Stage-Specific Alterations of DNA Methyltransferase Expression, DNA Hypermethylation, and DNA Hypomethylation during Prostate Cancer Progression in the Transgenic Adenocarcinoma of Mouse Prostate Model

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Abstract
We analyzed DNA methyltransferase (Dnmt) protein expression and DNA methylation patterns during four progressive stages of prostate cancer in the transgenic adenocarcinoma of mouse prostate (TRAMP) model, including prostatic intraepithelial neoplasia, well-differentiated tumors, early poorly differentiated tumors, and late poorly differentiated tumors. Dnmt1, Dnmt3a, and Dnmt3b protein expression were increased in all stages; however, after normalization to cyclin A to account for cell cycle regulation, Dnmt proteins remained overexpressed in prostatic intraepithelial neoplasia and well-differentiated tumors, but not in poorly differentiated tumors. Restriction landmark genomic scanning analysis of locus-specific methylation revealed a high incidence of hypermethylation only in poorly differentiated (early and late) tumors. Several genes identified by restriction landmark genomic scanning showed hypermethylation of downstream regions correlating with mRNA overexpression, including p16INK4a, p19ARF, and Cacna1a. Parallel gene expression and DNA methylation analyses suggest that gene overexpression precedes downstream hypermethylation during prostate tumor progression. In contrast to gene hypermethylation, genomic DNA hypomethylation, including hypomethylation of repetitive elements and loss of genomic 5-methylcytosine, occurred in both early and late stages of prostate cancer. DNA hypermethylation and DNA hypomethylation did not correlate in TRAMP, and Dnmt protein expression did not correlate with either variable, with the exception of a borderline significant association between Dnmt1 expression and DNA hypermethylation. In summary, our data reveal the relative timing of and relationship between key alterations of the DNA methylation pathway occurring during prostate tumor progression in an in vivo model system. (Mol Cancer Res 2008;6(8):1365–74)

Introduction
DNA methylation is deregulated in cancer such that the promoter regions of tumor suppressor genes become hypermethylated, resulting in gene silencing, whereas on a global level, DNA becomes hypomethylated, leading to genomic instability (1, 2). In human prostate cancer, both of these mechanisms have been observed (3-8). In addition, deregulated expression of DNA methyltransferase (Dnmt) proteins is seen in human prostate cancer (9). These data provide compelling circumstantial evidence of a role for these alterations in prostate cancer development. However, it is difficult to assess the functional contribution of these alterations to prostate cancer development using only human clinical samples. Moreover, the relative timing of and relationship between distinct DNA methylation pathway alterations during prostate tumor progression has not been assessed in an in vivo model system. To this end, we and others have recently established the transgenic adenocarcinoma of mouse prostate (TRAMP) model as a suitable mouse model to investigate the role of altered DNA methylation in prostate cancer development (10-13). We have shown that late stage primary tumors and metastases from TRAMP mice display increased Dnmt expression, locus-specific nonrandom CpG island hypermethylation, and hypomethylation of repetitive DNA elements (11, 13). In addition, using pharmacologic inhibition of Dnmt enzymes, Day and colleagues have shown that DNA hypermethylation contributes to the development of primary cancer in both intact and castrated TRAMP mice (10, 12). Taken together, these data suggest that the TRAMP model may be particularly useful to clarify the role of DNA methylation pathway alterations in prostate cancer development.

One notable finding of our previous study was that TRAMP tumors frequently display overexpression of p19ARF (p19) and
p16INK4a (p16), correlating with hypermethylation of a shared downstream region (exon 3) of the Cdkn2a locus (11). The relevance of this event to human prostate cancer is supported by the prior observation that p16 gene up-regulation and downstream hypermethylation also occur in human prostate cancer (14). Using restriction landmark genomic scanning (RLGS), we identified several other genes that were hypermethylated in downstream regions in TRAMP relative to normal prostate, suggesting that this phenomenon may be widespread (11). Previous work in other systems has also reported the hypermethylation of actively transcribed downstream gene regions in cancer (14-16). However, it remains unclear whether gene overexpression in cancer occurs prior to or subsequent to downstream DNA hypermethylation.

In the current study, we sought to define the relationship between disease stage, Dnmt expression, DNA hypermethylation, and DNA hypomethylation in prostate cancer. For this purpose, we selected TRAMP prostate samples from four distinct groups [prostatic intraepithelial neoplasia (PIN), well-differentiated tumors (WD), early poorly differentiated tumors (EPD), and late poorly differentiated tumors (LPD)] for analysis and comparison with nontransgenic strain-matched normal mouse prostates. In each sample set, we measured Dnmt1, Dnmt3a, and Dnmt3b protein expression by Western blot, locus-specific methylation using RLGS, and global methylation using liquid chromatography-mass spectrometry detection of 5-methyldeoxycytidine (5mdC), and bisulfite pyrosequencing of the B1 repetitive element. In addition, we examined the relationship between gene overexpression and downstream hypermethylation in TRAMP via comparative mRNA expression and DNA methylation analysis of p16INK4a, p19ARF, and Cacna1a in staged tumor samples. We also did statistical correlation analyses to determine the association between each of these variables during tumor progression. Our findings reveal key aspects of the relationship between distinct alterations of the DNA methylation pathway occurring during prostate tumor progression.

**Results**

**Multistage Prostate Cancer Progression in TRAMP**

We used prostate tumors from TRAMP mice, as well as normal prostates from nontransgenic, strain-matched mice (Fig. 1A). We grouped TRAMP samples based on differentiation status, age, and prostate weight into four categories: PIN (10-12 weeks, 0.008-0.04 g; n = 35), WD tumors (15-20 weeks, 0.03-0.09 g; n = 25), EPD tumors (15-20 weeks, 0.49-4.86 g; n = 12), and LPD tumors (22-28 weeks, 1.65-15.65 g; n = 12; Fig. 1A). This grouping is based on previous studies showing that age and prostate weight directly correlate with tumor progression in TRAMP (17). PIN samples are normal in weight, but microscopically display neoplasia and hyperplastic infolding of the epithelial layer into the luminal space of the gland (Fig. 1A and B). WD samples are larger than normal prostates, but were not palpable at necropsy. The majority of the disease in these samples is well-differentiated glandular epithelium (Fig. 1B). EPD samples were from the same age range as WD samples (15-20 weeks), but were palpable at necropsy and histologically showed sheets of predominantly poorly differentiated epithelial cells (Fig. 1A and B). LPD tumors, from 20- to 28-week-old mice, were very large and showed poorly differentiated late stage disease (Fig. 1A and B). H&E staining was used to stage a large subset of samples and confirmed the assigned groupings (Fig. 1B; data not shown).

**Dnmt Protein Expression during Multistage Prostate Cancer Progression**

We initially examined Dnmt1, Dnmt3a, and Dnmt3b protein expression in normal prostates and the four sets of TRAMP samples described above using Western blot analysis. Dnmt1
expression is significantly elevated in PIN and WD and its level increases further in late stage (EPD and LPD) samples (Fig. 2A and B). Dnmt3a and Dnmt3b show small increases in PIN and WD, which increases further in EPD and LPD tumors (Fig. 2A, C-D). As Dnmt expression is cell cycle–regulated with high-level expression restricted to S phase (18), we next measured cyclin A to normalize Dnmt expression. Notably, cyclin A expression is increased only in late stage (EPD and LPD) disease (Fig. 2A and E), suggesting that the increased expression of Dnmt proteins seen in PIN and WD is not related to increased cell proliferation. After normalization of Dnmt protein expression to cyclin A, expression of all three Dnmts are elevated in early stage lesions (PIN and WD) relative to normal prostate, but not in late stage tumors (EPD and LPD; Fig. 2F). These data suggest that increased Dnmt protein expression in TRAMP may be most biologically significant in the early stages of prostate cancer.

Locus-Specific DNA Hypermethylation during Multistage Prostate Cancer Progression

We next used RLGS to examine global CpG island methylation patterns in TRAMP samples of each progression stage. RLGS is a two-dimensional gel analysis of radio-labeled, methylation-sensitive, enzyme-restricted DNA fragments (19). When comparing RLGS gel patterns, spot loss and spot gain correspond to DNA hypermethylation and DNA hypomethylation events, respectively. RLGS allowed for the identification of hypermethylation events in TRAMP which, in the vast majority of instances, were confined to late stage (EPD or LPD) disease (examples shown in Fig. 3A and B). A low level of both hypermethylation and hypomethylation events were observed in PIN and WD samples, whereas EPD and LPD tumors showed a substantial increase in hypermethylation events (Fig. 3C and D). In addition, the number of hypermethylated loci from tumor to tumor was variable within the EPD, and particularly the LPD, groups (Fig. 3D). We identified the genes corresponding to different RLGS spots using cloning techniques described previously (ref. 20; Table 1). A number of these loci were hypermethylated at high frequency in EPD and LPD (Table 1), suggesting that methylation of these loci are under positive selection during prostate cancer progression in TRAMP.
Downstream Hypermethylation and Increased Gene Expression

We previously reported that overexpression of p19 and p16 correlated with the downstream hypermethylation at the shared exon 3 of the Cdkn2a locus in late stage TRAMP tumors (11). Several other genes also display hypermethylation in downstream regions in TRAMP tumors, providing further evidence of the potential importance of this phenomenon (Table 1). The staged progression model we describe here allows for an investigation of the relative timing of gene overexpression and downstream hypermethylation. We find that p19 and p16 are overexpressed in all stages analyzed, as compared with normal prostate, indicating that overexpression is an early event (Fig. 4A and B). In contrast, RLGS indicated that hypermethylation of the NotI site at exon 3 of the Cdkn2a locus was exclusively found in late stage (EPD and LPD) samples (Table 1). In addition, bisulfite sequencing further showed that this downstream region of Cacna1a, but not its promoter region, is methylated in TRAMP (Fig. 5B). Taken together, these data suggest that similar to Cdkn2a genes, overexpression of Cacna1a precedes its downstream hypermethylation. Interestingly, a low but significant level of methylation at the Cacna1a locus was seen in both normal prostates and early stage samples (Fig. 5B). This situation may be analogous with certain genes that are partially methylated in normal human prostate and become hypermethylated in human prostate cancer (21).

FIGURE 3. Locus-specific DNA hypermethylation during TRAMP tumor progression. A. RLGS analysis showing Spot 3C21, corresponding to Nrnx2 (solid circle). Dashed circle, the position of spot loss (hypermethylation event) seen exclusively in the EPD and LPD samples. B. RLGS analysis showing spots 3D22 (upper spot) and 3E30 (lower spot), corresponding to Cdkn2a and Gsc genes, respectively (solid circles). Dashed circles, the position of the spot loss (hypermethylation events), seen exclusively in the EPD and LPD samples. C. RLGS spot losses (hypermethylation events) and RLGS spot gains (hypomethylation events) in each sample group. Bars, 1 SD. D. Hypermethylation events in each sample analyzed by RLGS. Points, individual samples; bars, mean of each sample group.
DNA Hypomethylation during Multistage Prostate Cancer Progression

In addition to gene-specific DNA hypermethylation, global DNA hypomethylation contributes to oncogenesis (22-24). In TRAMP, we previously found increased variability but no consistent changes in 5mC levels in late stage TRAMP tumors and metastases as compared with normal strain-matched prostates (11). We hypothesized that global hypomethylation may be an early event during TRAMP tumor development that could have been missed in our previous study. To test this hypothesis, we measured 5mC levels by liquid chromatography-mass spectrometry as well as by the methylation level of the common murine repetitive element B1 using quantitative bisulfite pyrosequencing in the four stages of TRAMP samples described earlier (Fig. 1A). 5mC levels were significantly decreased in WD and EPD tumors (Fig. 6A). At the latest stage (LPD), this effect was lost; however, increasing variability from tumor to tumor was apparent (Fig. 6A). In contrast to 5mC levels, the B1 repetitive element is significantly hypomethylated in all four progression stages measured, but more dramatically in the later stages (Fig. 6B). Analyzed over the entire data set, 5mC levels directly correlated with B1 hypomethylation (one-tailed Spearman rank correlation, r = 0.30; P = 0.0214). These experiments show that genomic DNA hypomethylation occurs as an early event during prostate tumorigenesis in TRAMP, and persists and/or increases in advanced stages.

Relationship between DNA Methylation Pathway Alterations in TRAMP

We next took advantage of this unique data set to examine the relationship between Dnmt protein expression, DNA hypermethylation, and DNA hypomethylation during prostate tumorigenesis. To examine the link between DNA hypermethylation and DNA hypomethylation, we compared the extent of RLGS spot loss to 5mC levels or B1 element methylation status in all samples (Fig. 6C and D). Interestingly, we found no association between DNA hypermethylation and either variable of global DNA hypomethylation, suggesting that hypermethylation and hypomethylation are independently controlled in TRAMP. The lack of association was maintained when only late stage (EPD and LPD) samples, which show a much higher incidence of DNA hypermethylation (Fig. 3), were analyzed (Fig. 6E and F). Finally, we compared Dnmt1, Dnmt3a, and Dnmt3b protein expression to DNA hypermethylation and DNA hypomethylation. Because both protein and DNA could not be obtained from the same PIN sample, only WD, EPD, and LPD samples were part of this analysis. Expression of Dnmt3a and Dnmt3b did not correlate with either DNA hypermethylation or DNA hypomethylation (data not shown). In contrast, Dnmt1 expression showed a borderline significant correlation with DNA hypermethylation (two-tailed Spearman rank order correlation, P = 0.0546), but not with DNA hypomethylation (data not shown).

Discussion

We have used the TRAMP model to elucidate the nature and the temporal relationship of distinct DNA methylation pathway alterations occurring during prostate cancer development. A unified model encompassing the data presented here, as well that of our previous work (11, 13), is shown in Fig. 7. At the earliest stage analyzed (PIN), a number of alterations are already detected, including Dnmt protein overexpression, hypomethylation of the B1 repetitive DNA element and, to a far lesser extent, gene-specific DNA hypermethylation (Fig. 7). 5mC loss is substantial at the WD and EPD stages, but becomes highly heterogeneous later on (Fig. 7). DNA

### TABLE 1. RLGS Spot Loss in TRAMP Samples

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<th>Nor1 Site in CpG island</th>
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hypermethylation becomes highly prevalent only in late stage primary and metastatic tumors (Fig. 7). Similar to the heterogeneous 5-mdC levels seen in late progression stages, there is increased heterogeneity of DNA hypermethylation events in metastatic lesions (Fig. 7).

In general, we observed a lack of association between Dnmt expression, DNA hypermethylation, and DNA hypomethylation, suggesting that these three alterations largely make independent contributions to prostate cancer in TRAMP. The only exception to this was a borderline significant association between Dnmt1 expression and DNA hypermethylation (as determined by RLGS spot loss). This apparent association will be interesting to follow-up in studies using more comprehensive analyses of global DNA hypermethylation. However, the general lack of a strong association between Dnmt expression and DNA hypermethylation (as determined by RLGS spot loss) is consistent with previous studies showing that specific DNA motifs have an intrinsic propensity for aberrant DNA hypermethylation (27, 28).

In TRAMP, accumulating evidence suggests that aberrant DNA hypermethylation directly contributes to the disease progression. Treatment of TRAMP mice with the DNA methyltransferase inhibitor 5-aza-2\'-deoxycytidine delays tumor progression, without altering the incidence of early stage disease (10). Consistent with this finding, we observe that very few aberrant locus-specific hypermethylation events are detected in early stage tumors (PIN and WD), whereas a large number of these events are seen in late stage tumors (EPD and LPD). The fact that high-frequency gene-specific DNA hypermethylation occurs only at late stages of prostate cancer suggests that they may result from tumor selection and not simply transgene expression. This is in agreement with a recent study examining DNA hypermethylation in a murine lymphoma model, which found changes in DNA methylation only in late stage disease (29).

Increased expression of p19 and p16 occur as early as the PIN stage in TRAMP, whereas overexpression of Cacna1a occurs at later stage disease. For each gene, increased expression
coincides with regional downstream DNA hypermethylation. It is intriguing that downstream hypermethylation of overexpressed genes occurs at several loci in TRAMP (Table 1). The relative timing of these two events in vivo, for the genes studied here, suggests that gene overexpression occurs prior to and may facilitate downstream hypermethylation. However, in preliminary studies, we have observed that treatment of TRAMP cell lines with 5-aza-2'-deoxycytidine results in decreased expression of p19 and p16, coinciding with reduced downstream hypermethylation (data not shown). Taken together, these data seem to suggest that increased transcription facilitates downstream hypermethylation, which may then contribute to the maintenance of the transcriptionally active state. In vivo manipulation of DNA methylation levels in TRAMP mice will be required to adequately test this hypothesis. In any case, it is important to point out that p16 gene expression is also increased in human prostate cancer, in conjunction with hypermethylation of downstream regions (14), strongly supporting the relevance of our observations in the TRAMP model.

Significant reduction of global 5mC occurs only in the WD and EPD stages; in contrast, the B1 element is hypomethylated at all stages, including PIN. This result suggests that DNA hypomethylation of certain genomic regions is an early event during prostate tumor progression but is not uniform across the entire methylome. In the context of murine intestinal tumorigenesis, Jaenisch and colleagues have shown that DNA hypomethylation accelerates the formation of early stage microadenomas, but dramatically inhibits the formation of macroscopic polyps (30). Our findings suggest that an analogous scenario could occur in murine prostate cancer, with hypomethylation contributing to tumor initiation and hypermethylation contributing to tumor progression. The increased heterogeneity of both hypomethylation and hypermethylation in late stage prostate disease in TRAMP suggests a general decrease in the fidelity of DNA methylation in these tumors, which may serve as a source of tumor heterogeneity.

In summary, we have used a progression stage model of prostate cancer to decipher the temporal relationship between the three chief DNA methylation pathway alterations in cancer. Key aspects of this model will allow for the examination of the role of specific epigenetic defects to prostate tumor development in vivo.

Materials and Methods

Animals and Tissue Samples

Figure 1A summarizes the TRAMP samples used in this study. Normal prostate samples were obtained from F1 males generated by crosses of C57BL/6 and FVB strain mice. TRAMP prostate tissues were obtained from F1 males generated by crosses of C57BL/6 TRAMP males (homozygous for the Probasin-SV40 transgene) with wild-type FVB females. All prostate and tumor tissues were microdissected at necropsy. Samples were flash-frozen in liquid nitrogen, and stored at −80°C until use.

H&E Staining

Five-micron-thick tissue sections were cut from paraffin-embedded blocks and mounted on slides. Slides were deparaffinized and rehydrated with Xylene and graded alcohol and equilibrated with Tris-phosphate buffer. Samples were then stained with H&E, dehydrated through alcohol into xylene, and mounted with glass coverslips. Tissue sections were scored using a compound Olympus XI-50 microscope equipped with QCapture imaging software.
**Western Blot Analysis**

Nuclear proteins were extracted from mouse tissues using the Nuclear Extract kit (Pierce Biochemical). Protein concentrations were determined using the Lowry High system (Bio-Rad). Western blots were completed as described previously (11). Dnmt1 was detected using the NB 100-264 rabbit polyclonal antibody (Novus Biologicals). Dnmt3a was detected with ab14291 chicken polyclonal antibody (Abcam, Inc.).

**FIGURE 6.** DNA hypomethylation during TRAMP tumor progression. A. 5mdC levels in normal prostates and TRAMP samples. 5mdC levels were determined by liquid chromatography-mass spectrometry as described in Materials and Methods. Sample groups are the same as described in Fig. 1A. Points, individual samples; bars, mean of each sample group. Mann-Whitney test P values: **, P < 0.005; *, P < 0.05, for each group compared with normal prostate. B. B1 methylation in normal prostates and TRAMP samples. Methylation of the mouse B1 repetitive element was determined by quantitative bisulfite pyrosequencing as described in Materials and Methods. Points, individual samples; bars, mean of each sample group. Mann-Whitney test P values: **, P < 0.005; *, P < 0.05, for each group compared with normal prostate. Correlation analysis of RLGS hypermethylation events with global 5mdC levels or B1 repetitive element methylation in all TRAMP samples (C and D), or specifically in late stage (EPD and LPD) samples (E and F). Spearman rank-order correlation coefficients (r values) and P values are shown.

**FIGURE 7.** DNA methylation pathway alterations during prostate cancer progression in TRAMP. Timing and relative extent of distinct alterations in the DNA methylation pathway. Details of the model are explained in the Discussion.
Dnmt3b was detected using the NB 100-266 rabbit polyclonal antibody (Novus Biologicals). Cyclin A and E2F1 were detected using the sc-751 and sc-193 rabbit polyclonal antibodies, respectively (Santa Cruz Biotechnology), and Tag was detected with monoclonal mouse SV40 large T antigen antibody 554149 (BD PharMingen). Band density was analyzed using the Personal Densitometer SI instrument and ImageQuant 5.2 software (Molecular Dynamics).

RLGS and RLGS Spot Cloning
High molecular weight genomic DNA was isolated from TRAMP samples and nontransgenic control prostates as described previously (31). Individual tumor samples (~75 mg of tissue) were used for DNA isolation and 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 (19). Hypermethylated genes in TRAMP were identified by RLGS spot cloning as described previously (20, 32).

Quantitative Reverse-Transcriptase PCR
RNA samples were extracted from mouse tissues and converted to cDNA as described previously (11). PCR reactions were conducted using quantitative PCR SYBR MasterMix (Eurogentech) and the 7300 Real-time PCR System (Applied Biosystems). Primer sequences for analysis of p19, p16, Cacna1a, and 18s rRNA expression were designed using the Primer3 web-based program and are available upon request. SYBR green absolute quantification analysis was used to determine target gene copy number, which was normalized to 18s rRNA.

Sodium Bisulfite Sequencing
Genomic DNAs were isolated using the Puregene kit (Gentra Systems) and sodium bisulfite conversion was done using the EZ DNA Methylation Kit (Zymo Research). Sodium bisulfite sequencing primers were designed using MethPrimer (33) and are available upon request. Gradient PCR reactions were used to optimize annealing temperatures for each primer set. PCR products were directly cloned into the pTopoTA 4.1 vector (Invitrogen) and individual clones were sequenced at the Roswell Park Cancer Institute Biopolymer core facility, using an ABI prism automated DNA sequencer. DNA sequence information was analyzed using Lasergene (DNASTAR, Inc.). A minimum of 10 independent clones were sequenced per sample.

Determination of 5mdC Levels
5mdC levels were determined using liquid chromatography-electrospray ionization quadrupole mass spectrometry as described previously (34). Genomic DNAs were isolated using the Puregene DNA isolation kit (Gentra Systems) and 1 μg of genomic DNA samples were digested using four units of Nuclease S1 (Fermentas). All samples were analyzed in duplicate.

B1 Repetitive Element Pyrosequencing
Genomic DNA isolation and sodium bisulfite conversion were completed as described above. A bisulfite pyrosequencing assay for the murine B1 element was done as described previously (35), with slight modifications. The pyrosequencing primer (CpG2) was used (35). Pyrosequencing of the purified single-stranded PCR product was accomplished using the PSQ HS96 Pyrosequencing System (Biotage AB). The sequence analyzed contains two CpG sites (5'-CGAACCTCAGAAATCCG-3') and the mean methylation value of both sites was averaged for each sample. All samples were analyzed in duplicate.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Molecular Cancer Research

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