) developed invasive carcinoma (Table 1). Mice from line C (2 tg copies) developed hyperplasia (2/19), but none developed tumors over an 18-month observation period. All invasive tumors demonstrated muscle invasion. Histologically, tumors were indistinguishable from human urothelial carcinomas. Superficial papillary carcinomas were multifocal with fibrovascular stalks projecting into the bladder lumen similar to human tumors (Supplementary Fig. S2D). Muscle-invasive tumors consisted of malignant cells infiltrating into the muscularis (Fig. 1M). Tumors isolated from CAG-ATDC mice demonstrated elevated ATDC compared with littermate control bladders (Supplementary Fig. S1), and ATDC expression was similar to that seen in human bladder tumors (Supplementary Fig. S3). These results demonstrate that ATDC overexpression induces formation of noninvasive and muscle-invasive bladder cancer in a dosage-dependent manner.

ATDC is highly expressed in human bladder cancer and correlates with invasive disease

To confirm that ATDC is expressed in human bladder cancer, we analyzed mRNA expression in two large multicancer studies in the Oncomine database (28, 29). Human bladder cancer had the highest expression of ATDC compared with any other tumor type (P < 10^{-6}; Fig. 2A, lane 1). Furthermore, both noninvasive bladder cancers and muscle-invasive specimens had significantly higher ATDC expression than normal bladder tissues (Fig. 2B and C; ref. 18, 30).

To confirm ATDC protein levels were upregulated in human bladder cancer, we analyzed TMs of human primary bladder cancers (297 samples). The staining intensity of each tissue core was graded 0 (negative), 1 (low), 2 (moderate), or 3 (strong), and according to percentage of tissue positive (0%–100%) with a composite score (grade × percentage) generated (0–300) for each sample (representative images in Fig. 2D–G). ATDC was significantly overexpressed in both human noninvasive and muscle-invasive bladder cancers compared with normal human bladder specimens (Fig. 2D–G; Supplementary Table S2). ATDC was expressed predominately in the cytoplasm, although it was

<table>
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<th>Genotype</th>
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<th>ATDC, line B</th>
<th>ATDC, line C</th>
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<td>Transgene copy number</td>
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<td>2</td>
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<td>Noninvasive tumor</td>
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<td>2/2 (9%)</td>
<td>0/19 (0%)</td>
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<tr>
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<td>2/2 (10%)</td>
<td>0/2 (0%)</td>
<td>0/19 (0%)</td>
</tr>
<tr>
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<td>4/21 (9%)</td>
<td>1/22 (5%)</td>
<td>0/19 (0%)</td>
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also found in the nucleus in some samples (Supplementary Fig. S4; ref. 31). Among 283 human bladder cancer samples, 53% of muscle-invasive tumors and 50% of CIS specimens stained positively for ATDC. In contrast, 22% of the noninvasive bladder tumors demonstrated elevated ATDC staining.

To determine whether ATDC expression correlated with tumor behavior in patients, we next correlated ATDC expression levels with tumor pathologic stage. We found that increased ATDC expression correlated with invasive disease. Noninvasive tumors had lower scores \((n = 27, \text{mean} = 65)\) of ATDC expression as compared with invasive tumors \((n = 153, \text{mean} = 121, P < 0.05; \text{Fig. 2H})\), with no further increase in ATDC expression evident in metastatic lesions. These results are consistent with increasing ATDC expression in invasive bladder cancer.

**CAG-ATDC bladder cancers have a similar gene expression signature to human cancers**

To characterize the molecular signature of ATDC-induced tumors, we carried out Affymetrix gene expression profiling of muscle-invasive bladder cancers and matching normal bladder samples from control littermates \((n = 3 \text{ per group, Fig. 3A})\).
Selected genes that were the most up or downregulated in ATDC tumors are listed next to the heatmap and include VEGFR, connective tissue growth factor, insulin-like growth factor, and genes involved in the WNT1-inducible pathway (Fig. 3A). To establish a gene expression signature unique to CAG-ATDC bladder cancer tumors, we identified all of the genes upregulated >2-fold compared with littermate control bladders (Supplementary Table S3). Of these 77 genes, many, including CYR61, IGFBP2, LOXL2, and THBS1, have been previously reported to enhance migration of human bladder cancer cell lines and drive invasion (32). To determine whether there was an overlap between transcriptional programs in CAG-ATDC and human bladder cancers, we uploaded this 77 gene signature into Oncomine (28) as a custom concept. In Oncomine, this concept (↑ in CAG-ATDC BlCa tumors) was automatically evaluated for enrichment by disproportional overlap with over 10,000 molecular concepts, including other gene expression signatures and biologically annotated gene sets (27). The genes overexpressed in CAG-ATDC bladder tumors significantly overlapped with multiple expression profiling signatures of genes overexpressed in human bladder cancers and human infiltrating versus superficial human urothelial bladder cancers (Fig. 3B and C; Supplementary Tables S4 and S5). These data demonstrate that the CAG-ATDC tumors possess a morphologic and molecular phenotype typically seen in human muscle-invasive bladder cancer and that ATDC drives an invasive gene expression program.
ATDC is highly expressed in human bladder cancer cell lines and drives proliferation and invasion

To explore the functional role of ATDC in bladder cancer tumorigenesis, we next interrogated ATDC levels in human urothelial cell lines. ATDC mRNA and protein expression levels were elevated in the T24, UC9, and UC14 human bladder cancer cell lines, but not in 881 or PAM19 primary urothelial cells (Fig. 4A and B) or the SV-HUC1 immortalized human urothelial cell line (Fig. 5E). To assess the contribution of ATDC to the malignant phenotype of UC9 and UC14 cells, we transfected control or two ATDC shRNAs targeting distinct regions of the gene into these cell lines (Fig. 4C and D). We found that ATDC knockdown significantly inhibited proliferation in both UC14 and UC9 cell lines (Fig. 4E and F, n = 4; *, P < 0.01 for each cell line).

As gene expression profiling indicated that ATDC tg tumors harbored an invasive gene signature, we next assessed the role of ATDC in invasion using a modified Boyden chamber invasion assay. Knockdown of ATDC in UC14 and UC9 cells inhibited invasion compared with cells transfected with control shRNA (Fig. 4G and H, n = 4; *, P < 0.01). To confirm that ATDC promoted invasion, we overexpressed ATDC in the UC10 bladder cancer cell line, which has low endogenous ATDC expression (Supplementary Fig. S5A). ATDC overexpression in UC10 resulted in a 37% increase in invasion compared with control (Supplementary Fig. S5B). Therefore, ATDC promotes both proliferation and invasion of human bladder cancer cell lines.

To determine whether ATDC expression modulates bladder cancer growth in vivo, we established UC9 orthotopic xenograft tumors stably transduced with either control or ATDC shRNA vectors. All cells also stably expressed luciferase, allowing tumor monitoring using bioluminescence. ATDC silencing significantly decreased tumor growth in vivo (Fig. 4J–L, n = 6 animals per group; *, P < 0.05). These results demonstrate that ATDC is a determinant of growth and tumorigenicity in vitro and in vivo.
ATDC induces upregulation of DNMT3A and epigenetic silencing of PTEN

Loss of PTEN expression contributes to the development of both nonmuscle-invasive and muscle-invasive bladder cancers in tg mice (13, 16, 33). As ATDC overexpression induced both of these bladder tumor types, we assessed PTEN expression in both CAG-ATDC bladder cancers and bladder tissue from wild-type (WT) littermates by IHC and Western blotting. PTEN protein expression was lost in both noninvasive and muscle-invasive CAG-ATDC bladder cancers, but maintained in normal bladder tissue, indicating PTEN loss occurred during ATDC-induced bladder tumor formation (Fig. 5A and B; Supplementary Fig. S6).

Tumor suppressor gene expression can be lost by mechanisms including mutation, deletion, loss of transcription factors, histone modification, ncRNA interference, or epigenetic silencing by DNA methylation. PTEN promoter DNA methylation is a common mechanism by which expression is lost in many tumor types (34). We next hypothesized that ATDC overexpression might down-regulate PTEN expression in bladder cancers via promoter DNA methylation. To determine the methylation status of the Pten promoter in CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors, we extracted genomic DNA from 5 CAG-ATDC mouse tumors
DNA and performed methylation-specific PCR (MSP). Genomic DNA from CAG-ATDC–invasive bladder cancers, but not from normal littermate bladder tissue, revealed hypermethylation at the promoter region of PTEN (Fig. 5C). To determine whether ATDC expression correlated with methylation of the PTEN promoter in human cancer, we assessed methylation status of the PTEN promoter in invasive bladder cancer cell lines (UC14 and UC9) with and without ATDC knockdown and the immortalized normal urothelial cell line, SVHUC1 with and without ATDC overexpression using quantitative methylation-specific PCR. Knockdown of ATDC expression by shRNA reduced PTEN promoter methylation in both UC14 and UC9 and overexpression of ATDC in SVHUC1 increased PTEN promoter methylation (Supplementary Fig. S7). In ovarian cancer, reduction in DNA methylation can induce recovery of PTEN expression (35). To determine whether ATDC knockdown correlated with recovery of PTEN expression, we transfected ATDC targeting shRNA into the UC9 and UC14 cell lines with high ATDC levels. ATDC knockdown resulted in increased PTEN expression in these human bladder cancer cell lines (Fig. 5D and Supplementary Fig. S8). To confirm that this effect was attributable to ATDC, we next overexpressed ATDC in immortalized normal urothelial cells with low endogenous ATDC expression (SV-HUC1) and measured PTEN levels by Western blotting. ATDC overexpression decreased PTEN expression in SV-HUC1 cells (Fig. 5E).

DNA methyltransferases 3A and 3B (DNMT3A or DNMT3B) are responsible for most de novo DNA methylation (36). As DNMT3A has been previously implicated in PTEN silencing, we next examined the impact of ATDC expression on DNMT3A levels. ATDC silencing in UC9 cells decreased levels of DNMT3A and increased PTEN expression by Western blotting (Fig. 5D). Similarly, overexpression of ATDC in immortalized normal urothelial SV-HUC1 cells resulted in upregulation of DNMT3A and reduction of PTEN (Fig. 5E). In contrast, expression of DNMT1 and DNMT3B was not regulated by altered ATDC expression and did not correlate with PTEN loss (Fig. 5E and Supplementary Fig. S8). These results imply that ATDC upregulates DNMT3A, which induces methylation and silencing of PTEN expression.

To determine whether the effects of ATDC on PTEN expression, invasion, and proliferation are mediated by DNMT3A, we knocked down DNMT3A expression using targeting control shRNA in UC9 cells. DNMT3A knockdown increased PTEN expression (Fig. 5F) and reduced invasion and proliferation (Fig. 5G and H) when compared with control shRNA transduced cells. To determine whether the bladder cancer–invasive phenotype was mediated directly by PTEN loss, we next knocked down PTEN expression and observed a significant increase in invasive capacity when compared with cells transduced with control shRNA (Fig. 5I and J). To confirm that this was attributable to PTEN loss, we reintroduced PTEN expression in these cells using a lentiviral expression vector and were able to suppress the invasive phenotype induced by PTEN knockdown (Fig. 5I and J), suggesting that the dominant effect of ATDC is mediated through loss of PTEN expression. These results imply that ATDC drives bladder tumor invasion and proliferation via upregulation of DNMT3A, which silences PTEN, driving tumor invasion and proliferation.

To determine whether increased ATDC expression correlated with increased DNMT3A and loss of PTEN in patients, we measured expression of all three proteins by IHC in a TMA containing human muscle-invasive bladder cancer specimens (n = 120; each core was assigned a score 1–4 by a blinded pathologist). Using the Jonckheere–Terpstra test, we identified a statistically significant correlation between increased ATDC expression and DNMT3A expression in invasive human bladder cancer specimens (n = 120; P = 0.036; Fig. 5K; Supplementary Table S6). Most specimens also displayed loss of PTEN, but the correlation between DNMT3A and PTEN did not reach statistical significance (P = 0.07), possibly because these advanced human bladder tumors may have lost PTEN expression via other mechanisms.

To confirm that DNMT3A and PTEN expression was modulated by ATDC in our CAG-ATDC bladder tumors, we examined mRNA levels in three CAG-ATDC–invasive tumors and compared with three normal mouse bladders lacking ATDC using qRT-PCR. As anticipated, overexpression of ATDC resulted in loss of PTEN mRNA and upregulation of DNMT3A (P < 0.05, Supplementary Fig. S9A). These data demonstrate that ATDC overexpression in bladder cancer upregulates DNMT3A, silencing PTEN, and driving a malignant phenotype.

ATDC upregulates DNMT3A via MYC-mediated suppression of miR-29

In other tumor types, the miR-29 family (A, B, and C) has been shown to regulate the mRNA levels of DNMT3A by binding to complementary sequences in the 3′ UTR, leading to degradation of the mRNA message (38, 39). Furthermore, miR-29A and B1 are transcriptionally repressed by direct binding of MYC to the miR-29 promoter (40, 41). We have previously reported that ATDC binds to DVL2, resulting in stabilization of β-catenin and activation of the WNT pathway in pancreatic cancer (19). We observed that ATDC upregulated β-catenin and its target gene, MYC, in bladder cancer (Supplementary Fig. S9A and S9B), therefore, we hypothesized that ATDC-mediated upregulation of DNMT3A might be orchestrated by MYC-mediated suppression of miR-29 family members.

To determine whether miR29- A, B, or C regulated DNMT3A expression in urothelial cell lines, we transfected miR-29A, B, and C mimics into UC14 and UC9 cell lines and measured each in UC14 and UC9 cells transfected with control or ATDC-targeting shRNA and found that knockdown of ATDC resulted in increased miR-29A and B, but not miR-29C in both cell lines (Fig. 6D). ATDC knockdown also resulted in DNMT3A mRNA reduction similar to previous Western blotting data (Fig. 6E). To determine whether miR-29A and B mediated DNMT3A suppression in our ATDC knockdown cells, we transfected antisense oligonucleotides into UC9 and UC14 cell lines stably transfected with either control or ATDC-targeting shRNAs. miR-29A and B antisense oligos together, but not individually, rescued DNMT3A expression (Fig. 6F). These results demonstrate that ATDC can regulate DNMT3A expression by suppression of the miR-29 family members 29A and 29B.

To determine whether ATDC regulation of miR-29 was mediated by MYC or NFKB p65 (RELA) as previously reported in other cell types (40), we overexpressed ATDC in SV-HUC1 immortalized urothelial cells and then knocked down MYC or RELA expression using control or targeting siRNA and measured protein and miR-29 expression. ATDC expression increased β-catenin and
MYC expression in urothelial cells (Fig. 6G) and resulted in suppression of miR-29A expression (Fig. 6H). Interestingly, knockdown of MYC, but not RELA, abrogated ATDC-induced suppression of miR-29A (Fig. 6H). This result was also observed in the UC9 and UC14 bladder cancer cell lines (Supplementary Fig. S10). These results demonstrate that ATDC expression upregulated MYC, which repressed transcription of miR-29 and resulted in upregulation of DNMT3A.

Discussion

Although bladder cancer is common and can be deadly in its invasive form, the molecular drivers of bladder tumorigenesis are incompletely understood (42). Here, we find that overexpression of ATDC is sufficient to drive formation of both noninvasive and invasive bladder cancer in vivo. We report that ATDC is upregulated in the majority of human invasive bladder cancers and we elucidate a mechanism whereby ATDC expression drives MYC upregulation, repressing miR-29, allowing upregulation of DNMT3A and subsequent silencing of PTEN, which promotes urothelial tumorigenesis.

While PTEN is important in many tumor types, its contribution to bladder cancer is complex. 

Figure 6. ATDC suppresses miR-29 expression, resulting in increased expression of DNMT3A. A and B, transfection of miR-29A, B, and C into UC9 and UC14 bladder cancer cells lines suppresses DNMT3A protein and mRNA expression (mean ± SE; n = 3; * P < 0.05 vs. WT). C, transfection of miR-29A, B, and C antisense (AS) oligonucleotides into UC9 and UC14 cells results in increased DNMT3A protein expression. D, stable expression of ATDC-targeting shRNAs in UC9 and UC14 but not control shRNA resulted in increased miR-29A and B, but not miR-29C levels as measured by TaqMan qRT-PCR (mean ± SE; n = 3; * P < 0.05 vs. WT). E, knockdown of ATDC results in lower levels of DNMT3A mRNA as measured by RT-PCR (mean ± SE; n = 3; * P < 0.05 vs WT). F, transfection of both miR-29A and B antisense oligonucleotides allows recovery of DNMT3A expression in ATDC knockdown cell lines. NC, negative control AS; CS, control shRNA; AS, antisense miRNA (mean ± SE; n = 3; * P < 0.05 vs. CS). G, ATDC overexpression in SV-HUC1 cells induces increased MYC and β-catenin expression and transfection of siRNA targeting MYC and RELA knocked down protein expression. H, overexpression of ATDC suppressed miR-29A in SV-HUC1 cells and knockdown of MYC, but not RELA, eliminated ATDC suppression of miR-29A (mean ± SE; n = 3; * P < 0.05 vs WT).
Role of ATDC in Bladder Cancer

ATDC overexpression mediates tumorigenesis and invasion, whereas PTEN deletion driven by an adeno-Cre system may have resulted in atakin-5 (K5)-expressing basal cells (43, 44). Thus, PTEN and p53 expressing intermediate cells give rise to noninvasive tumors while muscle-invasive bladder cancer arise exclusively from keratin-5 (K5)-expressing basal cells (43, 44). This suggests that the distinction between CAG-ATDC tumors and other uroplakin-based systems may be related to the urothelial cell subpopulation in which these genes are altered. Recent work suggests that noninvasive and invasive bladder cancers arise from distinct cell populations with the urothelium. The uroplakin expressing intermediate cells give rise to noninvasive tumors while muscle-invasive bladder cancer arise exclusively from keratin-5 (K5)-expressing basal cells (43, 44). This hypothesis is further supported by the CAG-ATDC transgene was expressed in all urothelial cells inducing both noninvasive and muscle-invasive tumors, a unique aspect of this system.

Here we provide data demonstrating ATDC suppression of miR-29 as a novel mechanism by which DNMT3A and DNA methylation and thereby PTEN is regulated in bladder cancer. While miR-29 has been implicated in the regulation DNMT3A and DNA methylation in basal-like breast cancers (45), this is the first report to establish a role in bladder tumor cells. ATDC is highly expressed in multiple tumor types and PTEN loss and miR-29 suppression are increasingly recognized as important molecular events in tumor formation and growth. Therefore, because ATDC is highly expressed in multiple tumor types, including pancreatic, lung, head and neck, and cervical cancers, these findings establish a molecular mechanism of ATDC-mediated tumorigenesis, which is likely important across multiple tumor types.

Approximately 15% of noninvasive tumors progress to muscle-invasive disease but the mechanisms that drive invasive progression in this subset are unknown. Interestingly, we show here that ATDC is a driver in the development of both non-muscle-invasive and muscle-invasive bladder cancer in tg mice, with the distribution of tumors that develop dependent on the dose of the ATDC transgene. Furthermore, we provide data highlighting that ATDC is highly expressed in about 20% of human noninvasive bladder cancers. This raises the intriguing hypothesis that ATDC expression in noninvasive bladder cancer may predict progression from noninvasive tumors to muscle-invasive disease. This hypothesis is further supported by the invasive gene expression signature of bladder cancers derived from CAG-ATDC mice and by the loss of invasive capacity in bladder cancer cell lines when ATDC expression is downregulated. Although ATDC is beginning to be explored as a prognostic marker (46), additional studies are needed to determine whether it is biomarker for a subset of human bladder cancers that are at risk for invasive progression.

In summary, we have identified ATDC overexpression as a driver in bladder tumor formation and identified miR-29 suppression, DNMT3A upregulation, and PTEN silencing as a key mechanism by which ATDC exerts its oncogenic function in mouse models of bladder cancer and its human counterpart. These data place ATDC as a multifaceted player in bladder tumor formation and progression with important implications for human disease. Given its relevance to human bladder cancer, further studies of ATDC-induced tumorigenesis are likely to provide insight into the biology of invasive bladder cancer, and potentially provide novel diagnostic and therapeutic strategies for patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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