INTRODUCTION

Neuroendocrine (NE) cells may be involved not only in growth and differentiation of the normal prostate but also in carcinogenesis and development of prostate adenocarcinoma (Pca), including development of androgen resistance. However, the exact pathophysiology of NE cells in prostate cancer growth and metastatic development through induction of androgen-independent NE carcinoma has been postulated as one mechanism of development of NE carcinoma. Recent studies have reported that NE differentiation is present in normal and abnormal prostate tissue, including prostatic intraepithelial neoplasia (PIN) and invasive carcinoma. A recent study by the authors identified similar protein profiles between NE and ductal carcinoma in situ with NE features in human prostate tissue. These findings support the hypothesis that NE cells may be involved in prostate cancer growth and metastatic development.

The authors previously developed a transgenic mouse model that expresses human insulin-like growth factor-1 driven by a bovine keratin promoter linked to the SV40 early region. This model showed significant NE differentiation in the prostate with age, including increased NE cell numbers and expression of NE markers such as chromogranin A and synaptophysin. Additionally, the authors observed the development of NE carcinoma in these mice, which progressed to invasive adenocarcinoma and metastatic disease.

In this study, the authors further characterized the NE differentiation process in the transgenic mouse model and examined the role of NE cells in prostate cancer development. They found that NE cells are involved in the development of precursor lesions, such as PIN and invasive carcinoma, and that NE carcinoma cells are associated with increased proliferation and invasion. The authors also observed that NE cells may play a role in the progression of primary tumors and metastatic disease in the transgenic mouse model.

To better understand the role of NE cells in prostate cancer, the authors performed a comparative analysis of NE and ductal carcinoma in situ (DCIS) tissue from human prostate biopsies and xenografts. They found that NE cells are present in both human and transgenic mouse PIN and invasive carcinoma tissue, with increased expression of NE markers in the transgenic mouse model. These findings support the hypothesis that NE cells may be involved in the development of prostate cancer and provide new insights into the pathogenesis of NE carcinoma.
the prostate. At 24 weeks of age, 40% of the mice showed metastatic NE cancer in regional lymph nodes, liver, lung, and bone marrow (13). Although this promoter was subsequently shown to target an unrelated gene to the same cell population (13), its specificity for the prostate remains to be established.

The use of prostate-specific promoters is essential for limiting tumor development to the prostate and further assuring that metastatic tumors arise from prostate primaries versus other tumors that can occur with more widely expressed transgenes. The probasin promoter represents a useful prostate-specific promoter, specifically targeting transgene expression. Probasin is structurally related to the lipocalin protein superfamily (14). Although the ligand and the function of the protein remain unknown, probasin gene expression is prostate specific, regulated by androgen and zinc (15–17). Several transgenic mice that target transgene expression to the prostate have been generated using a small fragment of the probasin promoter (−426 to +28 bp) or a large fragment of the LPB promoter (−11,500 to +28 bp). Both probasin constructs confer androgen regulation and prostate-specific gene expression, although the level of transgene expression is greater with the LPB promoter (17–20). We have established seven transgenic mouse lines with a LPB promoter linked to SV40-large Tag (LPB-Tag), which contains a deletion in the early region to remove expression of the small Tag (17). In a previous study, we demonstrated that the rate of prostate growth varies among the individual lines, likely due to the level of transgene expression. The rapidly growing LPB-Tag lines exhibited similar histopathological alterations, which preferentially start in the dorsolateral prostate, characterized by prominent glandular proliferation and cytologic atypia and generally accompanied by stromal hypercellularity. The large size of the prostate precludes maintaining some of these lines to later ages, and invasive disease is limited. In this study, we have characterized in detail the LPB-Tag line that shows the slowest prostate growth rate. We report that this line develops precursor lesions more analogous to human HGPIN, without associated prominent stromal hypercellularity (also more similar to human Pca). Furthermore, this line predictably develops IC with glandular differentiation (adenocarcinoma) as well as NE Pca that commonly metastasizes.

MALDI-TOF-MS is an ideal tool for the rapid analysis and characterization of proteins (21, 22). In recent years, it has been used in combination with several types of separation methods to characterize and sequence proteins from complex biological mixtures. Some studies have focused on the identification of peptides and proteins involved in cancer (23, 24). Identical protein profiles on MALDI-TOF-MS between prostate primary and extraprostatic NE carcinoma support that NE carcinomas in the extraprostatic organs are metastases that originated in the prostate.

MATERIALS AND METHODS

LPB-Tag Transgenic Mouse Line. LPB-Tag transgenic mouse lines were established with the 5′-flanking region of the rat LPB gene (−11,500 to +28 bp; Ref. 20) linked to the SV40-Tag gene deletion mutant (dl 2005), which removed the expression of the small Tag. LPB-Tag transgenic mice were generated by microinjection of the DNA into the male pronucleus of a fertilized oocyte. Seven transgenic lines were established and maintained in the CD1 mouse strain (17). The 12T-10 transgenic line showed the slowest expression of the small Tag, which contains a deletion in the early region to remove expression of the small Tag (17). In a previous study, we demonstrated that the rate of prostate growth varies among the individual lines, likely due to the level of transgene expression. The rapidly growing LPB-Tag lines exhibited similar histopathological alterations, which preferentially start in the dorsolateral prostate, characterized by prominent glandular proliferation and cytologic atypia and generally accompanied by stromal hypercellularity. The large size of the prostate precludes maintaining some of these lines to later ages, and invasive disease is limited. In this study, we have characterized in detail the LPB-Tag line that shows the slowest prostate growth rate. We report that this line develops precursor lesions more analogous to human HGPIN, without associated prominent stromal hypercellularity (also more similar to human Pca). Furthermore, this line predictably develops IC with glandular differentiation (adenocarcinoma) as well as NE Pca that commonly metastasizes.

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Histological Classification of the 12T-10 Prostates. After an initial review of the slides as generated to characterize the evolution of the neoplastic lesions in question, the prostates of 12T-10 mice were classified in a blinded manner as containing LGPIN, HGPIN, MI, IC, UC, or combinations of those lesions by a single pathologist (S. B. S.; Fig. 1). LGPIN and HGPIN were characterized on the general histological and conceptual similarity to those found in human prostate (Fig. 1, A and B), as determined by stratification and crowding of the epithelial cells within basement membrane-bound preexisting ducts and glands and with cytological abnormalities, including nuclear and nucleolar enlargement (25). LGPIN showed round to oval slightly enlarged nuclei with stratification. HGPIN was distinguished from LGPIN primarily on the basis of accentuation of these features and particularly by the presence of marked nuclear atypia. In general, the nuclei in HGPIN in the mice are more hyperchromatic and have more chromatin clumping than the typically vesicular nuclei with prominent nucleoli in human HGPIN. In addition, the mitotic and apoptotic rates are greater than those typically seen in human HGPIN. The designation of HGPIN analogous to the more global concept of a cancer precursor in human prostate is further justified by the fact that IC develops in this model and always develops in association with this apparent precursor lesion, as described later. MI was recognized as foci of single cells or groups of cells breaking through the basement membrane of HGPIN-containing ducts/ glands into the thin rim of surrounding stroma (Fig. 1C). In addition, microacinar clusters at the base of HGPIN glands, often compressing the thin surrounding stroma, were strongly suspicious for invasion. The architecture of these foci was considered more than tangential sectioning of outgrowths of HGPIN. Criteria useful for recognizing invasion in the human prostate include sufficient spatial separation of small acini from adjacent HGPIN glands or infiltration among benign glands. However, these are not generally applicable in the murine prostate because HGPIN is present extensively or uniformly in this model (infiltration among benign glands is not observed), and the stroma surrounding the glands is extremely thin (composed of only one or two cell layers in wild-type mice or transgenic mice without stromal hypercellularity). Lesions were designated as IC when they were more extensive than those described above as MI, with foci unequivocally demonstrating stromal invasion and occasionally extending into surrounding periprostatic tissue. IC demonstrated multiple histological patterns, including small nests of cells with glandular differentiation (adenocarcinoma), small nests without discernible differentiation, and microscopic foci with cytological and histological features of NE differentiation (Fig. 1, D–F). Larger invasive lesions (>2–3 mm) often showing destructive overgrowth of normal prostate architecture essentially always lacked glandular differentiation and showed cytological and histological features typical of NE differentiation. These included scant cytoplasm, with high nuclear:cytoplasmic ratios, hyperchromatic or coarsely granular (“salt and pepper”) chromatin, nuclear molding, and rosette formation. Such foci were designated as UC (Fig. 1, F–H).

Immunohistochemistry. Immunostaining was performed on 5–μm-thick paraffin sections, which were deparaffinized and rehydrated, using standard techniques. For Tag, AR, and PCNA immunostaining, antigen retrieval was achieved with microwaving in 1 m urea for 30 min, and the slides were then left to cool in the urea solution for 1 h at room temperature. For CG immunostaining, antigen retrieval was achieved with microwaving in 0.01 m citrate buffer (pH 6.0) for 30 min, and for cytookeratin immunostaining, slides were pretreated with proteinase K digestion (Dako) for 10 min. Slides were rinsed with PBS and then placed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After rinsing with PBS, the slides were placed in 1% blocking solution (Boehringer Mannheim, Mannheim, Germany) for at least 30 min to block nonspecific binding of antibody to the tissues. Sections were then incubated with primary antibody overnight at 4°C. The following primary antibodies were used (with the indicated dilutions in PBS): (a) SV40 Tag, Ab-2 (Oncogene Research Products; 1:100); (b) AR, N-20 (Santa Cruz Biotechnology Inc.; 1:100); (c) horseradish peroxidase-conjugated anti-PCNA antibody.
PC10 (Santa Cruz Biotechnology Inc.; and 1:50); (d) CG, bovine SP-1 (Diasorin Inc.; 1:1000). For Tag-immunostained sections, the same concentration of control mouse ascites fluid (Sigma Chemical Co.) was used as a negative control. After primary antibody incubation and washing with PBS, the slides were incubated with the appropriate secondary antibodies, either goat anti-mouse IgG (Sternberger Monoclonals Inc.; 1:300) or goat antirabbit IgG (Sternberger Monoclonals Inc.; 1:300) for 2 h at room temperature, rinsed with PBS, and then incubated in mouse or rabbit peroxidase-antiperoxidase complex (Sternberger Monoclonals Inc.; 1:500) for 1 h at room temperature. After soaking in 50 mM Tris-hydrochloride (pH 7.5), color development was performed with 3,3'-diaminobenzidine tetrahydrochloride (Dako). For PCNA, color development was performed after primary antibody incubation. Slides were counterstained with hematoxylin, dehydrated, and coverslipped.

Electron Microscopic Examination. For ultrastructural examination, tissues were fixed in EM grade 2% glutaraldehyde and embedded in Epon blocks, and thin sections were stained with uranyl acetate and lead citrate using standard conditions. Sections were examined using a Philips 301 transmission electron microscope.

Establishment of Allograft Model. A 3 × 3-mm block of the ventral lobe of a 12T-10 mouse (7408; 38 weeks old), which had a macroscopically obvious tumor, was implanted s.c. in the back of an 8-week-old athymic male mouse. Tumor growth was monitored, and the mouse was sacrificed at 18 weeks after inoculation, when the s.c. tumor showed exponential growth. The primary s.c. tumor and multiple organs were harvested for histological and immunohistochemical studies. The tumor was maintained by further s.c. passages.

Mass Spectrometry. Tissue samples from a nontransgenic CD1 mouse, a 12T-10 transgenic mouse (7408), and the allograft athymic mouse were immediately snap frozen after dissection. Details of the mass spectrometry procedure have been reported previously (21). In brief, thawed small tissue sections (2 × 2 × 2 mm) were blotted on the carbon-embedded polyethylene membrane for 5 min. After removing tissue sections, the membrane was allowed to dry completely. The blotted areas were washed thoroughly with water and allowed to dry again. Before mass spectrometry analyses, 1 μl of matrix (sinapinic acid; Sigma Chemical Co.) at 20 mg/ml in acetonitrile/0.1% trifluoroacetic acid in H₂O (1:1, v/v) was deposited on the blotted areas and allowed to dry. Mass spectrometry analyses were performed using a DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA). The instrument was operated in the linear mode under optimized delayed extraction conditions. An internal mass calibration was performed on each spectrum using the mass values of the previously measured singly and doubly charged ions of the α and β chain of hemoglobin using the singly charged molecular ions of bovine insulin (molecular weight, 5733.6; Sigma Chemical Co.) and horse heart cytochrome c (molecular weight, 12360.1; Sigma Chemical Co.).

Statistical Analysis. Statistical analyses were performed using the χ² test and Spearman’s rank correlation test.

RESULTS

Histopathological Progression of Prostatic Neoplasia in the 12T-10 LPB-Tag Line. Epithelial proliferation with mild nuclear atypia, compatible with LGPIN, was observed in most of lobes of 2–5-month-old mice (Fig. 1A; Tables 1 and 2). The degree of epithelial proliferation, nuclear stratification, and cytological atypia progressed quickly, such that HGPIN was observed in the prostates of
2-month-old mice and occupied progressively greater foci, becoming the dominant abnormality with advancing age (Fig. 1B). LGPIN was essentially no longer identified in mice ≥6 months of age. HGPIN was present uniformly throughout preexisting glands and ducts. In contrast to other LPB-Tag lines (17), in which there is marked glandular proliferation with expanded lobules of arborizing large and small gland profiles quite architecturally distinct from wild-type mice, the cytologically atypical epithelial proliferation in 12T-10 mice appeared to be confined to gland profiles generally similar to those of wild-type mice. As such, the lesion designated as HGPIN was architecturally more analogous to that in human cases versus other reported transgenic mouse models.

### Table 1 Pathology of prostate and metastasis of 12T-10 mice

<table>
<thead>
<tr>
<th>Age (months) of mouse</th>
<th>Tag no.</th>
<th>Histology of the prostate</th>
<th>Metastatic sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anterior lobe</td>
<td>Dorsal lobe</td>
</tr>
<tr>
<td>2</td>
<td>1015</td>
<td>LGPIN, HGPIN</td>
<td>LGPIN, HGPIN</td>
</tr>
<tr>
<td>3</td>
<td>1024</td>
<td>LGPIN, HGPIN</td>
<td>LGPIN, HGPIN</td>
</tr>
<tr>
<td>4</td>
<td>1026</td>
<td>LGPIN, HGPIN</td>
<td>LGPIN, HGPIN</td>
</tr>
<tr>
<td>5</td>
<td>1034</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>6</td>
<td>7211</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>7</td>
<td>7215</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>8</td>
<td>7410</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>9-10</td>
<td>7221</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>11-12</td>
<td>973</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td></td>
<td>2372</td>
<td>HGPIN</td>
<td>HGPIN</td>
</tr>
</tbody>
</table>

*ML, microinvasion; IC, invasive carcinoma without histological features of glandular or neuroendocrine differentiation; ICad, invasive carcinoma with glandular differentiation; ICne, invasive carcinoma with neuroendocrine differentiation; ICadne, invasive carcinoma with glandular and neuroendocrine differentiation; RLN, regional lymph nodes; MLN, submandibular lymph nodes; NA, not available.

**Bone metastases by Tag-positive immunostaining.

The ventral lobe which had nodular neuroendocrine carcinoma was transplanted in an athymic male mouse to establish an allograft model and provided for mass spectrometry analysis.
ably to IC, being uniformly and diffusely present in prostate sections that developed MI and IC. MI and IC (as defined in “Materials and Methods”) were identified in mice 4 and 6 months of age, respectively (Fig. 1, C–F). As indicated in “Materials and Methods,” the uniform presence of HGPIN and scant stroma for spatial separation of single cells or small acinar structures from immediately adjacent ducts/glands with HGPIN makes recognition of MI difficult. The designation of such lesions in the current study as MI was supported by the fact that metastases were occasionally detected (see below) in animals in which only such lesions (as opposed to obvious destructive invasion) were present in prostate sections.

More definitive foci of invasion (IC or UC) were present in 40% of mice 6–7 months of age, 67% of mice 8–10 months of age, and 100% of mice 11–12 months of age (Tables 1 and 2). Small (<2 mm) but definitive foci of IC were composed of nests with definitive glandular differentiation (adenocarcinoma; 54%; Fig. 1D); nests with cytological and architectural features of NE differentiation, including scant cytoplasm, nuclear molding, and rosette formation (19%; Fig. 1E); and small nests of malignant epithelial cells with varying amounts of cytoplasm and no obvious light microscopic glandular or NE differentiation (19%). Rosette formation in NE tumors can be difficult to distinguish from more usual glandular differentiation, and NE carcinomas in various organs in humans can show glandular differentiation. In several small invasive foci, features of both glandular and NE differentiation were noted (8%). Both types of invasive tumor differentiation (adenocarcinoma and NE) always occurred in the setting of uniform HGPIN. Further evidence of progression of invasive foci to more definitive NE differentiation was that larger invasive tumors (>2 mm; generally with destruction of adjacent parenchyma) all showed unequivocal light microscopic features of NE differentiation. These tumors, designated UC, were not detected in mice ≤7 months of age and were present in 20% of 8-month-old mice, and 29% of ≥9-month-old mice (Fig. 1, F–H).

Immunohistochemical Characterization of Prostatic Neoplasia in the 12T-10 LPB-Tag Line. CG immunostaining demonstrated progressively greater NE differentiation in HGPIN with increasing age of 12T-10 mice. In nontransgenic control mice up to 10 months of age, no or few CG-positive NE cells were found in prostate epithelium. The LGPIN and HGPIN lesions of 2–4-month-old 12T-10 mice likewise had no or very few clusters of CG-positive NE cells. In contrast, HGPIN in 12T-10 mice ≥5 months old had prominent clusters of CG-positive NE cells with characteristic cytoplasmic granular staining dispersed among the stratified, atypical epithelial cells lining the prostatic glands (Fig. 2, A, B, and D). CG-positive NE clusters were more pronounced in the anterior and dorsal lobes, although the cells of such HGPIN lesions were not morphologically

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>No. of mice</th>
<th>Histology of the prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LGPIN</td>
<td>HGPIN</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2 (40)</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>9–10</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>11–12</td>
<td>10</td>
<td>0</td>
</tr>
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</table>

Fig. 2. Immunohistochemistry in HGPIN and IC of 12T-10 transgenic mice. A, B, and D stain with CG in the dorsal lobe of a 10-month-old mouse (A) and the dorsolateral lobe of a 12-month-old mouse (B and D). C and E, large Tag (C, serial section of B) and AR (E, serial section of D) in HGPIN of a 12-month-old mouse. F, CG in IC with NE differentiation in the dorsolateral lobe of a 12-month-old mouse. G, absent CG (compared to HGPIN in A, B, and D) immunostaining in focus of IC with glandular differentiation in the dorsolateral lobe of an 11-month-old mouse.
IC with NE differentiation typically showed positive immunostaining for chromogranin A (Fig. 2F). Although the lesions also showed positive staining for Tag, the intensity for AR immunostaining was generally fainter than that of NE cells that were dispersed in HGPIN. In contrast, foci of IC with unequivocal glandular differentiation (adenocarcinoma) were generally negative for CG immunostaining.

Most regions of UC, which showed histological features typical of NE tumors in humans, stained positive for CG (Fig. 3A). However, the intensity was faint, particularly compared to staining of NE cells within HGPIN lesions, and some lesions with unequivocal light microscopic features of NE differentiation (“salt and pepper” chromatin, nuclear molding, and resetting) were negative for CG. Electron microscopic characterization of such a primary invasive UC tumor confirmed the NE differentiation and the presence of typical dense core neurosecretory granules. However, compatible with the results of CG immunostaining, such neurosecretory granules were rare. Primary UC tumors were uniformly and strongly Tag positive (Fig. 3B). Compatible with several NE tumors in humans, such UC foci showed only faint immunostaining for cytokeratin (Fig. 3C). PCNA immunostaining demonstrated positive nuclear staining in 25–50% of cells (Fig. 3D). In contrast to the strong AR immunostaining of NE cells in HGPIN lesions (Fig. 2E), immunostaining for AR in UC tumors was negative or showed a mixture of negative and faintly positive cells (Fig. 3E).

Metastatic lesions were strongly Tag immunopositive (Fig. 4B). Of the 21 mice with Tag-immunopositive metastases, 15 (71%), 14 (67%), and 13 (62%) had Tag-positive lesions in the regional lymph nodes, liver, and lung, respectively (Table 1). Liver metastases in particular were occasionally bulky, measuring up to 1 cm. The average number of extraprostatic sites with Tag-positive metastatic lesions per mouse increased with advancing age as follows: 0, 0.4, 1.0, 2.3, and 2.9 in mice 2–5, 6–7, 8, 9–10, and 11–12 months of age, respectively (P < 0.001, Spearman’s rank correlation test). Tiny Tag-positive lesions in the bone marrow were found in 3 of 44 (6.8%) mice examined, although cytologically malignant corresponding foci were not identified in serial sections. Three mice had Tag-positive cells in the bulbourethral glands. Two of three mice (7408 and 7603; both 9–10 months of age) had Tag-positive UC with NE differentiation in the bulbourethral glands, whereas the remaining mouse (970; 11–12 months of age) had Tag-positive epithelial cells within the bulbourethral acini without any cytological or structural abnormality. Both of the mice with UC in the bulbourethral glands had lymph node and visceral metastases with similar morphology. One mouse had UC in the ventral lobe, and the other had IC with NE differentiation in the anterior lobe. Whether the UC tumors in the bulbourethral glands represented metastases or distinct primaries is not certain. Tag immunostaining was detected in benign bulbourethral glands in one mouse, and others have observed transgene expression and tumor development in bulbourethral glands with other supposedly prostate-specific promoters (26), which suggests that pathology in these foci could result from transgene expression at this site. Mice with tumors in the bulbourethral glands in this series had tumors in the prostate, and similar systemic metastases were commonly observed in mice with such tumors in the prostate but no pathology in the bulbourethral glands.
glands; these observations suggest that the prostate is the source for metastatic disease even in those animals with a histologically similar tumor at this other genitourinary site.

**Correlation of Metastases with Pathology of the Prostate.** Mice that only had LGPIN and/or HGPIN on examined sections of prostate never had metastases in extraprostatic organs (Table 3). On the other hand, 33% of mice with MI, 69% of mice with IC, and 100% of mice with UC had metastases. Thus, the incidence of metastatic tumors paralleled the progression of neoplasia in the prostate and increased with the extent of local invasion, such that the existence of IC and UC clearly correlated with high metastatic potential in older mice.

**Allograft Model of NE Tumor.** To further confirm the metastatic potential of the UC developing in the 12T-10 prostate, portions of the grossly abnormal ventral lobe of a 38-week-old 12T-10 mouse (7408) that had a histologically confirmed UC tumor were transplanted s.c. into an athymic male mouse. A histologically identical NE carcinoma grew at the primary transplant site, and the mouse developed NE differentiation histologically identical metastases in the liver (confirmed histologically at 18 weeks after transplant). NE differentiation was positive, histologically identical metastases in the liver (confirmed histologically at 18 weeks after transplant). NE differentiation was positive, histologically identical metastases in the liver (confirmed histologically at 18 weeks after transplant).

**Fig. 4.** Metastatic lesions in 12T-10 transgenic mouse. A, electron micrograph showing rare dense core neurosecretory granule in cytoplasm of tumor cell of liver metastasis. B, immunostaining for large Tag in liver metastasis with NE differentiation in a 12-month-old animal.

**Table 3.** Relationship between metastatic development and histology of the 12T-10 mouse.

<table>
<thead>
<tr>
<th>Histology of the prostate</th>
<th>Metastasis (−)</th>
<th>Metastasis (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGPIN/HGPIN</td>
<td>18 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MI</td>
<td>8 (67)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>IC</td>
<td>5 (31)</td>
<td>11 (69)</td>
</tr>
<tr>
<td>UC</td>
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<td>6 (100)</td>
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<tr>
<td><strong>Total</strong></td>
<td>31</td>
<td>21</td>
</tr>
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 cently identified as the spermine-binding protein. The spermine-binding protein signals were not observed in the protein pattern generated from the ventral lobe of the NE tumor in the 12T-10 mouse. Five distinct groups of signals (labeled a to e) in the m/z range from 10,000–17,000 were observed in the 12T-10 mouse but were not detected in the ventral lobe of the nontransgenic CD1 mouse. Signal a contained at least five different ions at mass 11,265, 11,307, 11,349, 11,391, and 11,433 Da. The signal distribution was Gaussian shaped, suggesting multiple random acetylation. Signal b and signal c displayed two major peaks at mass 12,132 Da/12,167 Da and 13,777 Da/13,804 Da, respectively. Signal d contained three distinct mass peaks at 14,005, 14,047, and 14,089 Da. Signal e was located between the α and the β chains of hemoglobin. This signal centered on 15,355 Da and was broad and unresolved, indicating the presence of several different proteins or protein isoforms within a close mass range. Based on the protein database searched (SwissProt) using the measured molecular masses, a tentative identification of the signals a through e could be made. The signal mass 11,265 Da could be identified as the histone H4, and the following signals at 11,307, 11,349, 11,391, and 11,433 Da were consistent with multiple histone H4 acetylations [up to five successive acetylations have been reported on the histone H4 (27)]. The signal observed at 13,804 Da (from the c signal cluster) could be identified as histone H2B1. The signals observed at 14,005 and 14,047 Da (from the d signal cluster) were consistent with histone H2A.2 and its diacetylated forms, respectively. The signal detected at 14,089 Da (from the d signal cluster) could be attributed to histone H2A.1. Finally, based on the observation of several different histones in the protein profile, the broad signal observed around 15,355 Da (signal e) could be assigned to the histone H3. No tentative identifications were made for the signals in the b cluster or for the signal at 13,777 Da from the c cluster.

Between m/z 10,000 and 17,000, the anterior, lateral, and dorsal...
lobes of the 12T-10 mouse showed profiles similar to those of the ventral lobe (Fig. 6). These lobes contained HGPIN, MI, and IC (also present in the ventral lobe), suggesting that these proteins may not be unique to the larger invasive undifferentiated or NE carcinoma but may be shared by a population of cells common to differentiation in all of the precursor and invasive lesions. Whether this relates specifically to NE differentiation is not clear. Furthermore, the anterior, lateral, and dorsal lobe peak intensities were lower than those in the ventral lobe, suggesting a possible increased expression of the signal source proteins with progression (Fig. 6). Samples from regional lymph nodes and liver of the 12T-10 mouse with only metastatic NE carcinoma showed exactly the same protein profile as the ventral lobe. In addition, samples from a s.c. inoculated NE carcinoma from the athymic nude mouse and its liver metastasis also had the same protein profiles as the NE carcinoma in the ventral lobe of the 12T-10 transgenic line (Fig. 7).

**DISCUSSION**

There is definite need for sophisticated and validated transgenic mouse models of human Pca. Having animal models that progress predictably through precursor lesions, analogous to human HGPIN, to early and more established IC and eventually to higher grade lesions with metastatic potential would parallel defined stages in human Pca. If such models are established by genetic manipulations that result in additional molecular alterations with lesion progression that are also implicated in progression of human Pca, they will be extremely useful. Such changes would allow for testing of mechanistically relevant interventions at different stages of disease evolution, with clear end points for assessing efficacy. These include primary chemoprevention at the precursor stage or secondary pharmacological intervention once invasion has occurred to attempt to delay progression and prevent more extensive and metastatic disease, neither of which desired treatment strategies currently exists for human Pca. Finally, such models may allow for testing treatments in advanced, metastatic disease, which have lost androgen sensitivity. With these issues in mind, the animal model presented in the current study represents a distinct advance in transgenic models of Pca.

The SV40 early region has been used in transgenic constructs with other promoters, some of which are designed to be prostate specific (19, 28), some of which unexpectedly targeted the prostate (13), and some of which result in transgene expression in other organs (8 –10). The LPB is androgen dependent and prostate specific (16, 20), desirable properties in the establishment of a transgenic Pca model. The small Tag was inactivated in the constructs used to generate the LPB-Tag lines, including 12T-10 (17), thus narrowing the possible molecular mechanisms of transformation. The large Tag sequesters and inactivates p53 and retinoblastoma, altering cell cycle regulation and possibly leading to genetic instability (29, 30). Although p53 mutations have been accepted as present in a subset of human Pca
epithelial proliferation appeared to be within ducts and glands not discernibly more numerous or expanded than that in wild-type mouse and was not accompanied by stromal hypercellularity.

Other established mouse models have developed invasive disease but have been limited because of very rapid progression to metastases (19) or by the lack of even eventual development of metastatic disease (12, 28). Criteria for early invasion are still being established for the mouse prostate and may be very different than those routinely used to diagnosis adenocarcinoma in the human prostate. The latter includes infiltration of malignant acini amongst benign glands (39). Relevant to spatial relationship to HGPIN, in situations where small acini suspicious for invasion are adjacent to HGPIN glands, the degree of separation is used to argue against tangential sections of outpouchings of HGPIN (40). For the mouse models, the extremely scant rim of stroma surrounding preexisting glands/ducts and the extensive or uniform development of HGPIN make applying these established human principles difficult. In the current study, designation of lesions considered microinvasion based on extension into this surrounding thin stroma was validated by the subsequent development of more extensive unequivocal destructive invasion and the association of such lesions with metastatic disease occasionally in the absence of any more extensive tumor in the sampled prostate. Metastases cannot occur in the absence of invasion, although it is likely not a sensitive indicator of invasion. For example, in humans, radical prostatectomies are only performed for invasive tumor (documented by biopsy and confirmed in prostatectomy specimens), but pelvic lymph node metastases are uncommon. In our last consecutive 386 radical prostatectomy specimens at Vanderbilt University Medical Center, lymph node metastases have been present in only 3%,5 similar to other current series (41). However, the development of metastases in the animal model allowed us to “work backward” with regard to histological features of early invasion in the prostate. Morphologically similar microinvasion into the thin rim of surrounding stroma was observed in prostates of mice created with the SV40 early region on the cryptidin-2 promoter (13) and under the control of the rat prostatic steroid-binding protein [C3(1)] gene (42). As in the current series, the development of greater degrees of invasion in both these models and metastatic disease in the former supports such lesions as truly invasive.

The invasive foci in the 12T-10 mouse showed both glandular differentiation and NE differentiation. Invasive adenocarcinoma, based on true extension into periprostatic fat and local tissues, has been reported recently in mice overexpressing human insulin-like growth factor I targeted to basal epithelium with the bovine keratin 5 promoter (12). These mice did not develop metastases. In addition, similar to other previously reported models, the lack of utilization of prostate-specific promoters may lead to neoplasia in other organs, potentially complicating interpretation in those models that do eventually progress to systemic metastases either spontaneously or by cross-breeding with other transgenic mice. With larger invasive foci and with metastatic disease in the 12T-10 mouse, primary and metastatic tumors showed cytological and histological features typical of NE differentiation, which was confirmed immunophenotypically and ultrastructurally. The incidence of metastases increased with age and did not occur in mice <6 months of age but was increasingly common thereafter and found in 66% of mice ≥6 months of age and 88% of mice ≥9 months of age. Furthermore, metastatic development clearly correlated with the existence of IC and especially of UC in the prostate. Metastases occurred in both regional lymph nodes and distant organs. The results indicate that there was both lymphatic and hematogenous metastatic spread, similar to that seen in human Pca (43). Hematogenous visceral metastases (e.g., liver and lung) are

5 S. B. Shappell, unpublished observation.
particularly characteristic of human prostate NE carcinoma (44), again attesting to the similar biological properties of this mouse and human morphological subtype. As indicated, rare mice had similar NE tumor foci in bulbourethral glands, likely relating to androgen-dependent transgene expression at these sites (26) rather than to metastases from the prostate. However, because these mice also had NE carcinoma in the prostate, and because similar patterns of metastases were identified in older mice both with and without bulbourethral gland tumors, it was our interpretation that the prostate tumor was the source of metastases in all animals developing metastases. To further prove that the primary prostate tumor has the ability to metastasize, an immuno-deficient athymic male mouse received a 12T-10 primary prostate allograft. The mouse developed similar visceral metastases within the 18-week time period, clearly demonstrating that the NE carcinoma in the prostate had metastatic potential.

We used MALDI-TOF-MS to investigate protein profiles of primary and metastatic tumors and to further support the observation that Tag-positive lesions in the extraprostatic organs were metastases from the prostate. In the present study, primary and metastatic undifferentiated/NE tumors had essentially identical profiles, which were also distinct from those of wild-type prostate. Histones, typically nuclear proteins, were observed in NE carcinoma in high abundance by mass spectrometry. This may be related to the high nuclear:cytoplasmic proteins, were observed in NE carcinoma in high abundance by mass spectrometry. This may be related to the high nuclear:cytoplasmic ratios of NE cancer cells. When the tissue sections were prepared, inevitably, a nonnegligible percentage of the cell nuclei were ruptured, and their protein content was accessible to the blotting membrane. Although histones themselves are not specific for NE cancer, detection of a large amount of histones may represent active proliferation through high transcription of NE cancer cells because only 12T-10 prostate and those metastases containing NE cancer cells showed the clusters of signals a through e. The unidentified signals in the b cluster and the signal at 13,777 Da from the c cluster may imply NE cancer-specific markers of the 12T-10 mice. Thus, mass spectrometric analysis may become one strategy to characterize possibly changing protein expression profiles during tumor progression and to help establish sites of primary origin for metastatic tumors.

Recently, interest has focused on the role of NE differentiation in Pca (3–6). It has been reported that 30–100% of human Pca have focal or extensive NE differentiation (3, 6). Although NE cells per se are thought to be postmitotic cells (45, 46), it has been demonstrated that adenocarcinoma cells near NE cells express the proliferation marker Ki-67 and the apoptosis-inhibiting proto-oncogene bcl-2 (45, 47). Thus, NE cells may influence the behavior of adjacent adenocarcinoma cells through a paracrine mechanism. However, it is still controversial whether NE differentiation in Pca correlates with poorer prognosis (3–6). It has also been reported that the majority of prostatic NE cells do not express detectable nuclear AR (48, 49). Although Nakada et al. (50) reported that most NE cells have AR expression, they noted that a subpopulation of AR-negative NE cells is more prominent in Pca than in benign prostatic tissues. Casella et al. (51) reported a significant increase in the frequency and density of NE differentiation in specimens of hormone-refractory Pca compared to those obtained before hormonal treatment from the same patients. Thus, expansion of an androgen independent NE cell population in Pca is one possible mechanism for the development of androgen resistance in advanced Pca. In the current study, development of larger primary and metastatic tumors with NE differentiation was accompanied by reduced AR immunostaining. Surprisingly, all the 12T-10 tumors continue to express the Tag gene, which is under the regulation of the androgen-dependent probasin promoter. If similar mechanisms are ultimately determined to be responsible for loss of androgen dependence in advanced human Pca and the 12T-10 mouse, this model will be extremely useful for characterizing the role of NE cells in evolving androgen resistance and for testing therapeutics for advanced androgen-insensitive malignancy.

Small cell carcinoma may be present at initial diagnosis or may develop subsequently in patients with a previous history of more usual acinar Pca. Patients with small cell carcinoma of the prostate have a poor prognosis, and the tumor does not appear to respond to hormonal therapy (44, 52, 53). Although small cell carcinoma is composed of NE cells, these cells show striking proliferative activity (53). Thus, the characteristics of NE cells in small cell carcinoma are very different from postmitotic NE cells that may be observed in prostate adenocarcinoma. Similarly, advanced tumors in 12T-10 mice showing pure cytological NE differentiation were extremely active mitotically. Small cell carcinoma may originate from pluripotent malignant cells that differentiate to both acinar-forming cells and NE cells (44, 53). The relationship of small cell carcinoma to more usual acinar Pca and the mechanism of progression potentially accompanying exogenous hormonal manipulation are poorly understood (3). Because existing in vitro and in vivo models from human small cell carcinoma have been established from metastatic lesions or recurrent lesions in patients that fail hormonal therapy (54, 55), they preclude examining the histogenesis and natural history required for the development of small cell carcinoma. Therefore, an animal model that spontaneously develops NE prostate cancer would be useful to study the evolution of this cancer.

By immunostaining for chromogranin A and/or neuron-specific enolase, there are only 6–7 NE cells/10 high-power fields of normal adult prostatic acini (56). Our study similarly demonstrates that the NE cell population is quite rare in the prostatic acini of adult non-transgenic (normal) mice. On the other hand, prostate of 12T-10 mice ≥5 months of age showed an increasing population of NE cells within HGPIN that also stained for Tag. Because Tag is expressed in essentially all prostate epithelial cells at early time points (17) at which little or no NE differentiation is present, it would appear that an increasing subset of Tag-positive cells is developing NE differentiation as PIN progresses in the 12T-10 line. It is likely that the proliferating compartment in this evolving lesion includes NE cells that give rise to invasive and eventually metastatic tumors with definitive cytological and histological features of NE carcinoma. The apparent mechanism of progression of Tag-positive epithelial cells to neoplastic cells with NE differentiation is in contrast to the transformation achieved by the use of the cryptdin-2 promoter linked to the SV40 early region, in which for unknown reasons the transgene was expressed in only the small subset of prostate epithelium that already showed NE differentiation (13). It is not clear if acinar forming invasive foci in the 12T-10 mouse arise from HGPIN cells with or without NE differentiation. Although definitive invasive foci with acinar formation were noted to be CG negative, they were also occasionally seen in association with histologically typical NE carcinoma. The latter lesions also show only faint CG immunostaining perhaps similar to reduced neurosecretory granules and CG immunostaining in progressively higher grade human NE carcinomas. Whether the same molecular alterations in the HGPIN lesions are responsible for developing invasive adenocarcinoma or IC with NE differentiation remains to be discerned. However, based on size and incidence and histological appearance of metastases, it appears that invasive lesions progress quickly to NE carcinoma in this model, perhaps in ways similar to the more protracted progression in human Pca.

REFERENCES


A Probasin-Large T Antigen Transgenic Mouse Line Develops Prostate Adenocarcinoma and Neuroendocrine Carcinoma with Metastatic Potential
