Hormone ablation is highly effective in the majority of patients with prostate cancer. However, it invariably fails over time, resulting in a state of androgen independence and disease progression. Thus, to improve treatment outcomes in prostate cancer, considerable efforts are being devoted to identifying and understanding key molecular pathways associated with the progression of prostate cancer after hormone ablation. The common genetic background, predictable tumor development, and multistage disease progression make the transgenic adenocarcinoma of mouse prostate (TRAMP) model highly useful for preclinical studies of prostate cancer, although, like all animal models, it has its limitations.

The TRAMP model has been used in prostate cancer studies for more than a decade. TRAMP mice express a PB-Tag transgene consisting of the minimal 426B2/8-bp regulatory element of the rat probasin promoter, which directs prostate-specific epithelial expression of the SV40 T antigen and c-myc were lower. These tumors also showed a reduction in proliferating cells compared with noncastrates and negatively responding tumors. Most of these changes disappeared 20 weeks after castration, by which time there was an increase in the size of primary tumors as well as in distant metastasis.

Conclusions: In TRAMP prostate cancer that responded positively to castration, different expression patterns of proteins involved in cellular apoptosis, stress, and proliferation occur ~10 weeks after castration. This may be an optimal time for targeting Bcl-2, and perhaps Grp78, to enhance the antitumor effects of androgen deprivation.

Abstract

Purpose: Divergent responses to androgen deprivation have been found in patients and in animal models of prostate cancer. The molecular basis for these different outcomes is unknown. Our aim was to identify the molecular responses of prostate cancer with divergent outcomes to androgen deprivation in TRAMP mice.

Experimental Design: Castrated and noncastrated B6xFVB TRAMP mice were evaluated for survival, tumor development, pathology, and expressions of specific proteins at different time points.

Results: TRAMP mice responded differentially to androgen deprivation. In the majority, primary tumors regressed after castration (positive response), whereas in others the tumors grew even more aggressively than in the noncastrated mice (negative response). Mice with regressed tumors had the highest survival rates. Androgen receptor was elevated in all tumors from castrated mice despite significant differences in tumor sizes. In positively responding tumors, expressions of Bcl-2 and Grp78 were greatly increased by 10 weeks after castration, whereas expressions of Bax, Bcl-xl, SV40 T antigen, and c-myc were lower. These tumors also showed a reduction in proliferating cells compared with noncastrates and negatively responding tumors. Most of these changes disappeared 20 weeks after castration, by which time there was an increase in the size of primary tumors as well as in distant metastasis.

Conclusions: In TRAMP prostate cancer that responded positively to castration, different expression patterns of proteins involved in cellular apoptosis, stress, and proliferation occur ~10 weeks after castration. This may be an optimal time for targeting Bcl-2, and perhaps Grp78, to enhance the antitumor effects of androgen deprivation.

Hormone ablation is highly effective in the majority of patients with prostate cancer. However, it invariably fails over time, resulting in a state of androgen independence and disease progression. Thus, to improve treatment outcomes in prostate cancer, considerable efforts are being devoted to identifying and understanding key molecular pathways associated with the progression of prostate cancer after hormone ablation. The common genetic background, predictable tumor development, and multistage disease progression make the transgenic adenocarcinoma of mouse prostate (TRAMP) model highly useful for preclinical studies of prostate cancer, although, like all animal models, it has its limitations.

The TRAMP model has been used in prostate cancer studies for more than a decade. TRAMP mice express a PB-Tag transgene consisting of the minimal 426B2/8-bp regulatory element of the rat probasin promoter, which directs prostate-specific epithelial expression of the SV40 early genes (T/t antigens; ref. 1). Spontaneous multistage tumor progression in the TRAMP prostate begins at the age of 8 to 12 weeks, and 100% of the mice develop prostate cancer by the age of 20 weeks. Subsequently, metastasis can occur in lymph nodes and distant sites as tumors progress (2–5).

Surgical castration has commonly been used in studies of prostate cancer response to androgen ablation (6–9). Several investigators have reported that primary TRAMP tumors show differential response to castration; some tumors regress, whereas the others continue to grow after castration (10, 11). These divergent results within mice of the same genetic background are not fully understood but likely reflect heterogeneity in primary tumors and underscore the complexity of cancer pathogenesis and progression.

In an effort to understand the divergent outcomes to androgen deprivation, we used B6xFVB TRAMP mice to study the response of prostate cancer to castration therapy. As reported previously (11), the primary tumors responded either positively (regressed) or negatively (continued to grow) to castration. Further, for tumors that initially regressed on castration, a state
of androgen independence eventually developed over time, thus defining several disease states of prostate cancer in the TRAMP model. The novel finding of the present study is that differential expression of specific proteins associated with cellular stress, proliferation, and apoptosis occurs in the various disease states of prostate cancer in this model, likely contributing to the different biological outcomes observed in TRAMP mice after castration. This study provides some rationale for targeting specific proteins in appropriately defined states of prostate cancer for enhanced antitumor effect.

Materials and Methods

Animals. C57B/6 TRAMP mice breeding pairs were provided by the NIH Animal Facility (Fredrick, MD). Crossbreeding was done in house to generate B6xFVB TRAMP mice. The probasin SV40 T antigen (PB-Tag) transgene was identified by PCR using tail DNA. Male mice that were heterozygous (+/-) for the transgene were randomly assigned to various groups. Body weight was measured weekly and the general condition of the mice was monitored daily up to 36 wk. As tumors progressed, some TRAMP mice were euthanized earlier based on whether they (a) developed significant regional tumor burden that compromised their mobility or (b) lost >20% of original body weight, combined with other symptoms such as labored breathing and/or decreased mobility. In addition, some mice were specifically sacrificed at defined time points for the purpose of sample collection.

Pathology. At necropsy, the genitourinary tract was dissected from the mice; this consists of seminal vesicles, prostate (including dorsal lateral, ventral, and anterior lobes), urethra, and ampullary and coagulating glands. The bladder was excluded. The genitourinary tract was weighed, and the ratio of genitourinary tract versus mouse body was calculated using the following formula: weight (G)/body weight (B) = genitourinary weight (G)/body weight (B)] × 100. The dorsolateral prostate lobe, which includes ventral, dorsal, and lateral prostate [according to the Pathological Classification of Prostate Lesions in Genetically Engineered Mice (12)], and visible tumors were further separated from the genitourinary tract under magnification (×4). Tissues for histopathology and immunohistochemistry were fixed in 10% paraformaldehyde for 4 h and then transferred to 70% ethanol before routine paraffin embedding and sectioning. Tissues for Western blot analysis were frozen immediately in liquid nitrogen and stored at -80°C for later use. Mouse organs, including lung, liver, kidney, bladder, and brain, were routinely collected at necropsy for histopathology.

Following the Bar Harbor Classification System for Genetically Engineered Mice (12), we graded the TRAMP tumors as follows: microinvasive, well differentiated, moderately differentiated, and poorly differentiated. Neuroendocrine differentiation was also observed in some cases (Supplementary Figure).

Castration. After anesthesia with ketamine/xylazine (80 mg/5 mg/kg), testes were gently pushed into the scrotum and a 0.5-cm incision was made under sterile conditions. Each testis was surgically removed, and the spermatic cord and vascular plexus were tied with sterile suture to prevent hemorrhaging. A wound clip applicer was used to close the skin incision and then was removed 1 wk after surgery.

PCR screening. Mouse DNA was isolated from tail tissue using the DNeasy Tissue kit (Qiagen, Inc.). The pair of synthetic primers used for PCR was 5'-CAGACGCAATTGCGCTTG-3' and 5'-GGACAAACCACAATAGACTGAG-3'. PCR conditions were as follows: initial denaturation at 94°C for 3 min, 35 cycles of amplification at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, followed by final extension at 72°C for additional 3 min.

Western blot. Twenty-five micrograms of protein extract from tissue samples were loaded onto each well of NuPAGE 4% to 12% Bis-Tris Gels (Invitrogen). After separation, proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp.). The membranes were incubated overnight at 4°C in appropriate concentrations of polyclonal antibodies, individually,
**Table 1. Prostate cancer development in 12- to 16-wk-old TRAMP mice**

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Total no.</th>
<th>G/B ratio</th>
<th>Pathology grade</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-16</td>
<td>14</td>
<td>2.0 ± 0.36</td>
<td>MI 4</td>
<td>WD 5</td>
</tr>
</tbody>
</table>

Abbreviations: MI, microinvasive; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; LN, lymph node.

against androgen receptor (AR), SV40 Tag, Bcl-2 (Santa Cruz Biotechnology), Bcl-xl (BD Biosciences), Bax (Upstate), c-myc, Grp78 (Cell Signaling), and β-actin (Sigma). Horseradish peroxidase–labeled secondary antibodies were then applied to the membranes for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence Western blotting detection reagents (GE Amersham Biosciences), digitized via densitometry (Molecular Dynamics), and normalized with respect to β-actin.

**Immunohistochemistry and image analysis.** Paraffin sections were dewaxed and heated by microwave for 20 min in Tris-EDTA (pH 9.0) for antigen retrieval. After quenching the endogenous peroxidases with 3% H₂O₂ for 5 min, blocking buffer (10% goat serum and 2% goat anti-mouse IgG in PBS) was applied to decrease nonspecific background. The tissue sections were incubated overnight at 4°C with antibodies against SV40 Tag (Santa Cruz Biotechnology), Grp78, and PCNA (BD PharMingen). Isotype-specific IgG for each antibody was used as negative control. After labeling cells with horseradish peroxidase–labeled secondary antibodies, Liquid DAB substrate Chromogen System (DaroCytoMation) was used to visualize the positive signals.

A digital image system was used to evaluate the signals. Briefly, 10 images of each tissue section were captured under a Nikon TE2000 microscope at a magnification of ×400 and saved as TIFF files using Act-1 software (Nikon Corp.). Images were analyzed with MICD software 7.0 (GE Amersham Biosciences) using a special function called automatic grain count. With this function, positively stained cells can be automatically detected and digitized based on their absorbance, color hue, color intensity, and saturation. The average grain count on each slide (10 images) was used to represent a particular sample.

**Statistical analysis.** The G/B ratios and survival data were analyzed for noncastrated and castrated mice. The Wilcoxon rank sum test was used to estimate and compare the average G/B ratios across treatment groups. The Kaplan-Meier method was applied to estimate survival times. Survival functions were assessed over the 36-wk study period. Animals sacrificed at 36 wk (end of study) were treated as censored (no event); animals that died for other reasons, including sickness and large tumor burden, were accounted for using the competing risks approach. All statistical tests were done at the 0.05 level of significance.

**Results**

A total of 54 mice were studied. Twenty-one mice were sacrificed at early time points for sample collection purposes, including 14 noncastrated mice at 12 to 16 weeks of age and 7 castrated mice at the 10-week postcastration time point. Thirty-three mice were followed longitudinally up to 9 months (36 weeks); these included 10 noncastrated and 23 castrated mice.

**Pathogenesis of prostate cancer in B6fxFVB TRAMP mice.** To obtain G/B ratios and background information about prostate cancer development at early time points, 14 TRAMP mice were sacrificed at 12 to 16 weeks of age. Histopathology showed prostate cancer in all mice at this stage, and neoplastic proliferation was restricted to the epithelia of dorsolateral prostate lobes. Immunohistochemistry showed that only proliferating cells were SV40 Tag positive (Fig. 1A). The G/B ratios among these mice ranged from 1.28 to 2.59 (2.0 ± 0.36), and the pathologic grades of the prostate tumors were microinvasive (two), well differentiated (six), moderately differentiated (five), and poorly differentiated (one; Fig. 1B; Table 1). Thus, tumors at 12 to 16 weeks of age were uniformly small in size and primarily well or moderately differentiated. Further, no local or distant metastasis was detected.

In the 10 mice followed longitudinally without castration, significantly enlarged abdomens were observed in all the noncastrated mice after they reached the age of 20 weeks. As tumors progressed, seven mice had to be euthanized before 36 weeks due to labored breathing and mobility; only three mice (30%) survived to 36 weeks. The average G/B ratio for the entire group was 18.81 ± 7.13, and the mean age at death was 28.9 ± 5.74 weeks (Table 2). At necropsy, tumors occupied most of the abdominal cavity, with or without hemorrhagic ascites. Local lymph node metastasis was seen in all but one mouse (90%); periaortic lymph nodes were usually the first to be involved. Distant metastasis occurred in 30% of the mice and was detected either at necropsy (gross metastasis) or by histopathology (micrometastasis; Table 2). The majority of tumors were poorly differentiated by histopathology (Table 2). Western blot analysis showed similar levels of AR in prostate tissues, but increased SV40 Tag expression was observed in mice after 18 weeks of age and remained elevated thereafter (Fig. 1C).

**Table 2. Comparison between noncastrated and castrated mice monitored up to 36 wk**

<table>
<thead>
<tr>
<th></th>
<th>Mean G/B ratio</th>
<th>Mean AAD (wk)</th>
<th>PD tumors</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LN</td>
</tr>
<tr>
<td>Noncastrated (n = 10)</td>
<td>18.81 ± 7.13</td>
<td>28.9 ± 5.74</td>
<td>7 (70%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>Castrated (n = 23)</td>
<td>25.43 ± 5.25</td>
<td>24.33 ± 5.07</td>
<td>7 (78%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>Cas-L (n = 9)</td>
<td>2.43 ± 1.31</td>
<td>30.5 ± 6.75</td>
<td>8 (57%)</td>
<td>13 (93%)</td>
</tr>
</tbody>
</table>

Abbreviation: AAD, average age at death.
Approximately 10% of the mice used in this study also developed neuroendocrine tumors (Supplementary Figure). To better understand the changes within the dominant non-neuroendocrine tumor components, we excluded neuroendocrine tumors from our protein studies.

Castration effects in TRAMP mice. Thirty mice were castrated around the age of 14 weeks. Twenty-three mice were followed longitudinally up to 36 weeks (the other 7 mice were sacrificed 10 weeks after castration; see below).

Based on G/B ratios, two categories of tumors were found among the 23 castrated mice ($\geq 5 = $ large, $< 5 = $ small; Fig. 2A). Fourteen mice (61%) had small tumors (Cas-S), with an average G/B ratio of 1.41 ± 1.31 at the time of death. Other mice (39%) developed large tumors (Cas-L) with an average G/B ratio of 25.43 ± 5.25, which was 1.4-fold higher than even the noncastrated mice (18.81 ± 7.13; $P = 0.03$; Fig. 2A; Table 2). In addition, the average age at death of the Cas-L mice was 24.33 ± 5.07 weeks, which is 4.5 weeks shorter than that of the noncastrated mice ($P = 0.04$), and no Cas-L mouse survived to the end of the experiment (36 weeks). In contrast, the Cas-S mice had the longest survival times, with an average age at death of 30.5 ± 6.75 weeks ($P = 0.01$, compared with Cas-L), and 7 of 14 mice (50%) survived to the end of the experiment. Statistical analysis showed that there was a significant difference in survival rates between Cas-S mice and the others (Cas-L and noncastrated mice; $P = 0.01$; Fig. 2B, top).

However, when comparing the noncastrated mice with all castrates (Cas-S and Cas-L as a group), only borderline difference in survival was observed ($P = 0.06$; Fig. 2B, bottom).

Histopathology showed that all castrated mice developed prostate cancer in their dorsolateral prostates by the time of death, and the majority of the cancers were poorly differentiated. No significant difference in lymph node metastasis was observed between the noncastrated and castrated mice (Table 2). However, distant metastases were more common in the castrated mice (18 of 23, 78%) compared with the intact mice (3 of 10, 30%; Table 2). Among the castrates, although a similar incidence of distant metastasis occurred in the Cas-L mice (89%) and the Cas-S mice (71%), 7 of 8 Cas-L mice had gross distant metastasis at the time of death, whereas only 4 of 10 Cas-S mice had visible metastatic tumors at necropsy. Thus, gross metastasis was more common in Cas-L mice, whereas micrometastasis occurred with greater frequency in Cas-S mice (Table 2). Consistent with previous reports, lungs were the most common site of distant metastasis, although other organs could also be involved (2–5).

The above data show that, on androgen withdrawal, our TRAMP mice could be divided into two categories. In the majority, the primary tumors regressed (Cas-S), but in the others, tumors continued to grow (Cas-L) and grew even faster than in the noncastrated mice. Overall, the proportion of mice with distant metastasis was higher in the castrated mice than in

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Fig. 2. Castration effects on TRAMP mice. A, comparison of G/B ratios between noncastrated mice and castrated mice. Tumors can be divided into two groups after castration based on the G/B ratio: $\leq 5 = $ small tumor (Cas-S); $> 5 = $ large tumor (Cas-L). B, Kaplan-Meier estimates of survival among noncastrated mice and mice with Cas-S or Cas-L tumors.
the noncastrated mice, with more gross metastasis observed in Cas-L than in Cas-S mice. Significantly longer survival was associated with Cas-S mice, whereas most Cas-L mice died even earlier than the noncastrated mice.

**Cas-S tumors at early and late time points.** As noted above, 60% of primary tumors regressed after castration, with G/B ratios remaining low throughout (Cas-S mice). Furthermore, these mice had the longest survival, with 50% (7 of 14) living until the end of the experiment (36 weeks). Mice surviving ≥20 weeks after castration were designated as Cas-S20. In an effort to understand the biological properties of the Cas-S tumors, an additional seven mice that were castrated around the age of 14 weeks were sacrificed at 10 weeks after castration. The average G/B ratio among these seven mice was 0.37 ± 0.14; these mice were designated as Cas-S10.

Histopathology showed that four of the seven Cas-S10 mice had developed poorly differentiated primary tumors but none had gross metastasis, except one mouse with micrometastasis in the lungs and one with local lymph node metastasis.

**Immunohistochemistry and Western blot analysis.** Immunohistochemistry and Western blot were used to analyze the expressions of several proteins in tumor tissues. Based on the response to castration, tumors were grouped into four categories: intact (noncastrated), Cas-S, Cas-S10, and Cas-L. Because the majority of cancers in all four groups were poorly differentiated, to maintain uniformity, we restricted our analysis to poorly differentiated tumors. The intact, Cas-S, and Cas-S10 tumors were from mice 24 to 26 weeks of age, whereas the Cas-S20 tumors were from mice 34 to 36 weeks of age.

**Expressions of AR, Tag, and markers of proliferation and stress.** The expression of AR increased in both Cas-S and Cas-L tumors. On Western blot, the average AR band density in tumors from castrated mice (n = 8) was ~3-fold higher than...
that from noncastrated mice \( (n = 4) \) after normalization to β-actin (Fig. 3A). This is consistent with the known effects of androgen ablation on AR expression.

Tag expression in intact mice increased and then remained elevated as they matured (Fig. 1C). After castration, minimal levels of Tag were seen in Cas-S10 tumors \( (n = 4) \) by both Western blot (Fig. 3A) and immunohistochemistry (Fig. 3B). In contrast, high levels of Tag protein were observed in Cas-L \( (n = 4) \) and Cas-S20 tumors \( (n = 5) \); Fig. 3A and B).

Among these four groups of tumors, PCNA staining showed that only the Cas-S10 tumors \( (n = 4) \) had a significantly lower number of proliferating cells (Fig. 3C). Consistent with this result, c-myc was undetectable in Cas-S10 tumors \( (n = 4) \) by Western blot, whereas higher levels of c-myc were noted in other groups of tumors (Fig. 3A), in keeping with their higher proportions of proliferating cells.

The reduced numbers of proliferating cells in Cas-S10 tumors that responded positively to androgen withdrawal suggest that the cancer cells in these tumors were under cellular stress. To test this hypothesis further, expression of cell stress protein Grp78/Bip was evaluated by immunohistochemistry andWestern blot. The results showed prominent overexpression of Grp78 only in the Cas-S10 tumors (Fig. 3A and D). The Grp78 band density on Western blots was >100-fold higher in Cas-S10 tumors after β-actin normalization compared with all other groups. No significant differences in Grp78 expression were found among Cas-S20, Cas-L, and intact tumors (Fig. 3A and D).

**Apoptotic proteins in tumors.** The same tissue samples used in the above experiments were used for testing apoptotic proteins by Western blot. Compared with intact and Cas-L tumors, Bcl-2 was highly expressed in all the Cas-S10 samples examined (Fig. 4). Interestingly, Cas-S20 tumors showed a mixed response with respect to Bcl-2 expression in that only two of the five tumors evaluated had elevated Bcl-2 levels, whereas minimal expression of Bcl-2 was observed in the others (Fig. 4). Notably, expressions of the antiapoptotic protein Bcl-xl and the proapoptotic protein Bax decreased to almost undetectable levels in the Cas-S10 tumors, in sharp contrast to the other groups in which the tumors expressed high levels of both proteins (Fig. 4).

**Discussion**

In this study, we focused on the effects of androgen ablation on primary tumor development in TRAMP mice. Consistent with prior reports, in mice followed longitudinally after castration, primary tumors could be grouped into two categories: large tumors (Cas-L; G/B ratio, 25.43 ± 5.25) or small tumors (Cas-S; G/B ratio, 1.41 ± 1.31; Table 2). Because all noncastrated mice \( (n = 14) \) before the age of 16 weeks had small G/B ratios \( (2.0 ± 0.36; \text{Table 1}) \), it is highly unlikely that Cas-L tumors developed before castration (which was done around week 14). Of note, the average G/B ratio of mice with Cas-L tumors \( (25.43 ± 5.25) \) was even higher \((14.4 \text{-fold})\) than the noncastrated mice \( (18.81 ± 7.13; P = 0.03; \text{Table 2}) \). Thus, all primary tumors responded either positively (Cas-S) or negatively (Cas-L) to castration. Mice with tumors that responded positively to castration maintained low G/B ratios throughout, whereas tumors that responded negatively seemed to grow even more rapidly than those in the noncastrated mice (Table 2). Although similar rates of distant metastasis were observed in the Cas-L and Cas-S tumors \( (89\% \text{ versus } 71\%) \), not unexpectedly, mice with Cas-L tumors developed more gross metastatic disease and had the shortest survival (Table 2; Fig. 2B, top). In contrast, most mice with Cas-S tumors lived longer and had primarily microscopic metastases. These results suggest that, despite having the same genetic background, TRAMP mice can respond differently to castration. Although a lower incidence of distant metastasis might be expected for Cas-S mice, their longer survival may have allowed more time for distant metastases to develop. This might account for the overall similar metastatic rates between Cas-S and Cas-L mice at necropsy. Interestingly, although the majority of TRAMP tumors exhibited poorly differentiated histology, most noncastrated mice had locoregional disease, whereas castration seemed to trigger metastatic spread, which is consistent with previous reports (2–5).

The expression of AR increased in all castrated mice tested irrespective of whether they responded positively or negatively to androgen deprivation (Fig. 3A), as has been observed in other preclinical models (6) of prostate cancer as well as in the clinical setting (13, 14). In TRAMP mice, expression of the oncoprotein Tag is responsible for prostate cancer initiation and development. The gene encoding T/t antigen is under the control of the probasin promoter, which harbors AR response elements. Thus, probasin promoter activation is regulated by androgen, and the levels of androgen are a key regulator in tumor development and growth. Tag was highly expressed in intact, Cas-L, and Cas-S20 tumors. In contrast, only minimal levels of Tag were detected in Cas-S10 tumors (Fig. 3A and B). This indicates that the expression of Tag in Cas-S10 tumors is androgen dependent because removal of androgens by castration decreased probasin promoter-directed Tag expression in these tumors. In Cas-S20 tumors, on the other hand, Tag was highly expressed despite androgen ablation, which suggests that these tumors had become androgen independent. Although mice with both Cas-S10 and Cas-S20 tumors had low G/B ratios, the average G/B ratio of mice with the Cas-S20 tumors was ~4-fold higher than those with the Cas-S10 tumors \( (1.41 ± 1.31 \text{ versus } 0.37 ± 0.14) \). Further, 10 of 14 (71\%) mice with Cas-S20 tumors had distant metastasis (gross or microscopic) compared with only 1 of 7 (14\%, microscopic) of the mice with Cas-S10 tumors. Taken together, these data indicate that although tumors within the Cas-S group responded positively to castration, the Cas-S20 tumors primarily exhibit an androgen-independent, more aggressive biological phenotype.
manifested with androgen-independent Tag expression, larger G/B ratios, and a higher incidence of distant metastasis.

Consistent with the inhibitory effects of castration, Cas-S10 tumors had the lowest proportion of proliferating cells among the four tumor groups (Fig. 2C). There is a strong correlation between c-myc family proteins, especially c-myc, and cell growth, differentiation, and proliferation (reviewed in ref. 15). Overexpression of c-myc can elicit a proliferative response, whereas low levels of c-myc are generally characteristic of quiescent cells (15). As another measure of cell proliferation, we also evaluated c-myc expression in the above tumors. c-myc protein was essentially undetectable in Cas-S10 tumors, whereas higher levels were observed in Cas-L and Cas-S20 tumors (Fig. 3A). Further, prominent expression of the stress response protein Grp78 was only seen in the Cas-S10 tumors (Fig. 3A), which suggests that most of the low-proliferating/growth-arrested cells in these tumors were under stress as a result of androgen withdrawal. In recent reports, the stress protein Grp78 has been shown to have antiapoptotic properties (16), and increased levels of Grp78 were observed in castration-resistant prostate cancer (17). In our study, high expression of Grp78 was observed in tumors at the "early" (10 weeks) but not later time points (≥20 weeks) after castration. Because androgen ablation inflicts a form of cellular stress, higher levels of Grp78 would be expected in cancer cells that respond to androgen ablation, as opposed to cells that have escaped its suppressive effects.

Although androgen-dependent and androgen-sensitive prostate cancer cells initially undergo growth arrest and/or apoptosis on androgen withdrawal, cancer cells not removed from the population by cell death can potentially serve as a nidus for subsequent androgen-independent growth. Notably, Bcl-2 was highly expressed in the Cas-S10 tumors (Fig. 4) at a stage when they were still biologically responsive to castration (as shown by low G/B ratios coupled with low Tag expression and low numbers of proliferating cells).

A consequence of enhanced Bcl-2 expression at the 10-week postcastration time point is that it allows at least some of the Cas-S10 cancer cells to survive the stress and proapoptotic effects of androgen withdrawal. Also of note is that expression of both Bcl-xL and Bax decreased sharply in Cas-S10 tumors, whereas these proteins were expressed in both Cas-S20 and Cas-L tumors at levels similar to those in the noncastrated mice (Fig. 4). Although mechanisms underlying the differential regulation of these proteins in our TRAMP mice are presently not clear, the down-regulation of Bax that was specific to the Cas-S10 tumors would enhance the protective effects of Bcl-2 due to favorable antiapoptotic Bcl-2 to Bax ratios. The other antiapoptotic protein, Bcl-xL, is unlikely to fulfill this protective role in Cas-S10 tumors because it is minimally expressed in them, further underscoring the relevance of Bcl-2 at the 10-week postcastration time point. Increased levels of Bcl-2 after androgen ablation have been shown to occur in several in vitro and in vivo systems of prostate cancer (18). Higher Bcl-2 levels are also detected in cells under stress (19), cancer stem cells (20), and basal cells of the prostate epithelium (18). In addition, overexpression of Bcl-2 has been shown to result in abnormal accumulation of noncycling cells (21, 22). Ultimately, a population of Cas-S10 cells surviving the effects of androgen withdrawal could, over time, provide a pathway for androgen-independent growth and progression, as occurred at later time points after castration (Cas-S20 tumors).

It is apparent that within the group of mice responding positively to castration, a transformation occurs from a state that is responsive to androgens (Cas-S10) to a state that is essentially independent of androgens. Although the Cas-S20 tumors still had relatively small G/B ratios, they escaped the stress of androgen withdrawal (Grp78 returned to basal levels) and were in a proliferative state (Fig. 3). In contrast to Cas-L tumors, the Cas-L tumors represent the other end of the spectrum in terms of response to androgen withdrawal, as shown by their even more aggressive locoregional and systemic growth than the noncastrated tumors. The biological behavior of Cas-L tumors was different from the noncastrated tumors because they seemed to be triggered on castration. In addition, although both Cas-L and Cas-S20 tumors showed androgen independence, they were quite different: Cas-L tumors showed continued progression after castration, whereas Cas-S20 tumors evolved from an intermediate (Cas-S10) stage. Consistent with the androgen independence of Cas-L and Cas-S20 tumors, their expression profiles with respect to Grp78, PCNA, c-myc, and apoptotic proteins, particularly Bcl-xL and Bax, were similar and paralleled those of intact tumors not subjected to any androgen withdrawal (Figs. 3 and 4). It is likely that other, perhaps differentially regulated, pathways were being recruited, including possibly stromal and/or neuroendocrine paracrine effects, which could account for the different growth patterns of Cas-L and Cas-S20 tumors.

Interestingly, in a study by Eng et al. (23), in which TRAMP mice were castrated much earlier (i.e., at 4 weeks) than in our study, some mice developed aggressive tumor growth, similar to Cas-L mice, whereas most had small prostates up to 37 weeks of follow-up. Thus, although early castration can prevent cancer development in a proportion of mice, it is still permissive in other mice, allowing cancer to develop despite the hormone ablation (23). In the Eng et al. study, a statistically significant survival difference was noted between the castrated and noncastrated mice, whereas in our study borderline difference in survival occurred between the two groups (Fig. 2B, bottom). This may, in part, be due to the differences in the time of castration in the two studies (4 weeks versus 14 weeks).

In summary, despite the complexity in the response to androgen withdrawal in the TRAMP model, several noteworthy features have emerged from this study. It is apparent that the primary tumors responded either positively or negatively to castration. For tumors that responded positively to castration, androgen-independent growth emerged from tumors that were initially responsive to androgen withdrawal. In the negatively responding tumors, androgen-independent growth seems to be triggered soon after castration. Potentially, Cas-L and Cas-S20 tumors can serve as two, but not necessarily exclusive, models of androgen-independent growth. In contrast to Cas-L and Cas-S20 tumors, consistent expression profiles with respect to Grp78, c-myc, Bcl-2, Bcl-xL, and Bax were observed in all Cas-S10 tumors examined. Increased expression of Bcl-2 and Grp78 occurred within a particular time frame (10 weeks after castration) in tumors that responded positively to androgen ablation.

Clinically, after their initial response to androgen deprivation, prostate cancers generally progress despite the androgen
suppression. Promising results from preclinical studies provide a rationale for targeting Bcl-2 to suppress its antiapoptotic role in cancer; this approach is actively undergoing clinical testing (reviewed in refs. 24–26). Antisense oligonucleotides to Bcl-2 mRNA have also been evaluated in pilot studies on hormone-refractory prostate cancer (reviewed in ref. 27). Our results suggest that a strategy of targeting Bcl-2, and perhaps Grp78, while tumors are still under the effect of androgen deprivation may enhance the outcome of hormone therapy. The data indicate that targeting Bcl-2 may be less useful for tumors that are unresponsive to androgen ablation (like Cas-1 tumors in our animal model).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

Divergent Effects of Castration on Prostate Cancer in TRAMP Mice: Possible Implications for Therapy

Yao Tang, Linbo Wang, Olga Goloubeva, et al.

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