Tumorigenesis and Neoplastic Progression

Dissociation of Epithelial and Neuroendocrine Carcinoma Lineages in the Transgenic Adenocarcinoma of Mouse Prostate Model of Prostate Cancer

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The transgenic adenocarcinoma of mouse prostate (TRAMP) model is widely used in prostate cancer research because of rapid tumor onset and progression. The transgenic mouse is on a C57BL/6 (B6) background and expresses SV40 T-antigen under the probasin promoter. The strong genetic component of susceptibility to prostate cancer in humans prompted us to investigate the effect of mouse strain background (FVB and B6) on incidence, progression, and pathology of prostate cancer in this model. Because TRAMP lesions are unique but differ from conventional prostatic intraepithelial neoplasia because the epithelium and stroma are affected diffusely, we designated them as “atypical hyperplasia of Tag.” Although the incidence and severity of atypical hyperplasia of Tag is similar, FVB-TRAMP mice live significantly shorter lives than B6-TRAMP mice because of the rapid development and progression of neuroendocrine carcinomas. This is associated with an increased frequency of neuroendocrine precursor lesions in young TRAMP mice, detectable at 4 weeks after birth. These lesions show properties of bipotential stem cells and co-express markers of epithelial (E-cadherin) and neuroendocrine (synaptophysin) lineages, as well as the transcription factors Foxa1 and Foxa2. Transplantation studies using TRAMP prostatic ducts suggested that neuroendocrine carcinomas arise independently from atypical hyperplasias or other epithelial lesions. Adenocarcinomas were not seen in our cohort. Thus, neuroendocrine

carcinomas are the principal malignancy in this model and may develop from bipotential progenitor cells at an early stage of prostate tumorigenesis. (*Am J Pathol* 2008, 172:236–246; DOI: 10.2353/ajpath.2008.070602)

Prostate cancer is the most frequently diagnosed cancer in men living in the United States and the second leading cause of cancer-related mortality, accounting for more than 29,000 deaths annually. The causes of this high incidence are unknown, but include both environmental factors and a genetic component.^{1,2} Numerous experimental systems have been developed to study the genetic and biological aspects of prostate cancer development including cell culture systems, xenografts, and genetically engineered mouse models.^{3–10} Animal models that faithfully recapitulate the stages of human cancer progression are valuable but rare. In comparison to cell culture, mouse models are advantageous because they offer a system in which all aspects of disease progression can be studied in a setting where both genetic background and environment can be controlled.¹¹ The use of such models can facilitate dissection of the multiple steps of cancer initiation and progression, as well as the identification of genetic modifiers of disease phenotypes.

Transgenic adenocarcinoma of mouse prostate (TRAMP) is a transgenic mouse engineered to express the SV40 virus large T and small t oncoproteins in the secretory epithelial cells of the prostate under the control of the androgen-responsive minimal rat probasin promoter.^{12,13}

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The TRAMP mouse has been widely used to study molecular events important in prostate cancer progression because it develops prostatic tumors that have been reported to share many features important in human cancer, including metastasis to distant sites, progression to androgen independence, and neuroendocrine differentiation. The model has been used in preclinical and chemopreventive studies, and to derive cell lines currently used in numerous research laboratories.¹⁴ TRAMP was originally produced and characterized on a C57BL/6 (B6) inbred background strain, although subsequent study revealed that tumors arise more quickly in B6 × FVB F₁ mice, and many published studies that followed were performed on F₁ animals.¹⁵ These early data suggested that genetic background may have a profound effect on some aspect of tumor initiation or progression in the TRAMP model. In this study we have examined the steps of prostate tumor development in both B6 and FVB mouse strains. We crossed the probasin-SV40 transgene onto the FVB background and then followed cohorts of TRAMP mice from B6 and FVB strains for a minimum of 20 weeks. Our data show a major difference in average survival between FVB and B6 TRAMP mice. This is mainly attributable to rapid growth of neuroendocrine carcinomas in FVB mice, rather than to altered incidence of atypical hyperplasia of Tag, which was slightly more severe in B6 mice. Studies involving transplantation of TRAMP prostatic ducts under the renal capsule of nude mice, or of TRAMP prostatic epithelium combined with normal embryonic rat urogenital sinus mesenchyme, suggested that neuroendocrine carcinomas do not arise by progression from pre-existing atypical hyperplasias or other epithelial lesions. Rather, the incidence and progression of neuroendocrine carcinomas in FVB mice are associated with an increased frequency of neuroendocrine precursor lesions in young TRAMP mice, detectable as early as 4 weeks after birth. Some of these lesions showed properties of bipotential stem cells, and co-expressed markers of the epithelial (E-cadherin) and neuroendocrine (synaptophysin) lineages. In addition, the neuroendocrine carcinomas co-expressed the Forkhead box factors Foxa1 and Foxa2, which also support a bipotential stem cell origin. Interestingly, neuroendocrine carcinomas commonly arose in the ventral prostate (VP), which is the prostatic lobe less prone to the development of atypical hyperplasia of Tag. We conclude that neuroendocrine carcinomas in this model are not likely to arise from pre-existing committed epithelial cells, but may develop from bipotential stem or progenitor cells at an early stage of prostate tumorigenesis.

Materials and Methods

Animal Breeding

C57BL/6NHsd (B6) TRAMP mice were obtained from Dr. Norman Greenberg (Fred Hutchinson Cancer Center, Seattle, WA) and were subsequently bred in our facility by continued backcrossing to B6 mice obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). FVB/NTac mice

were obtained from Taconic (Germantown, NY). Congenic FVB TRAMP mice were generated as follows: B6 TRAMP females were mated with FVB males to generate B6FVBF₁ TRAMP animals and the F₁ TRAMP females were backcrossed to FVB males; this scheme of backcrossing TRAMP females to FVB males was continued. The FVB TRAMP mice used in this study were generation N9-N12. A congenic N20 strain has been deposited at the National Cancer Institute Mouse Models of Human Cancers Consortium Repository (Frederick, MD). Genotyping was performed according to a protocol developed in the laboratory of Dr. Norman Greenberg (Fred Hutchinson Cancer Research Center). Polymerase chain reaction was used to detect a 600-bp product using the following set of primers: 5'-CCGGTCGACCGGAAGCT-TCCACAAGTGCATTTA-3' (Pb-1, forward) and 5'-CTC-CTTTCAAGACCTAGAAGGTCCA-3' (SV40Tag, reverse). The thermocycler was run for 29 cycles at 94°C (1 minute), 94°C (1 minute), 60°C (2 minutes), 72°C (3 minutes), and 4°C (end). Animals were housed in plastic cages on newspaper bedding and fed PicoLab Rodent Diet 20 (PMI Nutrition International, Richmond, IN) and tap water *ad libitum*. Animals were bred, housed and used in accordance to the Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, National Institutes of Health, Bethesda, MD).

Tissue Dissections

Mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation; prostate glands were removed and placed in Dulbecco's modified Eagle's medium on ice. For the time course experiment, prostates were trimmed under a dissecting microscope, fixed in 4% paraformaldehyde overnight at 4°C, and then stored in 70% ethanol until processed. For the grafting experiments, prostatic lobes were microdissected as previously described.^{16,17} Tissues harvested from graft experiments and tissue recombinants were fixed in neutral buffered formalin for 1 to 2 hours at room temperature. After fixation all tissues were dehydrated, cleared in HistoClear (National Diagnostics, Atlanta, GA), and embedded in paraffin. Hematoxylin and eosin (H&E)-stained, 6-μm-thick sections were used for basic morphological examination.

Time Course Experiment

The mice used in all experiments were hemizygous for the SV40 transgene. B6, FVB, and B6FVB F₁ TRAMP mice were assigned to groups at weaning, with littermates distributed throughout several time points. Each group consisted of 10 mice of each strain designated for evaluation at 4, 8, 12, 16, and 20 weeks. Additional B6 mice were evaluated between 20 and 38 weeks of age (no FVB mice were evaluated in this age group because they died earlier with neuroendocrine carcinomas). Our institutional criteria for euthanasia required mice to be sacrificed as soon as a palpable tumor was identified. In this study many mice developed palpable tumors begin-

ning at 12 weeks and were therefore sacrificed at times between the designated time points.

Grafting and Tissue Recombination

To investigate whether lesions from the two strains would progress to more severe degrees of abnormality, ductal segments from TRAMP prostates were grafted under the kidney capsule of intact, 45- to 60 day-old nude male mice (Charles River, Wilmington, MA). Prostatic ducts were microdissected from adult B6 or FVB TRAMP mice as previously described, but no enzymatic treatment was used.^{16,17} Ductal segments were cultured overnight on agar before grafting.^{18,19} In some experiments TRAMP prostatic epithelial tissues were recombined with rat urogenital sinus mesenchyme (rUGM). Pregnant Sprague Dawley rats were obtained from Simonsen Laboratories, Inc. (Gilroy, CA), and rat UGM was obtained from 18-day-old embryos by trypsin separation as previously described.^{18,19} Tissue recombination and subcapsular renal grafting were performed as previously described and allowed to grow *in vivo* for up to 2 months.²⁰

Immunohistochemistry

Immunostaining was performed on paraffin sections that had been deparaffinized and rehydrated. Antigens were unmasked by boiling sections in citrate buffer (antigen unmasking solution; Vector Laboratories, Burlingame, CA), pH 6, for 30 minutes in a microwave oven. Blocking solution (Superblock; Pierce, Rockford, IL) was applied to sections followed by the primary antibody [anti-SV40 T-antigen, 1:2000, from BD Transduction Laboratories, San Diego, CA; anti-androgen receptor (AR), 1:50, from Affinity Bioreagents, Golden, CO; anti-p63 antiserum, 1:100, from Santa Cruz Biotechnology, Santa Cruz, CA; anti-synaptophysin, 1:100, from BD Bioscience, San Jose, CA; anti- α -smooth muscle actin, 1:1000, from Sigma, St Louis, MO; anti-E-cadherin, 1:200, from BD Bioscience; and anti-Foxa1 (C-20) and anti-Foxa2 (P-19), 1:1000, from BD Transduction Laboratories, San Diego, CA]. Antibody binding was detected using secondary anti-rabbit or anti-mouse IgG antibodies conjugated to biotin (Amersham, Piscataway, NJ) and the ABC peroxidase method with 3',3'-diaminobenzidine or NovaRED color reagent (Vector Laboratories).

Histopathological Examination

Prostates from TRAMP mice of both B6 and FVB background strains were characterized in detail based on H&E-stained slides and on a panel of immunohistochemical stains as listed above. Our goal was to identify strain-dependent lesions, and therefore prostates of B6FVB F₁ mice were not examined for the sake of simplicity. Whenever possible the prostatic lobes were dissected and labeled as coagulating gland, dorso-lateral prostate, and VP. The same histopathological criteria were used to examine tissues obtained directly from the animals and from renal capsule grafts. A set of slides representative of

both strains at all ages were first reviewed by a pathologist (S.S.C.) to develop a detailed classification system using criteria in accordance with previously published guidelines but modified to reflect the lesions seen in our cohort.²¹ All identifiers were then removed from the slides, and the entire slide set was graded for lesion type and severity. The lesion classification criteria are described in detail in the Supplemental Appendix (see <http://ajp.amjpathol.org>).^{22–24}

Prostatic Maturation in Young TRAMP Mice and Tag Expression Scores

The stage of maturation of 4-week-old prostates from both strains were scored on a scale from 1 to 5 as follows: 1, the connective tissue was all mesenchyme, and there were still some solid cords among the ducts; 2, the connective tissue was all mesenchyme, all ducts had a lumen, and the epithelium was cuboidal; 3, the connective tissue consisted of some mesenchyme and some smooth muscle, and the epithelium was a mixture of cuboidal and columnar cells; 4, the connective tissue was mostly smooth muscle, and the epithelium was mostly columnar; 5, the connective tissue was all smooth muscle, the epithelium was columnar, and the lumen contained some secretion. Tag expression in young TRAMP mice was scored on a scale from 0 to 2 (0, not expressed; 1, low heterogeneous expression; or 2, uniformly high expression).

Statistical Analysis

Strain differences in survival were analyzed using the Kaplan-Meier method and the SPSS package (SPSS, Chicago, IL). Tumor or lesion prevalence differences between strains were tested using the Mantel-Haenszel test at six different age groups, 4 to 8, 8 to 12, 12 to 16, 16 to 20, 20 to 24, and >24 weeks. The prostatic maturation stages of the two strains were compared using the Wilcoxon rank sum test and the correlation between maturation stage and Tag expression was assessed using Kendall's test.

Results

Survival Time and Tumor Development Are Strain-Dependent

We followed the survival time of TRAMP mouse groups on the B6 ($n = 18$), F1 ($n = 10$), and N5 FVB ($n = 15$) backgrounds (Figure 1A). The median survival time for B6 mice was 41 weeks [95% confidence interval (CI) = 35, 47] compared to survival times of 19 weeks (95% CI = 14, 24) and 28 weeks (95% CI = 21, 35) for the F₁ and FVB strains, respectively (Figure 1A). The differences in survival times between B6 and F1 groups ($P = 0.037$), as well as between B6 and FVB ($P = 6 \times 10^{-7}$) and F₁ and FVB ($P = 0.0029$), were statistically significant. Subsequent backcrossing to FVB past N5 did not further decrease the survival time (data not shown).

Figure 1. Survival curves and lesion incidence graphs for FVB, B6, and B6 × FVB F₁ TRAMP mice. **A:** Survival curves for B6, FVB, and B6 × FVB F₁ mice. Histograms showing the incidence of neuroendocrine (NE) carcinomas by age for B6, FVB, and B6 × FVB F₁ mice (**B**); severe atypical hyperplasia of Tag by age for B6 and FVB mice (**C**); and synaptophysin-positive foci by age in B6 and FVB mice (**D**).

This difference in survival between the FVB and B6 strains was attributable to a striking difference in the age of onset and incidence of neuroendocrine (NE) carcinomas (Figure 1B). Although all FVB mice developed NE carcinomas by 21 weeks, the lifetime incidence of NE carcinomas in B6 mice was only 20% ($P = 3.3, e-11$). Tumors also developed earlier in FVB mice. Half of the 10- to 12-week-old FVB mice had prostatic NE carcinomas, whereas none of the B6 mice had developed a NE carcinoma at that age. By 16 weeks, 87% of FVB had developed NE carcinomas, compared to only 11% of age-matched B6 animals. Of 42 B6 mice examined between 16 to 37 weeks, only 8 (19%) had a NE carcinoma. In both strains, NE carcinomas were most prevalent in the VP lobe. We determined the lobar location of macroscopic tumors in a subset of 13- to 20-week-old mice (B6 = 6, FVB = 9) and found that in B6 mice there were five VP tumors, three coagulating gland tumors, and one dorso-lateral prostate tumor. All nine FVB mice had a VP tumor, six of which also had a coagulating gland tumor and one also had a dorso-lateral prostate tumor.

Terminology of Prostatic Histopathology in TRAMP Mice

Although a general account of lesions of the TRAMP prostate has been previously published, the survival data indicated that tumor progression in FVB mice either proceeded at a different rate or through an alternative pathway compared to B6 mice.²¹ We therefore evaluated the histopathological characteristics of lesion progression ranging from normal prostate to malignant tumors in both strains. We found that lesions in the TRAMP model differ significantly from human prostatic intraepithelial neoplasia and mouse prostatic intraepithelial neoplasia in other models. In the TRAMP model, the epithelium and stroma are simultaneously and diffusely affected, and although there is nuclear atypia, this too is a diffuse lesion. In

addition, because experimental evidence suggests that luminal/stromal lesions in these animals do not progress to invasive and metastatic disease, we have opted to regard these lesions as a form of atypical hyperplasia. To differentiate this specific lesion from atypical hyperplasia described elsewhere, we will refer to TRAMP lesions as “atypical hyperplasias of Tag” from here onwards. A detailed description of the grading system for atypical hyperplasia of Tag can be found in the Supplemental Appendix (see <http://ajp.amjpathol.org>).

The lesions observed in this study were classified as atypical hyperplasia of Tag (mild, moderate, and severe), papillary tumors, and NE carcinomas (Supplemental Figure 1S, Supplemental Appendix, see <http://ajp.amjpathol.org>). In prostate samples from 160 mice, none of the lesions examined met our criteria for adenocarcinoma (true invasion of transformed epithelium with glandular differentiation, desmoplastic stromal reaction, or local/distant metastasis). In both strains, we observed many NE carcinomas, poorly differentiated and highly invasive lesions, forming large masses that distorted the ductal architecture, entrapped normal or hyperplastic glands, usually involved multiple lobes, and often had large areas of necrosis. The NE carcinomas were composed of highly vascularized sheets of pleomorphic cells with scant cytoplasm that occasionally formed pseudo-rosettes (Figure 2, A and B).

To characterize the lesions we used a panel of immunohistochemical markers that included antibodies to SV40 Tag, differentiation markers (synaptophysin for NE cells and E-cadherin for epithelial cells), smooth muscle actin to evaluate the stroma, and the nuclear transcription factors AR, p63, Foxa1, and Foxa2. The staining patterns in normal control prostate epithelium and TRAMP lesions are summarized in Table 1. NE carcinomas were positive for Tag (Supplemental Figure 2S and Supplemental Appendix, see <http://ajp.amjpathol.org>) and synaptophysin (Figure 2C) and either were negative for AR or showed weak and diffuse cytoplasmic staining for AR (Figure 2D). The NE pattern contrasted sharply with normal and hyperplastic prostatic epithelia, which were positive for Tag (Figure 3B) but negative for synaptophysin (Figure 2C) and showed strong nuclear staining for AR (Figure 2D). The neuroendocrine carcinomas tended to arise in the VP where atypical hyperplasia was relatively mild.

The high incidence of NE carcinomas in the FVB background prompted us to look for differences in the prostate maturation, and in histopathology of early lesions in both strain backgrounds. No strain differences were seen in the incidence, time of onset, or lobe distribution of atypical hyperplasias or papillary adenomas (Supplemental Figure 3S and Supplemental Appendix, see <http://ajp.amjpathol.org>). Severe atypical hyperplasias were seen as early as 8 weeks in both strains and were more prevalent in B6 mice ($P = 0.004$). In FVB mice, the incidence increased from 30% at 8 weeks to 50% at later time points, whereas the incidence was 50% at 8 weeks in B6 mice and this increased to 80% at 20 weeks (Figure 1C). In summary, there were no differences in development or progression of atypical hyperplasias that could

Figure 2. Neuroendocrine carcinoma in the coagulating gland of an FVB mouse. **A:** Notice discrete tumor (arrow) within the coagulating gland epithelium. The tumor appears hyperchromatic because of the high nuclear:cytoplasmic ratio of neoplastic cells, H&E. **B:** Detail of neuroendocrine carcinoma cells adjacent to mildly hyperplastic prostatic glandular epithelium indicated by arrows. Note loss of glandular differentiation and marked cell pleomorphism, H&E. **C:** Positive synaptophysin staining (brown) of tumor cells (asterisk) in contrast to surrounding glandular epithelial tissue (asterisk), anti-synaptophysin antibody and hematoxylin. **D:** AR staining of glandular epithelial cells (asterisk) is intense and localized to the nucleus in contrast to the diffuse and weak staining seen in the adjacent NE carcinoma, anti-AR antibody, and hematoxylin.

account for the large difference in the incidence of neuroendocrine carcinomas in the same animals.

We assessed prostate maturation in 4-week-old TRAMP mice of both background strains on a scale of 1 to 5, and Tag expression on a scale of 0 to 2. Interestingly, prostates from FVB mice were more mature than those from B6 mice (means of 4.6 and 3.6, respectively; $P = 0.04$) and expressed Tag earlier. There was a positive correlation between prostatic maturation stage and Tag expression ($P = 0.001$). We also observed Tag expression in the prostatic stroma of both strains as early as 4 weeks.

Tumorigenic Potential of Early-Stage Epithelial Lesions from B6 and FVB Mice

One possible explanation for the above data was that neuroendocrine carcinomas of the prostate could arise from pre-existing epithelial lesions by a process of trans-differentiation, or by selection of a prostate stem cell population with subsequent commitment to the neuroendocrine lineage. To test these possibilities we performed grafting studies of microdissected prostate ducts from 17-week-old B6 and FVB TRAMP mice. Abnormalities in ductal morphology visible in whole-mount preparations

Table 1. Summary of Immunohistochemical Marker Expression in Prostatic Epithelium

IHC marker	Normal prostate			Atypical hyperplasia		NE foci and tumors	
	Embryonic	Neonatal	Adult	Early	Late	Early	Late
Tag	—	—	—	+	++	+	++
AR	+/-	+	+	+	+	+	+/-
p63	+	+ in basal cells	+ in basal cells	+ in basal cells	+ in basal cells, but fewer basal cells present	—	—
E-cadherin	+	+ in luminal cells	+ in luminal cells	+	+	Some cells co-express with synaptophysin	—
Synaptophysin	—	—	—, except for an occasional NE cell	—, except for an occasional NE cell	—, except for an occasional NE cell	+, co-express with e-cadherin	+
Foxa1	+	+	+	+	+	+	+/-
Foxa2	+, cells near basal lamina and ductal tips	+/-, positive cells near basal lamina and ductal tips	—, except for an occasional NE cell	—, except for an occasional NE cell	—, except for an occasional NE cell	+/-	+

Figure 3. Prostate whole mounts and histology of pregraft tissues. **A:** Whole mount of prostatic ducts (dorso-lateral prostate) used to isolate tissue for subcapsular renal grafting. Note abnormal duct (box), which is thickened and fails to *trans*-illuminate. **B:** Tissue used for grafting was bisected, and half was used for histological and immunohistochemical analysis. This section shows a duct stained for anti-smooth muscle actin (purple) to outline the intact smooth muscle stroma, and anti-Tag (nuclear stain, brown) to highlight the transformed epithelium. Note intact smooth muscle sheath around focus of glandular herniation (*inset*). **C:** H&E-stained section of pregraft duct showing atypical hyperplasia of Tag.

(Figure 3A) were dissected and grafted under the renal capsule of intact, male nude mice and harvested after 2 months. We tested the tumorigenic potential of lesions that originated in all lobes. The majority (95%) of grafted ducts ($n = 85$) that were abnormal but not yet neoplastic yielded small pieces of tissue that showed different degrees of atypical hyperplasia of Tag (Figure 3, B and C). The remaining four ducts judged to be abnormal based solely on whole-mount morphology became NE carcinomas.

We then designed a follow-up study to assess the progression potential in conjunction with pregraft histology. In the second study visibly abnormal ducts were bisected, and half of the ductal fragment was examined histologically while the other half was grafted. Forty-two ductal fragments that exhibited atypical hyperplasia of Tag but no NE features were grafted and when harvested displayed minimal growth and slightly more severe atypical hyperplasia of Tag. There were no NE carcinomas in the resulting grafts. In contrast, 90% (19 of 21) of the grafted fragments of NE carcinomas or ducts within or

adherent to the carcinomas grew quickly and yielded large NE carcinoma grafts (data not shown). These NE carcinoma grafts grew at identical rates, independently of the strain of origin. The two grafts that did not yield tumors were later verified to be small stromal remnants. No strain differences were noted in these grafting experiments. These data suggest that, although NE carcinomas develop well underneath the renal capsule, they do not appear to arise from transdifferentiation of pre-existing atypical hyperplasia of Tag.

Previous work has demonstrated that embryonic rat urogenital sinus mesenchyme (rUGM) is a potent inducer of proliferation of prostate epithelial cells. In an attempt to promote neuroendocrine carcinoma progression by stimulating proliferation, normal rUGM was combined with abnormal ductal fragments from 17-week-old mice. After 4 weeks of growth tissues were recovered, a portion was taken for histological analysis, and a portion was re-grafted with or without fresh rUGM. No NE carcinomas were found after the initial 4 weeks of growth ($n = 5$) or in the regrafts ($n = 46$). The recovered tissues had less advanced atypical hyperplasia of Tag than *in situ* prostates from 21- or 25-week-old TRAMP mice. To understand this apparent regression, we harvested three UGM plus TRAMP ductal tissue recombinants 2 weeks after grafting and observed that epithelial outgrowths from the ductal fragments did not express SV40 Tag and resembled embryonic prostatic buds; they were solid and contained a high proportion of p63-expressing cells (Figure 4, A and B). These data indicate that the rUGM, rather than inducing proliferation and further progression of the TRAMP epithelium, had a normalizing effect, allowing the outgrowth of prostatic buds with normal morphology and normal gene expression pattern. These results show that Tag expression in the stroma is a critical step in lesion progression in the TRAMP model and emphasize the important role of the tumor microenvironment, and in particular the stromal cells, in control of tumor cell growth.²² These transplantation studies of prostate tissue from TRAMP mice support a separate and distinct lineage of origin for neuroendocrine carcinomas.

Existence of Bipotential Epithelial/Neuroendocrine Cells in the TRAMP Prostate

The results described above suggested differences in the frequency or location of neuroendocrine precursor cells in B6 and FVB mice before tumor development. Small foci of intraepithelial neuroendocrine proliferation were frequently found in both B6 and FVB samples, within normal and mildly hyperplastic glands (Figure 5). These foci were readily identified in sections stained for synaptophysin (Figure 5, B and D; and Supplemental Figure 4S, see <http://ajp.amjpathol.org>). In areas of normal prostate, atypical hyperplasia of Tag, and papillary tumors, the epithelium stained strongly with anti-E-cadherin, especially on the lateral cell borders, but not with anti-synaptophysin. The neuroendocrine foci (NE foci) were Tag-positive (data not shown), and some showed a pattern of

Figure 4. Tissue recombinants composed of rUGM and TRAMP prostate epithelium harvested 2 weeks after subcapsular renal grafting. **A:** Nuclear SV40 Tag expression is turned off in prostatic outgrowths induced by rUGM (left) when compared to original epithelium grafted (right), anti-Tag antibody and hematoxylin. **B:** The new prostatic outgrowths express nuclear p63 (left) simulating early prostatic duct development. Note that original epithelium grafted (right) is mostly negative for p63. **C:** Detail of p63 nuclear expression in continuous basal layer of prostatic outgrowths in tissue recombinants. Note cuboidal epithelium characteristic of immature ducts. **D:** Detail of original prostatic epithelium negative for p63 with the exception of scattered basal cells (arrows). **B–D:** Anti-p63 antibody and hematoxylin.

transitional or concomitant expression of E-cadherin and synaptophysin (Figure 5, A–D). In these NE foci, the more cuboidal or columnar cells with epithelial morphology expressed E-cadherin (Figure 5, A and C) whereas the smaller, darker, pleomorphic cells expressed synaptophysin (Figure 5, B and D). Interestingly, the areas of transition between cells of the two morphologies frequently co-expressed E-cadherin and synaptophysin (Figure 5, C and D; arrows). Synaptophysin-expressing cells were often within the basal layer of the epithelium, supporting the existence of a stem cell niche at this location (Figure 5C, unstained cells). However, some cells with purely epithelial morphology (columnar and indistinguishable from surrounding epithelia) also expressed synaptophysin (Supplemental Figure 4S, see <http://ajp.amjpathol.org>).

Analysis of the expression patterns of these two markers demonstrated that there may be an intraepithelial population of bipotential cells in the developing TRAMP prostate that expressed both synaptophysin and E-cadherin. The loss of E-cadherin expression was accompanied by a change of cell morphology in these NE foci (Figure 5C, unstained cells). Most importantly, NE foci appeared earlier than and unrelated to invasive NE car-

Figure 5. Staining of NE precursor foci in the FVB-TRAMP VP with E-cadherin and synaptophysin. **A:** Normal gland staining brightly for E-cadherin. **B:** Serial section of the same gland showing small intraepithelial clusters of synaptophysin-positive NE cells (arrows). **C:** Another glandular duct showing a larger NE precursor focus. Note intense E-cadherin stain of epithelium with the exception of purely NE cells (asterisk) that have a more basal location. **Arrow** indicates an area of transition between epithelial and neuroendocrine morphology that expresses both E-cadherin and synaptophysin. **D:** Synaptophysin staining of serial section of the gland depicted in C showing purely neuroendocrine cells (asterisk) and an area of transition that expresses both synaptophysin and E-cadherin (arrow).

cinomas, and it arose in glands virtually unaffected by atypical hyperplasia of Tag.

The identification of these bipotential lineages in the prostate was further investigated using antibodies to the transcription factors Foxa1 and Foxa2. Normal prostatic epithelium expressed Foxa1 but not Foxa2 (Supplemental Figure 5S, A and B, arrowheads; see <http://ajp.amjpathol.org>). Areas of atypical hyperplasia were positive for Tag, strongly expressed Foxa1, similarly to normal epithelium but not Foxa2 (Figure 6, A and B; arrows). In contrast, all small NE foci (Supplemental Figure 5S, A and B, arrows; see <http://ajp.amjpathol.org>), as well as overt NE carcinomas, strongly expressed Foxa2 as well as Foxa1, although less intensely (Figure 6, A and B; and Supplemental Figure 5S, A and B, see <http://ajp.amjpathol.org>). All cells positive for Foxa2 were also positive for synaptophysin (Figure 6C; and Supplemental Figure 5SC, see <http://ajp.amjpathol.org>). In the proliferative intraepithelial neuroendocrine foci, some of the Foxa2-positive cells had an apparent basal distribution (Supplemental Figure 5SB, see <http://ajp.amjpathol.org>).

There was a significant difference in the number and age of onset of NE foci between strains ($P = 0.034$, Figure 1D). Overall, 61% of FVB prostate samples had NE foci, compared to only 15% of B6 prostate samples. In FVB mice, NE foci were found as early as 4 weeks, whereas the first NE foci in the B6 prostatic tissues were

Figure 6. NE carcinoma and adjacent hyperplastic gland in FVB TRAMP mouse showing Foxa1, Foxa2, and synaptophysin expression. **A:** Strong nuclear expression of Foxa1 in NE carcinoma cells (**asterisk**) and adjacent hyperplastic glandular epithelium (**arrows**), anti-Foxa1 and hematoxylin. **B:** Foxa2 expression is limited to neuroendocrine carcinoma cells (**asterisk**). Note that the glandular epithelium does not express Foxa2 (**arrows**), anti-Foxa2 and hematoxylin. **C:** NE carcinoma cells (**asterisk**) also express synaptophysin in contrast to the negative hyperplastic glandular epithelium (**arrows**), anti-synaptophysin and hematoxylin.

seen at 12 weeks. NE proliferative foci were not found in wild-type mice.

The fact that prostates from FVB mice appear to mature faster and express Tag earlier than prostates from B6 mice may help to explain the predominance and early onset of NE foci in FVB compared to B6 TRAMP mice (Supplemental Figure 6S, Supplemental Appendix, see <http://ajp.amjpathol.org>). The early emergence and the increased incidence of NE foci in FVB when compared to B6 mice parallel the early onset and high prevalence of NE carcinomas in this strain.

Discussion

This study of the influence of genetic background in prostate pathology between FVB and B6 TRAMP mice reveals that FVB mice have a significantly higher incidence of malignant neuroendocrine carcinomas and a significantly shorter survival time compared to B6 mice. All FVB mice developed malignant NE carcinomas by 20 weeks compared to a lifetime incidence of 20% in B6 mice. We evaluated prostatic tissues from 160 mice and established the lobar location, age of onset, and incidence of the different types of lesions in both strains and found that all 77 malignant tumors examined had histological and immunohistochemical features of neuroendocrine carcinomas. By our criteria, no adenocarcinomas were found in the samples from 160 mice examined. We also found that epithelial lesions caused by Tag expression in the prostate of TRAMP mice are morphologically unique and do not resemble human or mouse prostatic intraepithelial neoplasia. To more accurately reflect these differences and to convey the fact that these lesions do not seem to progress to invasive cancer, we opted to use the terminology “atypical hyperplasia of Tag” to designate lesions of the TRAMP model. We propose that the prostate pathology in TRAMP mice can follow two independent pathways (Figure 7). Both strains develop benign and atypical hyperplasia of Tag affecting all prostatic lobes with the exception of the VP, which remains

relatively normal or is mildly affected. Atypical hyperplasias become slowly more severe but rarely progress to invasive adenocarcinoma. In parallel, small foci of NE cells arise independently from other lesions and can proliferate to form NE carcinomas. NE carcinoma formation is strain-dependent, occurs in 100% of FVB mice, and is most prevalent in the VP lobes.

All lesions, whether epithelial/stromal or neuroendocrine, expressed SV40 Tag suggesting that Tag is required for initiating transformation. The invasive NE carcinomas had characteristic morphology confirmed by positive staining for synaptophysin and Foxa2. The earliest NE carcinomas in both strains were found between 12 and 16 weeks. Other epithelial and stromal abnormalities were negative for synaptophysin and Foxa2 and were not associated with invasion. We found no significant strain differences in the incidence or age of onset of mild or moderate atypical hyperplasia of Tag. Severe atypical hyperplasia of Tag, on the other hand, was more frequent in B6 mice, which further supports the dissociation between NE carcinomas and epithelial lesions.

To identify precursors to NE carcinomas, we quantified synaptophysin-positive NE foci. Two differences between the FVB-TRAMP and B6-TRAMP strains were observed: NE foci, which were located within the basal and luminal compartments of the glandular epithelia, appeared earlier in FVB mice than in B6 mice, and FVB mice had a significantly higher incidence of NE foci than B6 mice. In addition, synaptophysin-positive NE foci were more common in the VP lobe, which is the lobe where most NE carcinomas were found and where atypical hyperplasia of Tag was either nonexistent or mild. These observations support the hypothesis that synaptophysin-positive NE foci are the precursors to NE carcinomas in the TRAMP model.

Given that mature prostatic epithelium consists of luminal, basal, and neuroendocrine cells, we considered that NE lesions could originate from pre-existing neuroendocrine cells, pluripotent progenitor or stem cells, or transdifferentiation of mature luminal epithelial cells. Cur-

Figure 7. Schematic representation of dissociation of epithelial and neuroendocrine lineages in early prostatic tumorigenesis in TRAMP mice in our cohort. In the neuroendocrine carcinoma pathway (**B1** and **B2**) a pluripotential stem cell of the normal prostate (**A**) is transformed by SV40 Tag expression and starts to proliferate. Initially these proliferative foci maintain a transitional epithelial/NE phenotype co-expressing E-cadherin, synaptophysin, and both Foxa1 and Foxa2 (orange cells with solid nuclei). These bipotential cells continue to proliferate, undergo full NE differentiation with loss of E-cadherin, and then progress to an overt NE carcinoma. NE carcinomas quickly invade the surrounding stroma and have the potential to metastasize. NE carcinomas maintain Foxa1 expression reminiscent of this bipotential stem cell origin. In the atypical hyperplasia of Tag pathway (**C1** and **C2**), luminal cells of the normal prostate (**A**) are transformed by SV40 Tag expression (yellow cells with solid nuclei), proliferate, and form focal areas of hyperplasia. These foci expand and soon involve the entire glandular lumen. These lesions become large, complex, and dysplastic, but they do not invade the surrounding stroma and do not metastasize. Epithelial cells in these lesions continue to express E-cadherin, Foxa1, and SV40 Tag.

rent models of prostate development propose the existence of a stem cell compartment within the basal layer of the prostatic epithelium, which gives rise to all cells that compose the glandular epithelium, including basal, luminal, and neuroendocrine; however, the exact lineage relationships are unknown. Despite extensive investigation, no single protein is currently accepted as a definitive stem cell marker in the prostate.^{25,26}

Because we could not directly trace the lineage of cells in the prostate, we used the immunohistochemical distribution of cytoplasmic and nuclear markers to infer lineage relationships. In the mature normal prostate, luminal cells express E-cadherin and AR, basal cells express p63, and neuroendocrine cells express synaptophysin. Many of the neuroendocrine foci in the TRAMP mice had dual expression of synaptophysin and E-cadherin in areas of transition between epithelial and neuroendocrine cell morphologies. It seems likely that these double-positive cells represent a progenitor cell population that can give rise to the rapidly growing neuroendocrine carcinomas. The alternative scenario of transdifferentiation from atypical hyperplasias is less likely given the results of the grafting experiments and the lobe distribution of tumors, which are more common in VP, in which atypical hyperplasia is less frequent and NE precursor foci are often found. However, this possibility cannot be definitively excluded based on our current data. In humans, neuroendocrine carcinomas, such as small cell carcinoma of the lungs and breasts as well as some cutaneous Merkel cell tumors, can co-express E-cadherin and synaptophysin.^{27–29} Our results differ from these reports in that we

only observed E-cadherin and synaptophysin co-expression in transitional areas within neuroendocrine foci, not in overt neuroendocrine carcinomas.

This interpretation of lineage relationships is also supported by the observed expression patterns of the Forkhead box transcription factors Foxa1 and Foxa2. FOXA1, FOXA2, and FOXA3 are required for normal endodermal and mesodermal development; FOXA1 and FOXA2, specifically, play an important role in prostatic morphogenesis and function. Foxa1 is expressed in the developing and mature mouse prostate whereas Foxa2 is present in prostatic buds early in development but disappears shortly after birth.^{30,31} In the adult mouse prostate, Foxa2 is confined to sparse synaptophysin-positive neuroendocrine cells within periurethral prostatic ducts and is thought to be a marker of neuroendocrine differentiation.³¹ In humans, FOXA1 is expressed in all prostate adenocarcinomas, regardless of Gleason grade, whereas FOXA2 is expressed only in high-grade prostatic carcinomas with neuroendocrine differentiation and in neuroendocrine small cell carcinomas of the prostate.³⁰ In the 12T-10 LADY model, prominent Foxa1 and Foxa2 expression was observed in neuroendocrine carcinomas.³¹ Similarly, both Foxa1 and Foxa2 are expressed in neuroendocrine foci and neuroendocrine carcinomas of TRAMP mice. The co-expression of Foxa1 and Foxa2 in NE foci also supports a model of descent from a bipotential or multipotent precursor cell.

Although small cell prostate carcinomas (pure neuroendocrine carcinomas) are rare in the human prostate, neuroendocrine differentiation occurs in ~10 to 30% of

cases and is associated with poor prognosis. Tumors with neuroendocrine characteristics are usually androgen-independent and refractory to treatment, therefore understanding their pathogenesis is essential in improving therapy. In men, prostate neuroendocrine differentiation is thought to occur within focal areas of pre-existing adenocarcinomas, resulting in the development of a more aggressive clone that becomes invasive and metastatic. Our findings, however, suggest that in the TRAMP model, neuroendocrine differentiation may arise independently from adenocarcinoma formation.

SV40 Tag blocks both the *RB* and *TP53* pathways, and because loss of *RB* or *TP53* function alone does not drive the neuroendocrine phenotype, the simultaneous loss of both genes may be an essential step for neuroendocrine differentiation.^{32–34} This hypothesis is supported by observations of other human neuroendocrine carcinomas, as well as neuroendocrine carcinoma models. For instance, 90% of human small cell cancers of the lung (a neuroendocrine carcinoma) have inactivation of both *RB* and *TP53*.^{35,36} The mouse model counterpart of small cell cancer of the lung also depends on simultaneous *Rb* and *Trp53* inactivation.³⁷ Furthermore, neuroendocrine carcinoma formation in the context of simultaneous *Rb* and *Trp53* loss is illustrated by a colon cancer model in which SV40 is expressed in the mouse intestinal epithelium under the Trefoil promoter.³⁸ In addition, NE carcinomas have been described in a recently published mouse model created by means of prostate-specific deletion of both *Rb* and *Trp53*.³⁹ Simultaneous deletion of these two tumor suppressor genes in the prostate leads to benign proliferative epithelial-stromal lesions in the terminally differentiated, distal regions of prostatic ducts and to invasive NE carcinomas in the stem cell-rich, proximal ducts of the prostate. Similarly to our findings, early preneoplastic lesions in this model have features of stem cells and also co-expressed luminal and neuroendocrine markers such as cytokeratin 8, synaptophysin, and chromogranin A. We conclude that the simultaneous loss of function of *RB* and *TP53* tumor suppressor genes is essential in early cell fate decisions and NE carcinoma formation in multiple organs. Furthermore, combined *Rb* and *Trp53* inactivation in the prostate appears to affect stem cells and terminally differentiated cells differently, resulting in either NE carcinomas or benign atypical hyperplasias, respectively.

Gene expression profiling experiments of neuroendocrine prostate tumors from the Cr-2 Tag mouse, which expresses SV40 Tag driven by the cryptidin promoter, and of tumors from a mouse model of neuroendocrine gastric cancer (SV40 Tag under the *Atp4b* promoter) have shown increased mRNA of several transcription factors related to neuroendocrine differentiation including *Foxa2*, *mASH1*, *Sox 1*, *Sox 2*, and *Gad1*.^{36,38,40} Investigation of the functions of these factors will further the understanding of neuroendocrine carcinoma development in the prostate. These data suggest that the TRAMP model may not be an appropriate model for initiation and progression studies of human prostate cancer but may be a valuable tool in chemotherapeutic trials targeting the aggressive neuroendocrine variant of the human disease.

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