DNA Methylation Pathway Alterations in an Autochthonous Murine Model of Prostate Cancer

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Abstract
We examined the DNA methylation pathway in an autochthonous murine prostate cancer model, transgenic adenocarcinoma of mouse prostate (TRAMP). We observed that, compared with strain-matched normal prostates, primary and metastatic TRAMP tumors display increased cytosine DNA methyltransferase (Dnmt) activity, Dnmt1 and Dnmt3b protein expression, and Dnmt1, Dnmt3a, and Dnmt3b mRNA expression. Increased expression of Dnmt genes correlates with increased expression of cyclin A and E2F target genes, implicating increased cell proliferation and Rb inactivation in Dnmt overexpression. We analyzed DNA methylation in TRAMP and found that global levels of 5-methyl-2′-deoxycytidine are unaltered, whereas specific tumors display centromeric repeat hypomethylation. To interrogate locus-specific methylation, we did restriction landmark genomic scanning (RLGS) on normal prostates and primary tumors. In primary tumors, 2.3% of ~1,200 analyzed loci display aberrant DNA hypermethylation, whereas a considerably smaller number of events show hypomethylation. The pattern of RLGS changes was nonrandom, indicating a coordinated methylation defect. Two specific genes identified by RLGS were studied in detail. Surprisingly, methylation of a downstream exon of Two specific genes identified by RLGS were studied in detail.

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

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expression, along with global and locus-specific methylation changes that correlate with altered gene expression. The overall pattern of methylation changes in TRAMP is nonrandom, suggesting a coordinated methylation defect. Our findings thus establish TRAMP as an appropriate in vivo system for dissecting the functional role of epigenetic alterations in prostate cancer.

Materials and Methods

Animals and tissue samples. Supplementary Table S1 summarizes the samples used in this study. Normal prostate samples were obtained from male mice generated by crosses of C57BL/6 and FVB strain mice. Primary and metastatic tumor tissues were obtained from male mice generated by crosses of wild-type FVB with homozygous C57BL/6 TRAMP. Compared with the more commonly used pure C57BL/6 TRAMP, 50:50 FVB × C57BL/6 TRAMP mice show less seminal vesicle invasion and more localized disease (26).

The majority of the primary prostatic tumors of the age collected (average age, 26 ± 4.9 weeks; Supplementary Table S1) are poorly differentiated, late-stage tumors (26). All prostate and metastatic tumor tissues were microdissected at necropsy. Samples were flash frozen in liquid nitrogen and stored at −80°C until use.

Dnmt activity assay. Dnmt activity was measured in nuclear protein extracts obtained from mouse tissues as described previously, with minor modifications (27). TriPLICATE samples containing 10 μg nuclear extract, 0.5 μg poly(dexoyinosinic-deoxycytidylic acid) [poly(dI-dC); Amersham Biosciences, Piscataway, NJ], and 1.5 μCi [3H]SAM (Perkin-Elmer Life Sciences, Boston, MA) were incubated for 2 hours at 37°C, and DNA was recovered by phenol extraction and ethanol precipitation. Unincorporated [3H]SAM was removed using the QIAquick PCR Purification kit (Qiagen, Valencia, CA), and radiolabeled poly(dI-dC) was quantified by scintillation counting. In positive control reactions, 2 units of the prokaryotic CpG methylase Ssml (New England Biolabs, Ipswich, MA) were used.

Western blot analysis. Nuclear and cytosolic proteins were extracted from mouse tissues using the Nuclear Extract kit (Pierce Biosciences, Rockland, IL). Protein concentrations were determined using the Lowry procedure (Fermentas). PCRs were conducted using the qPCR MasterMix Plus UNG (Eurogentec, San Diego, CA) and the 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). Primer and probe sets for specific amplification and detection of murine Dnmt1, Dnmt3a, Dnmt3b, CyclinA1, and 18S rRNA were obtained from Applied Biosystems. Primer and probe sets for linc3 and p16 were obtained from Roche Applied Science. In all cases, target gene expression was normalized to 18S rRNA and the ΔCt method was used to compare fold changes of gene expression relative to a pooled normal prostate sample from strain-matched mice.

Standard end point reverse transcription-PCR (RT-PCR) was used to measure Dnmt3b expression using the following primers: 5′ end, 5′-AGATGATTGAAATGCTCTG (forward, +400) and 5′-AACATGCGCATCACCAACC (reverse, +958) and 3′ end, 5′-GCGATGAGGCACGATCAAT (forward, +2,220) and 5′-GAGCTGACGGAACCAAAAAA (reverse, +2,784). 18S rRNA expression was measured using primers reported previously (28). Product band intensity was analyzed using Quantity One image analysis software (Bio-Rad).

Northern blot analysis. Northern blotting was done as described previously (29) and used to measure Dnmt1, Dnmt3a, and 18S rRNA expression. Northern blot probes were prepared using gel purification of end point RT-PCR products (primer sequences available on request). Band

Figure 1. Dnmt1, Dnmt3b, and 18S rRNA expression are increased in TRAMP primary tumors and metastases. A, nuclear protein extracts from the samples listed in Supplementary Table S1 were used to analyze Dnmt protein expression as described in Materials and Methods. Dnmt activity is expressed relative to the activity obtained from 2 units of purified bacterial CpG methyltransferase Ssml. B, representative nuclear protein extracts from the samples listed in Supplementary Table S1 were used to measure the expression of Dnmt1, Dnmt3b, and Tag. C, Dnmt1, Dnmt3b, and Tag by Western blot analysis. Ponceau S staining confirmed equivalent protein loading.

**P < 0.01, Wilcoxon rank sum, compared with normal prostate.
density of target genes on exposed films was analyzed by densitometry using the Personal Densitometer SI instrument with ImageQuant 5.2 software.

**Genomic DNA methylation.** Specific quantification of 5-methyl-2'-deoxycytidine (5mdC) levels in mouse tissue samples was determined by liquid chromatography (LC)-electrospray ionization quadrupole mass spectrometry (MS) as described previously (30), with slight modifications. Genomic DNAs were isolated using the Puregene DNA isolation kit (Genta Systems, Minneapolis, MN). Genomic DNA samples (1 µg) were digested using 4 units of Nuclease S1 (Fermentas). All samples were analyzed in duplicate.

**Southern blot analysis.** Southern blot analysis of mouse minor centromeric repetitive sequences was performed as described previously (31). Briefly, 5 µg of high molecular weight genomic DNA samples were digested with restriction enzymes HpaII and separated on a 1.2% agarose gel. As a control, samples were digested with MspI. DNA was transferred by capillary action to nylon membranes (Millipore, Billerica, MA) and probed with the pMR66 probe end labeled with [γ-32P]ATP using the DNA 5' End-Labeling kit (Promega, Madison, WI).

**Restriction landmark genomic scanning and restriction landmark genomic scanning spot cloning.** High molecular weight genomic DNA was isolated from 15 TRAMP primary tumor samples and 14 nontransgenic C57BL/6:FVB 50:50 control prostates using DNA isolation methods described previously (32). Individual tumor samples (~75 mg tissue) were used for DNA isolation and restriction landmark genomic scanning (RLGS) analysis. Normal prostate samples were segregated into four pools of three to four prostates to allow for isolation of sufficient high molecular weight DNA for RLGS. RLGS was done as described previously (32). Hypermethylated genes in TRAMP were identified by RLGS spot cloning as described previously (33).

**Sodium bisulfite sequencing.** Genomic DNAs were isolated using the Puregene kit, and sodium bisulfite conversion was done as described previously (34) or using the EZ DNA Methylation kit (Zymo Research, Orange, CA). Sodium bisulfite sequencing primers were designed using MethPrimer (35). Primers for p16 are as follows: start site, 5'&GTGTTGGAGTTAG (forward, +54) and 5'&AATTAAAAAATCTTTAACACATTTT (reverse, +514) and NotI site, 5'&TTAGAGTTTATGAAAGGGGTTG (forward, +19,660) and 5'&ATTCTAAAAAACAAAAATTTCCA (reverse, +20,204). Primers for Irx3 are as follows: start site, 5'&GATGGAATTGGATGAAAT (forward, +42) and 5'&AAACCTCACCCTGCTTCTCTCT (reverse, −121) and NotI site, 5'&GAGGTGTTTACATTTGGGT (forward, +1,343) and 5'&AAAAACATTTTTAACACAATCC (reverse, +1,873). Gradient PCRs were used to optimize annealing temperatures for each primer set. PCR products were directly cloned into the pTopoTA4.1 vector (Invitrogen), and individual clones were sequenced at the Roswell Park Cancer Institute Biopolymer core facility (Buffalo, NY) using an ABI prism automated DNA sequencer. DNA sequence information was analyzed using DNASTar (Lasergene, Madison, WI).

**Results**

**Dnmt activity and Dnmt gene expression in TRAMP.** To begin to examine whether the DNA methylation pathway is altered in TRAMP primary tumors and metastases, we first determined the overall levels of Dnmt enzymatic activity in TRAMP prostates and strain-matched normal prostates. We found that Dnmt activity was significantly increased in both primary and metastatic TRAMP tumors compared with normal prostates (Fig. 1A). The robust increase in Dnmt activity in TRAMP tumors suggested that the expression of one or more catalytically active Dnmt proteins is induced. Therefore, we examined Dnmt1 and Dnmt3b protein expression by Western blot analyses (Fig. 1B). We were unable to identify an antibody that recognized murine Dnmt3a; thus, we only measured mRNA expression of this gene (see below). Dnmt1 and Dnmt3b protein expressions are robustly induced in both primary tumors and metastases compared with normal prostate (P < 0.01; Fig. 1B-D). As expected, Tag expression was detected in TRAMP but not in control tissues (Fig. 1B). Moreover, we identified a significant correlation between Dnmt enzymatic activity and the expression of Dnmt1, Dnmt3b, or combined Dnmt1 plus Dnmt3b protein expression in TRAMP [Kendall Tau b correlation coefficients of 0.195 (P < 0.05), 0.189 (P < 0.05), and 0.242 (P < 0.01), respectively]. These data suggest that increased expression of Dnmt1 and Dnmt3b proteins contributes to increased Dnmt activity in TRAMP.

Increased Dnmt1 protein expression in TRAMP could result from increased Dnmt1 mRNA expression. To test this, we measured the expression of Dnmt1, Dnmt3b, and Dnmt3a mRNAs in TRAMP using quantitative real-time PCR. Similar to the corresponding protein, Dnmt1 mRNA expression was increased in primary and metastatic tumors compared with normal prostates (P < 0.01; Fig. 2A). Dnmt3a mRNA expression was significantly elevated in primary tumors and liver and kidney metastases (Fig. 2B). There was a trend toward increased Dnmt3b expression in all tumor types, but this only reached statistical significance in liver and kidney metastases (Fig. 2C). To confirm the quantitative real-time PCR method with alternative assays, we did Northern blotting to measure Dnmt1 and Dnmt3a expression, and, because of the lack of a suitable Northern probe, end point RT-PCR to measure Dnmt3b expression. These experiments confirmed that Dnmt mRNA expression is up-regulated in TRAMP primary tumors and metastases (Supplementary Fig. S1).
To test whether Dnmt1 and Dnmt3b protein and mRNA expression were correlated, we did Kendall Tau correlation testing of our quantitative real-time PCR data with our Western blot data. This analysis revealed that Dnmt1 protein and mRNA expression are weakly correlated (correlation coefficient = 0.2; \( P < 0.04 \)), whereas Dnmt3b protein and mRNA expression are not correlated (correlation coefficient = 0.04; \( P = 0.66 \)). These data suggest that increased Dnmt mRNA expression may only partially account for increased Dnmt activity and protein expression in TRAMP.

Dnmt1 and Dnmt3b, and to a lesser extent Dnmt3a, show cell cycle-regulated gene expression, with levels peaking during S phase (9). Thus, increased cell proliferation could contribute to Dnmt gene overexpression in TRAMP. Additionally, E2F activation may contribute to Dnmt induction in TRAMP because the Dnmt1 promoter is directly activated by E2F transcription factors, which are normally negatively regulated by Rb (11). To examine the potential contribution of increased cell proliferation and E2F target gene activation to Dnmt gene overexpression in TRAMP, we measured cyclin A protein expression as a marker of cell proliferation and E2F1 and E2F3 protein expressions as markers of E2F pathway activation [E2F genes are autoregulated (36)] in normal prostates and TRAMP tumors. We find that cyclin A is robustly induced in TRAMP relative to normal prostate (Fig. 3A and B). Similar to cyclin A, E2F1 and E2F3 expressions are increased in TRAMP primary tumors and lymph node metastases but less so in liver and kidney metastases (Fig. 3A, C, and D). We used correlation testing to examine the association between Dnmt gene expression and the expression of cyclin A, E2F1, and E2F3 in TRAMP tumors. This analysis revealed that Dnmt1 and Dnmt3a expression closely correlates with the expression of cyclin A, E2F1, and E2F3, whereas Dnmt3b shows a mild correlation with cyclin A and does not correlate with expression of E2F1 or E2F3 (Supplementary Table S2). Consistent with these results, Dnmt1 and Dnmt3a expression is significantly correlated, whereas Dnmt3b expression was only weakly correlated with Dnmt1 and did not correlate with Dnmt3a expression (Supplementary Table S2). These data suggest that induction of all three Dnmt genes at least in part reflects increased cell proliferation in TRAMP, whereas activation of Dnmt1 and Dnmt3a, but not Dnmt3b, is also closely linked to activation of the E2F pathway.

To further test the relationship between Dnmt expression and cell proliferation in TRAMP, we directly normalized Dnmt expression to cyclin A expression. Normalization of Dnmt1, Dnmt3a, or Dnmt3b mRNA expression to cyclin A mRNA expression negated statistically significant Dnmt overexpression in each tumor type, with the exception of liver metastases (data not shown). However, Dnmt1 and Dnmt3b proteins remained overexpressed in primary and metastatic TRAMP tumors after normalization to cyclin A protein expression (Supplementary Fig. S2). This result suggests that Dnmt1 and Dnmt3b protein overexpression in TRAMP is not solely accounted for by increased cell proliferation.

**Global genomic DNA methylation in TRAMP.** The above data indicate that Dnmt enzymatic activity, protein expression, and mRNA expression are increased in TRAMP. To examine whether these alterations are associated with aberrant DNA methylation, we first determined the levels of 5mdC in TRAMP prostates compared with strain-matched normal prostates (Fig. 4A and B). In normal prostates and each TRAMP tumor type, the percentage 5mdC/dG was \(-3.0\), indicating that overall levels of genomic DNA methylation are unaltered (Fig. 4B). To further examine global methylation in TRAMP, we used Southern blot analysis to determine the methylation level of minor centromeric repeat elements, which are normally heavily methylated in the mouse genome (37). Southern blotting revealed that 8 of 11 (73%) primary tumors and 5 of 11 (45%) liver metastases tested showed hypomethylation of centromeric repeats compared with normal prostates (data not shown; Fig. 4C). The lack of overall genomic DNA hypomethylation in tumors showing evidence of hypomethylation in centromeric repeats suggests that hypomethylation at these regions may contribute to overall 5mdC levels or that hypomethylation at these regions is offset by hypermethylation at other regions of the genome.

**Figure 3.** Cyclin A and E2F gene expression is increased in TRAMP primary tumors and metastases. A, representative nuclear protein extracts from the samples listed in Supplementary Table S1 were used to measure the expression of cyclin A, E2F1, and E2F3 by Western blot analysis. Ponceau S staining confirmed equivalent protein loading. B to D, densitometric analysis of cyclin A (B), E2F1 (C), and E2F3 (D) protein expression by Western blot analysis of the samples listed in Supplementary Table S1. Bars, SD. ***, \( P < 0.01 \), Wilcoxon rank sum test; *, \( P < 0.05 \), compared with normal prostate.
Locus-specific DNA methylation in TRAMP. To further define the pattern of DNA methylation in TRAMP, we used RLGS. RLGS analysis combines methylation-sensitive restriction digestion by NotI with radiolabeling and two-dimensional gel electrophoresis to reveal DNA methylation patterns primarily at single-copy loci contained within CpG islands (32). A typical gel profile from a TRAMP tumor sample analyzed by RLGS is shown in Fig. 5A. Validating this experimental approach in TRAMP, we observed that RLGS spot patterns were identical in four different pools of normal prostates (Fig. 5B). In contrast, primary TRAMP tumors showed numerous RLGS spot alterations compared with normal prostates, including spot losses and spot gains, corresponding to hypermethylation and hypomethylation events, respectively (Fig. 5C and D). In total, we profiled 15 primary TRAMP tumors by RLGS, and the mean number of spots examined per sample was ~1,200. An average of 28 spots (2.3%) was lost and an average of 9 spots (0.8%) was gained in the primary tumor samples. We used an applied Friedman's test to determine whether the RLGS spot loss pattern in primary TRAMP tumors was nonrandom, which yielded a P value of <0.01. This result indicates that the RLGS spot loss in TRAMP is nonrandom, suggesting an underlying DNA methylation defect. Interestingly, we did not observe a correlation between Dnmt expression level and RLGS spot loss in TRAMP tumors (data not shown), suggesting that hypermethylation patterns in specific tumors are not simply a function of the extent of Dnmt expression. Quantitative RT-PCR and Western blot analyses revealed that both p16 mRNA and protein expression are strongly induced in TRAMP tumors (Fig. 6B and C). Furthermore, sequencing of the p16 mRNA in two primary tumors and one metastatic TRAMP tumor revealed that the expressed sequence is wild-type (data not shown). Taken together, these data indicate that aberrant hypermethylation of downstream regions of the p16 gene is linked to increased expression of wild-type p16 mRNA and protein in TRAMP. Remarkably, a similar scenario has been previously reported for human prostate cancer (39).

To investigate the significance of Hox gene hypermethylation in TRAMP, we examined the methylation and expression pattern of Irx3. As was seen for p16, sodium bisulfite sequencing of the CpG island region flanking the NotI site of Irx3 in normal prostates and primary tumors confirmed the RLGS data (Fig. 6D). For the tumors showing hypermethylation at the NotI site (P13 and P8), hypermethylation did not extend into the core promoter (Fig. 6D). To determine whether methylation of the Irx3 gene is related to altered gene expression, we did quantitative real-time RT-PCR (no antibody was available for Irx3 protein analysis). As shown in Fig. 6E, methylation of Irx3 was associated with a reduced level of Irx3 mRNA expression in TRAMP tumors. However, some tumors...
showed low expression of Irx3 despite having low methylation levels (e.g., tumor P11 in Fig. 6D and E), suggesting that multiple mechanisms may contribute to down-regulation of Irx3 expression in TRAMP.

Discussion

We have characterized DNA methylation pathway alterations in a widely used autochthonous murine model of prostate cancer. Our data show that primary prostatic tumors and metastases in TRAMP are characterized by increased Dnmt activity, Dnmt1 and Dnmt3b protein expression, and Dnmt1, Dnmt3a, and Dnmt3b mRNA expression. Activation of Dnmt1 and Dnmt3a gene expression closely correlates with increased expression of cyclin A, E2F1, and E2F3, suggesting a link with increased cell proliferation and Rb inactivation mediated by Tag. Despite a robust increase in Dnmt expression, global genomic DNA methylation is unaltered in TRAMP tumors compared with normal

Table 1. RLGS spot loss in TRAMP primary tumors

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<th>Spot ID</th>
<th>No. times hypermethylated, n (%)*</th>
<th>Gene name</th>
<th>NotI site in CpG island</th>
<th>Gene context of NotI site</th>
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<td>5' End</td>
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<td>Xra2</td>
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<td>Body</td>
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<td>3' End</td>
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<td>—</td>
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*Of 15 primary tumors analyzed.
prostates, and the majority of tumors show hypomethylation at minor centromeric repeats. In contrast, RLGS revealed that single-copy genomic loci are frequently and nonrandomly hypermethylated in TRAMP. These hypermethylation events were associated with gene repression, as seen for Irx3, or gene activation, as seen for p16. We did not observe a direct connection between the level of Dnmt gene overexpression and the extent of gene hypermethylation, as measured by RLGS spot loss, in TRAMP.

In TRAMP, the probasin promoter drives the specific expression of the SV40 early genes in hormonally responsive prostate epithelial cells (25). Expression of Tag is thought to drive tumor initiation by binding to and functionally inactivating Rb and p53. A connection between Rb disruption and alterations of DNA methylation has been suggested in previous studies (8, 11, 40, 41). Szyf et al. (8) observed that Tag expression in murine 3T3 cells leads to increased Dnmt1 expression and both global and gene-specific DNA hypermethylation. Notably, we find that Tag expression in TRAMP leads to locus-specific hypermethylation but does not lead to global DNA hypermethylation. This result suggests that, unlike in tissue culture, Rb inactivation in vivo, in the context of murine prostate cancer, is incompatible with global genomic DNA hypermethylation. A potential mechanism whereby loss of Rb leads to locus-specific DNA hypermethylation in TRAMP is suggested by studies showing that Rb associates directly with human Dnmt1 and disrupts its enzymatic activity (40, 41). Additionally, McCabe et al. (11) recently showed that the Dnmt1 promoter is directly activated by E2F1 in Rb-null human prostate epithelial cells. Along with the data presented in the current study, these observations suggest a functional connection between Rb inactivation, one of the most common alterations in human cancer, and altered Dnmt1 function. As Dnmt mutations have not been discovered in human cancer (42), it is plausible that

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**Figure 6.** DNA hypermethylation and gene expression in TRAMP. **A,** methylation analysis of p16 (RLGS spot 3D22). Top, schematic of the p16 gene and the regions analyzed by bisulfite sequencing (horizontal lines bordered by ovals). Bent arrow, transcriptional start site; lines, introns; boxes, exons; gray bar, 5' CpG island; vertical arrow, position of the NotI site. Bottom, sodium bisulfite sequencing of p16 from one representative normal prostate and two primary tumors (P5 and P9) found to be methylated by RLGS. Vertical arrow, position of the NotI site; rows, individually sequenced alleles; open circles, unmethylated CpG sites; filled circles, methylated CpG sites. **B,** p16 mRNA expression in normal prostates and TRAMP primary tumors. Samples, p16 mRNA expression was measured by quantitative real-time PCR using the ΔCt method as described in Materials and Methods. **C,** p16 protein expression was measured by Western blot analysis of available tumor samples as described in Materials and Methods. Ponceau S staining confirmed equivalent protein loading. **D,** methylation analysis of Irx3 (RLGS spot 4G73). Top, schematic of the Irx3 gene indicating the regions analyzed by bisulfite sequencing (horizontal lines bordered by arrows). Vertical arrow, transcriptional start site; lines, introns; boxes, exons; gray bar, 5' CpG island; down arrow, position of the NotI site. Bottom, bisulfite sequencing of Irx3 from one representative normal prostate, two primary tumors unmethylated by RLGS (P11 and P1), and two primary tumors methylated by RLGS (P13 and P8). Vertical arrow, position of the NotI site; rows, individually sequenced alleles; open circles, unmethylated CpG sites; filled circles, methylated CpG sites. **E,** Irx3 mRNA expression in normal prostates and TRAMP primary tumor samples. Irx3 mRNA expression was measured by quantitative real-time PCR using the ΔCt method as described in Materials and Methods. Irx3 expression was normalized to 18S rRNA and is expressed relative to a pooled sample of four strain-matched normal prostates. Expression in 4 normal prostates and 15 primary tumors analyzed by RLGS is indicated. All tumor samples except tumor P8 (labeled “M.”) were analyzed by bisulfite sequencing of p16 from one representative normal prostate and two primary tumors unmethylated by RLGS (P11 and P1), and two primary tumors methylated by RLGS (P13 and P8). Vertical arrow, position of the NotI site; rows, individually sequenced alleles; open circles, unmethylated CpG sites; filled circles, methylated CpG sites.
disruption of Rb, which is exceedingly common in cancer, could be a key contributor to aberrant DNA methylation in human cancer. The link between Rb inactivation and Dnmt1 overexpression is particularly noteworthy based on the recent demonstration that Dnmt1 may constitute the primary de novo methyltransferase activity in human cancer cells (43). Interestingly, in addition to Dnmt1, we observed that Dnmt3a overexpression in TRAMP is also correlated with activation of E2F target gene expression. Whether Dnmt3a is a downstream target for E2F-mediated transactivation is currently unknown.

Our RLGS studies indicate that downstream Cpg island hypermethylation of p16 is a very frequent event in TRAMP, and this correlates with increased expression of both p16 mRNA and protein. This observation was unexpected based on the fact that p16 is a frequent target of promoter hypermethylation and transcriptional silencing in cancer (38). However, p16 promoter hypermethylation is rare in human prostate cancer (18). Moreover, Nguyen et al. (39) have reported that exon 2 of p16 is hypermethylated in human prostate cancer and that p16 mRNA expression is increased in this malignancy. These observations are strikingly similar to our data in TRAMP, although it is unknown whether p16 protein is also up-regulated in human prostate cancer (39). Consistent with our methylation and expression data for p16, increased transcription has been proposed to facilitate hypermethylation in downstream regions of genes (44). Thus, the increased methylation of p16 exonic regions in TRAMP may be a consequence, and not a cause, of increased p16 gene expression. Although the mechanistic basis for increased p16 expression in TRAMP and other Rb compromised settings remains unknown, previous studies using other model systems have also observed this connection (45, 46).

Remarkably, >20% of the most frequently hypermethylated loci in TRAMP identified by RLGS were Hox genes. We found that hypermethylation of one of these genes, Irx3, in TRAMP, correlates with reduced mRNA expression. The prevalence of Hox gene hypermethylation in TRAMP suggests that Hox family genes may function as tumor suppressors in prostate cancer. Consistent with this idea, the Hox gene Nkx3.1 is an established prostate tumor suppressor in mice and humans (47, 48). Interestingly, Nkx3.1 is also epigenetically inactivated in prostate cancer, although this seems to primarily occur at the level of protein expression (47).

There is also a precedent for aberrant hypermethylation of Hox genes in other malignancies. Shiraishi et al. reported that Hox gene clusters are hotspots of de novo methylation in lung cancer, and Hox genes are transcriptionally repressed in breast cancer (49, 50). These observations suggest important additional roles for Hox proteins outside of development (e.g., as tumor suppressors in differentiated tissues, such as the prostate). Using human mammary epithelial cells, it was recently shown that transcriptional silencing of p16 indirectly leads to Hox gene repression (51). These observations contrast with our findings using TRAMP, in which p16 gene induction and Hox gene silencing are coincident.

Our data further validate the usefulness of murine models for defining the causes and consequences of epigenetic change in cancer. The prevalence of Rb pathway deregulation in cancer and its close link to Dnmt activation suggest TRAMP and similar models as ideal tools to dissect the relationship between Rb loss and DNA methylation changes. Notably, it was recently shown that 5-aza-2′-deoxycytidine (DAC) treatment has a strong anticancer effect in TRAMP on the pure C57BL/6 background, with prevention of both primary tumors and lymph node metastases (52). The nonspecific Dnmt inhibitory effect of DAC, coupled with our observations that multiple Dnmt genes are up-regulated in TRAMP, indicates that genetic studies will be required to dissect the critical Dnmt(s) contributing to prostate cancer etiology in TRAMP. However, the currently available data suggest that targeting of Dnmt enzymes, either by drugs, such as DAC, or by other agents, should be explored as potential chemopreventive or therapeutic intervention strategies in prostate cancer. In this regard, murine models will provide key systems in which to evaluate the use of these approaches.

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References

DNA Methylation Changes in TRAMP


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Shannon R. Morey, Dominic J. Smiraglia, Smitha R. James, et al.


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