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Transgenic *Polyoma Middle-T* Mice Model Premalignant Mammary Disease[1]

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ABSTRACT

Mice transgenic for the *Polyomavirus middle T* (*PyV-mT*) gene have been widely used to study mammary tumorigenesis and metastasis. Although numerous molecular insights were gained from the analysis of these transgenic malignant tumors, the early events leading to malignant transformation have not been systematically investigated nor has the biological potential of hyperplastic lesions been documented. This paper presents the first comprehensive histopathological characterization of transgenic PyV-mT hyperplasias together with classical transplantation experiments designed to test the growth potential of these lesions. Moreover, stable hyperplastic outgrowth lines were established as a tool to study premalignant PyV-mT-induced hyperplasias in detail. Each line has a different tumor latency, indicating that PyV-mT-induced hyperplasias, like early proliferative lesions seen in the human breast, are heterogeneous with respect to their malignant potential. Our results settle a controversy; they establish that *PyV-mT* gene expression alone is insufficient to induce tumors and that additional events are required for tumorigenesis and metastasis. These results support the use of *PyV-mT* transgenic mice as a model for investigating the multistep progression of malignant mammary tumorigenesis and metastasis.

INTRODUCTION

A more complete understanding of the biology of hyperplastic epithelial lesions is clearly essential for improving intervention and treatment of human breast disease. The current method for diagnosing breast disease is based on histopathology (1), whereas prognosis and decisions concerning treatment rely on epidemiological studies that correlate specific histopathological findings with clinical outcomes (2, 3). The lack of knowledge regarding the malignant potential of hyperplastic lesions continues to hamper accurate prediction of clinical outcomes. The lack of certainty regarding which cells will become malignant impedes the development of treatments designed to target premalignant cells. However, human investigations remain limited to retrospective study designs that provide only relative risk statistics. Therefore, it is critical to augment human retrospective studies with detailed analyses of premalignant lesions that include both histology and biological experiments testing growth behavior (4–7).

Genetically engineered mice have emerged as powerful tools for investigating neoplastic progression within an intact array of host factors that are not present in *in vitro* models (8–10). An important goal of the research community is to translate information gained from studying transgenic mouse models into useful clinical applications. To address the need for a more comprehensive and uniform analysis of mammary lesions in transgenic models, a panel of expert pathologists was convened by the National Cancer Institute at Annapolis, MD (6). The panel recommended that diagnostic nomenclature of transgenic lesions in mice be based on similar morphological, immunohistochemical, and biological criteria so that direct comparisons among mouse models and with humans would be possible. The use of the term MIN[4] was proposed for premalignant lesions associated with cancer, with the *proviso* that their premalignant properties be confirmed using classical mammary gland transplantation experiments (6).

Although multistep transgenic models of tumorigenesis and progression are particularly useful in the study of premalignant lesions, characterization of early proliferative lesions is not yet complete. A recent literature review of the histopathogical and biological properties of premalignant mammary lesions in transgenic mice identified 70 papers describing potential premalignant lesions (5). Few contain mammary transplantation experiments and none fulfill the rigorous requirements proposed by the pathology panel for the diagnosis of MIN (6).

One widely used transgenic model of mammary tumorigenesis expresses the PyV-mT antigen (10). Tumor induction is so rapid in *PyV-m*T transgenic mice that some investigators have postulated that there is no intermediate stage of tumorigenesis, and transgene expression alone is sufficient for mammary epithelial cell transformation (11–15). Further, the expression of the *PyV-mT* transgene in endothelial cells has been reported to cause a single-step transformation (16). However, the presence of focal atypias within the *PyV-mT* mammary gland is consistent with a multistep model in which additional molecular events are required (17). The *in vivo* growth properties of these early proliferative lesions had not previously been studied (5) and it has not been clear whether *PyV-mT* transgenic mice serve as a model for single-step or multistep transformation (10, 13, 15). In fact, these focal hyperplastic PyV-mT lesions had not been investigated for potential heterogeneity in their capacity to produce malignancy.

This paper provides a comprehensive histopathological and biological analysis of early proliferative lesions found in this widely used mammary cancer model. It is the first, in a transgenic model, that completely fulfills the Pathology Panel criteria for diagnosing MIN, and it provides a baseline for comparison with premalignant lesions in other models and in humans (5). Furthermore, a set of five different stable, HPO lines were established. The method of establishing HPO lines was pioneered by Kenneth B. DeOme *et al.* more than four decades ago (18), and it has been extensively used by Dan Medina *et al.* to isolate and study premalignant mammary tissues in other models of mammary disease (reviewed in Ref. 4). Thus far, HPO lines have not been used to study premalignant tissues in genetically engineered mouse models (5). The HPO lines are amenable to direct experimental

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[4] The abbreviations used are: MIN, mammary intraepithelial neoplasia; PyV-mT, Polyomavirus middle-T; HPO, hyperplastic outgrowth; IHC, immunohistochemistry; CK-8, cytokeratin 8; SMA, smooth muscle actin; ER, estrogen receptor; WAP, whey acidic protein; OPN, osteopontin; PgR, progesterone receptor; CK-14, cytokeratin 14.

manipulation and will facilitate analyses that are needed to augment data from human retrospective studies. Distinct tumor incidence among these lines reveal that PyV-mT-induced MIN, such as early proliferative lesions seen in the human breast, are heterogeneous with respect to their malignant potential. Our results provide comprehensive evidence that PyV-mT mice have premalignant hyperplastic foci that require additional "hits" before malignancy is acquired. The data firmly establish the utility of the PyV-mT system for the study of the multistep progression of mammary lesions from normal through hyperplasia to malignancy and metastasis. The growth behavior of the individual HPO lines clearly document that they are heterogeneous with respect to their malignant potential.

MATERIALS AND METHODS

Mice. FVB mice, expressing PyV-mT antigen under control of the mammary-specific mouse mammary tumor virus long terminal repeat promoter, were provided by Prof. William J. Muller (McMaster University, Hamilton, Ontario, Canada) All mice were bred and maintained in a University of California, San Diego (San Diego, CA), vivarium according to NIH guidelines and were killed by CO_2 inhalation.

Whole Mounts and IHC. Mammary whole mounts were prepared as described (19). For microscopic analyses, glands fixed overnight in formalin, paraformaldehyde, or Bouin's reagent, were paraffin-embedded and sectioned to 5 μm. H&E staining and appropriate IHC or *in situ* hybridization were performed. Antigen retrieval was accomplished by microwaving in citrate buffer. IHC studies used anti-CK-8 (PH19211.xs; Binding Site; 1:300), anti-CD14 (PH504; Binding Site; 1:200), anti-SMA (A-2547; Sigma Chemical Co.; 1:1000), antilaminin (L-9393; Sigma Chemical Co.; 1:1000), anti-ERα (SC542; Santa Cruz Biotechnology; 1:1000), anti-OPN (AF808; R&D Systems; 1:800), anti-WAP (Dr. Lothar Henninghausen; NIH; 1:4000; Ref. 20), anti-p53 (PH507.xS; Binding Site; 1:1000), anti-Ki-67 (CNLki67p; Novo Castro; 1:1800), anti-CD31 (SC-1506; Santa Cruz Biotechnology; 1:200), anti-Polyomavirus T-ag (Ab-4; Oncogene Research; 1:15), and anti-PGr (A0098; Dako; 1:1800; Ref. 21). Anti-SMA was amplified and detected using ARK (Dako). Other antibodies were amplified and detected using the Vector ABC kit. A digoxiginated PyV-mT antisense probe (Dr. Robert Oshima, Burnham Institute, La Jolla, CA), was detected as a blue signal using anti-digoxigen-coupled alkaline phosphatase (22). Images were captured using a Kontronic camera model 8102 on an Olympus BH2 microscope, digitized using Photoshop 6.0 with the Kontron ProgRes "plug-in" module, color enhanced, balanced for contrast, and printed using a Kodak 8650 dye sublimation printer. Images of nuclei were captured with a Kontron digital camera and analyzed using Image Pro Plus 4.1 (Media Cybernetics), internal programs, and statistical packages.

Primary Lesion Transplantation. Donor tissue from zones 3 and 4 of the inguinal mammary glands of anesthetized PyV-mT$^{+/-}$ female mice 8 weeks of age was isolated with IP injections of xylazine/ketamine. One mm^3 tissue segments were transplanted into the inguinal mammary fat pads of nontransgenic FVB female mice 3 weeks of age that were either cleared of epithelium as described (18, 23) or left with intact growing epithelium.

Generation and Characterization of HPO Lines. Specific mammary gland lesions from zone 4 of a female PyV-mT$^{+/-}$ mouse 8 weeks of age were visualized under a dissecting scope, and 1-mm^3 tissue segments were surgically isolated and transplanted into cleared mammary fat pads as described above. Recipients were monitored weekly for palpable tumors. At 6–12-week intervals, nonmalignant tissue was selected and retransplanted into cleared mammary fat pads and also s.c. at intervals to test malignant potential. At the fourth generation, each of five established HPO lines were transplanted bilaterally into 10–11 gland-cleared recipient fat pads and monitored weekly for palpable tumors, and fat pads bearing palpable tumors were surgically removed. At 14 weeks and at 21 weeks, all surviving mice were surgically examined under a dissecting microscope for evidence of malignancy. If no tumor was evident, weekly examination resumed. Twenty tumors emerging from HPO tissues were s.c. transplanted to test malignant status. Lungs were examined grossly at autopsy for evidence of metastasis.

Angiogenesis. Anesthetized mice were exsanguinated with whole-body PBS perfusion using a flow pump (Control Co., Houston, TX) and, without pause, reperfused with black India ink (Sanford No. 4418; 1:10 in PBS). Fat pads were prepared as whole mounts (19). Selected tissues were embedded in paraffin, sectioned, and counterstained with eosin. For anti-CD31 staining, 5-μM frozen sections were fixed in 2% formalin and stained with anti-CD31 (01951A; PharMingen) at 1:200. Images ($\times$20; Leica DMLB microscope) were captured with a SPOT RT Real Time digital camera (Diagnostic Instruments, Sterling Height, MI), imported into Photoshop 6.0 (Adobe, Inc.), and analyzed as described (24), except the data were normalized to the epithelial area.

RESULTS

Development of Focal Atypical Lesions. *PyV-mT* transgenic female mice exhibited palpable tumors beneath the nipple of several mammary glands by the fifth week of life (25). Multifocal areas of mammary hyperplasia with atypia were described previously in the PyV-mT gland (13). To study these hyperplasias, whole-mount preparations of inguinal mammary glands from virgin females were analyzed. For descriptive purposes, mammary glands were divided into four topographical zones (Fig. 1).

Regardless of age, a gradation of focal atypical lesions was always apparent. The advancing ductal tree seemed normal, whereas the older, more proximal ducts exhibited hyperplastic foci. Zones 1 and 2 generally had more and larger foci than zones 3 and 4. However, by 9 weeks, the entire gland sometimes had too many coalescing cellular masses to count. A majority of the small lesions visible in zones 3 and 4 were cystic out-croppings arising along or at the ends of ducts (Fig. 1*B*). Most were lined by a thickened, papillary epithelium that had smooth outlines, suggesting noninvasive growth (Fig. 1*C*, *arrow C*). In older animals, larger lesions were also present in zones 3 and 4. These lesions were frequently solid (Fig. 1*C*, *arrow S*).

Microscopic and Gene Expression Analysis. To identify the cell types present in the focal lesions and document their relative differentiation, inguinal mammary glands of PyV-mT mice 3, 5, 7, and 9 weeks of age were assessed by a combination of microscopic, morphometric, and immunohistochemical analyses and compared with

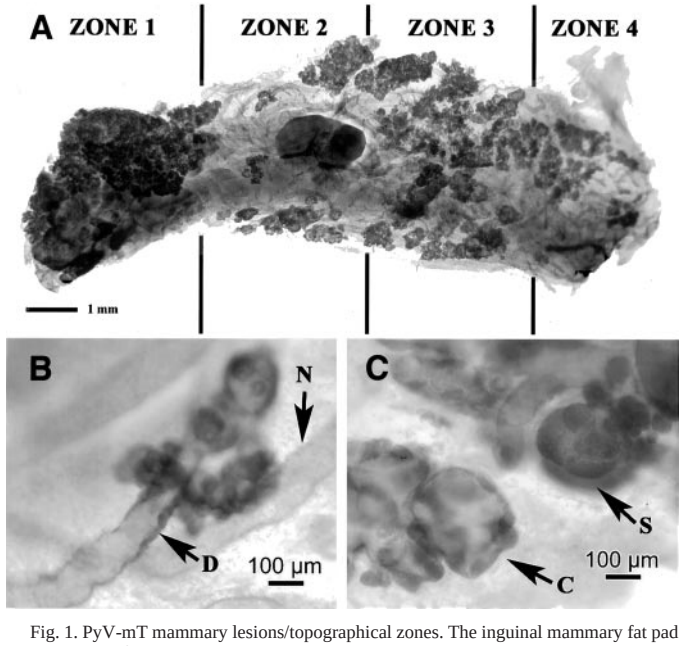

Fig. 1. PyV-mT mammary lesions/topographical zones. The inguinal mammary fat pad is divided into four topographical zones. The lymph node serves as a convenient guide, dividing the rest of the mammary fat pad roughly into halves. Note the solid invasive lesion in *zone 1*. *Two bottom images*, enlarged areas of the whole mount showing a cystic lesion (*arrow C*) and a solid lesion (*arrow S*). Early lesions start at the terminal ducts, which may have thickening of the duct lining (*arrow D*). Compare the thickness of this duct with that of a normal duct (*arrow N*).

normal nontransgenic virgin ducts, end buds, and prelactating mammary acini. To establish a baseline for comparison with other transgenic models as well as human breast disease, a modification of the recommended immunohistochemical panel was used (6). The antibodies were chosen to yield information about (*a*) transgene expression; (*b*) cytoplasmic differentiation; (*c*) cell proliferation; (*d*) nuclear characteristics; and (*e*) nonepithelial components including basement membrane and microvasculature. CK-8 is a differentiation marker used to identify epithelial cells (26). WAP (27) and OPN (28) are proteins expressed by differentiated secretory mammary epithelial cells and are commonly increased in prelactating mammary acinar tissue (Table 1). Several proteins commonly used in the diagnosis, grading, and prognosis of human breast cancer were included. For example, expression of the nuclear antigen Ki-67 is commonly assessed in both humans and mice to identify proliferating cells (29–31). ER, PgR, and p53 expression are routinely assessed in humans because they are important prognostic and predictive factors in human breast disease (31–33). Normal nontransgenic virgin ducts and end buds were compared with nontransgenic hyperplastic (prelactating) mammary acini, PyV-mT-induced MIN, and PyV-mT-induced tumors. Table 1 summarizes the results of immunohistochemical stains that identify changes in gene expression between normal and transgenic mammary tissue. Several pertinent findings are highlighted in the text below.

Mammary Epithelium: Morphological Heterogeneity. Noninvasive focal lesions were well developed by 5 weeks and could be classified into four subsets: (*a*) simple; (*b*) solid; (*c*) cystic; and (*d*) "mixed," solid and cystic (Fig. 1). Simple lesions were observed microscopically as semicystic bulges along the ducts, containing one to several layers of atypical cells (Figs. 2 and 3). Occasionally these lesions had small glandlike structures with secondary lumens that were positive for WAP and OPN, indicating mammary secretory epithelial differentiation (Fig. 3). The configuration and staining characteristics of these lesions suggest that they may be abortive side buds.

Solid lesions were generally larger foci containing dense masses of atypical cells organized in nodular sheets. These lesions also contained OPN-positive cells. However, they did not contain central fluid-filled spaces. Cystic lesions varied in size and complexity (Figs. 2 and 3). Most were lined by a multilayered epithelium that was frequently papillary and contained WAP- and OPN-positive cells (Fig. 3). These lesions were uniformly associated with significant amounts of clear fluid containing both WAP and OPN (Fig. 3). Some cystic lesions were directly adjacent to or connected to one or more solid cell masses and were therefore classified as mixed solid and cystic.

Table 1 *Relative expression of mammary markers*

Immunohistochemical stains were performed to detect the presence of selected antigens, as noted. The relative intensities are recorded in a 4+/4+ scoring system based upon the intensity in a positive control slide for indicated tissue from nontransgenic FVB mammary glands (not bold) and from PyV-mT-induced MIN and tumors (bold).

	Duct	Terminal end bud	Prelactating acinus	**Dysplasia (MIN)**	**Tumor**
CK-8	4	4	4	**3**	**var**[a]
CK-14	1	1	1	**2**	**2**
WAP	±	−	4	**1**	**−**
OPN	−	−	4	**2**	**2**
SMA	1	−	1	**−**	**−**
PgR	3	4	3	**−**	**−**
ER	1	2	2	**2**	**var**
p53	−	−	−	**−**	**±**
Ki-67	4	4	2	**4**	**4**
PyV-mT	−	−	−	**2**	**4**
c-erbB2	−	1	2	**var**	**var**
CD-31	−	−	−	**var**	**−**
Laminin	1	1	4	**1**	**var**

[a] var, variable intensity.

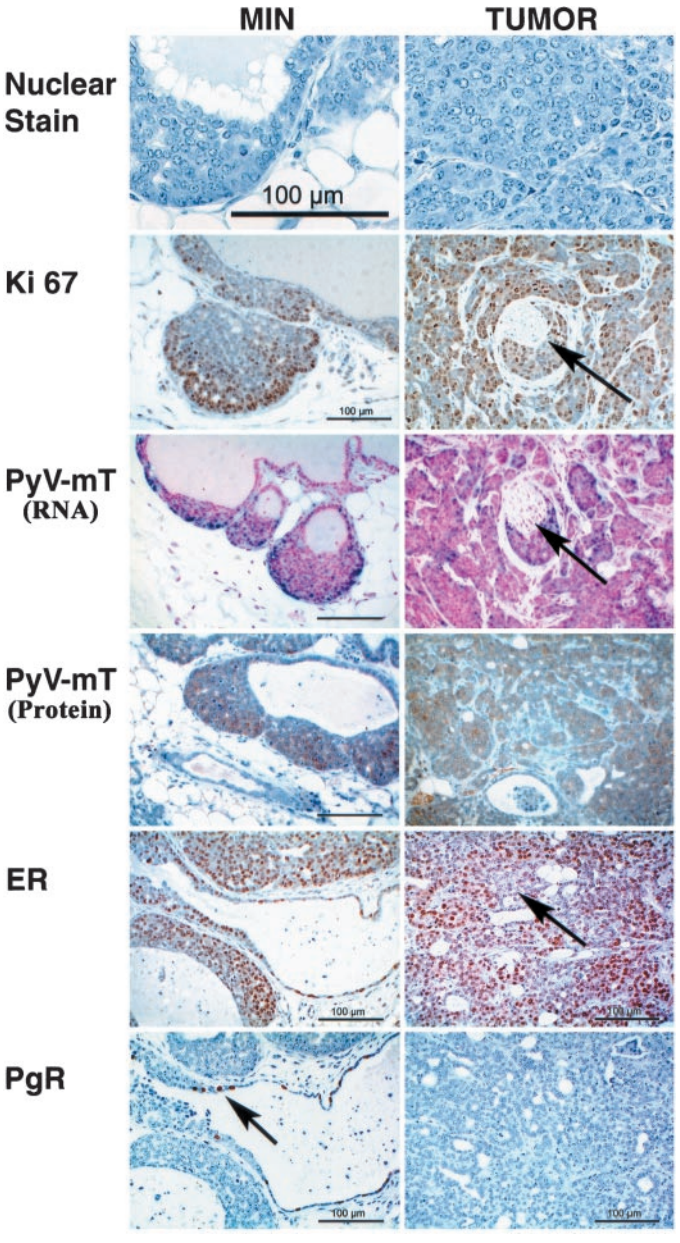

Fig. 2. MIN-tumor comparisons. Photo images of MIN and tumor in virgin female mice 9 weeks of age carrying the *PyV-mT* transgene. *Top row*, the cytological and organizational detail of PyV-mT MIN and tumor. Note that MIN cells are confined within a basement membrane but are multilayered and form ill-defined glands, whereas tumor cells are not surrounded by a basement membrane. The cycling Ki-67-positive cells in MIN lesions tend to be concentrated at the periphery. This expression pattern corresponds with the distribution of cells that are most highly positive for the PyV-mT RNA/antigen. Note that the tumor cells in sections identified with the PyV-mT RNA probe *(blue staining)* and Ki-67 surround a nerve fiber (*arrows*). Tumors have a more random PyV-mT RNA/protein and Ki-67 distribution pattern. The row of images stained for ER and PgR demonstrate that the MIN and tumor cells contain identifiable ER antigen within the nuclei. The ER pattern is variable, with patches of ER-negative cells (*arrow*). Some ductal cells stain for PgR (*arrow*), however, neither the MIN nor tumors have detectable levels of PgR antigen.

Transgene Expression. Regardless of morphology, the hyperplastic foci contained a peripheral, multilayered rim of solid cells that expressed higher levels of PyV-mT. Interestingly, the pattern of Ki-67-positive proliferating cells was similar to the pattern of transgene expression (Fig. 2).

Myoepithelial Changes. SMA and CK-14 expression were used to identify the myoepithelial cells surrounding normal mammary epithelium (Refs. 34 and 35; Table 1). However, CK-14 is also expressed in

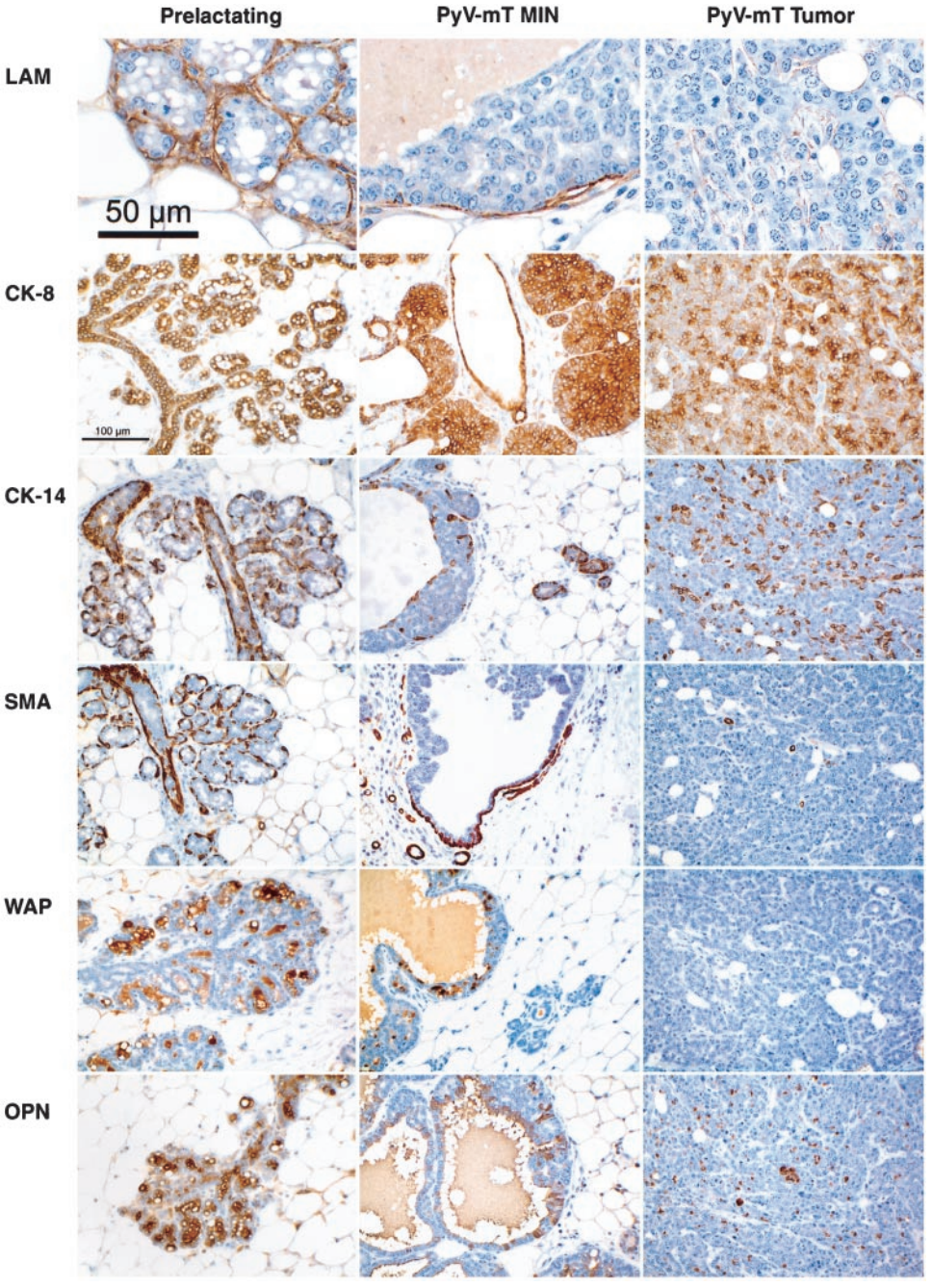

Fig. 3. IHC of PyV-mT MIN/tumors. Photo images of prelactating mammary gland (*column 1*), PyV-mT MIN (*column 2*), and PyV-mT mammary tumor (*column 3*) show the patterns of laminin staining, CK-8, CK-14, smooth SMA, WAP, and OPN. Note that CK-8 stains all epithelial cells in the prelactating and hyperplastic mammary gland but is variable in tumors. CK-14 is largely limited to the basal myoepithelium in normal mammary gland tissue. However, CK-14 appears in all layers, including the luminal surface, of MIN lesions and is randomly distributed in the tumor. In contrast, SMA-positive myoepithelium is absent in MIN and mammary tumors. SMA-positive cells remain only in the smooth muscles of the blood vessels and myoepithelium surrounding normal ducts. Both WAP- and OPN-positive cells are found in MIN. However, WAP falls below detectable levels in tumors.

some proliferating tumor cells (36). Without exception, atypical foci lacked detectable myoepithelium as measured by SMA (Fig. 3). Consistent with the hypothesis that CK-14 is a proliferation cytokeratin, some CK-14-positive cells were found scattered in the more solid lesions and tumors (Fig. 3) and within the myoepithelium of normal tissue (Table 1).

Basement Membrane. Laminin expression, used to identify the basement membrane surrounding noninvasive tissues, was present in nontransgenic ducts, end buds, and prelactating acini (Table 1), although it was reduced but detectable at the expansile margins of atypical foci (Fig. 3). In contrast, laminin stains revealed no basement membranes around tumor cells (Fig. 3). The presence of laminin suggests that the lesions are *in situ*, restrained by a basement membrane.

Nuclear Changes. Nuclear characteristics are used to describe and grade mammary lesions (37). Both AgNOR and Feulgen stains, as well as analysis by flow cytometry, suggested that atypical hyperplastic lesions were primarily composed of diploid cells. The tumor cells analyzed by flow cytometry were also primarily diploid (data not shown). Focal PyV-mT-induced lesions generally contained relatively large nuclei with a coarse chromatin pattern and somewhat irregular nuclear outlines (Fig. 2 and 3). Some overlap in morphometric parameters was noted when these nuclei were measured and compared with nuclei in normal or malignant areas in the same tissue section. However, morphometric measurements were consistent with the trend toward increased size and pleomorphism observed by the pathologist. For example, the modal (mean) area of normal, hyperplastic, and tumor nuclei is 24.9 (23.0) μm^2, 26.8 (30.0) μm^2, and 39 (35.9) μm^2, respectively. Furthermore, when compared with the nuclei of proliferating acinar cells in the prelactating glands of nontransgenic mice, nuclei in PyV-mT-induced atypical lesions had greater size variation and a higher proportion of Ki-67 positive cells.

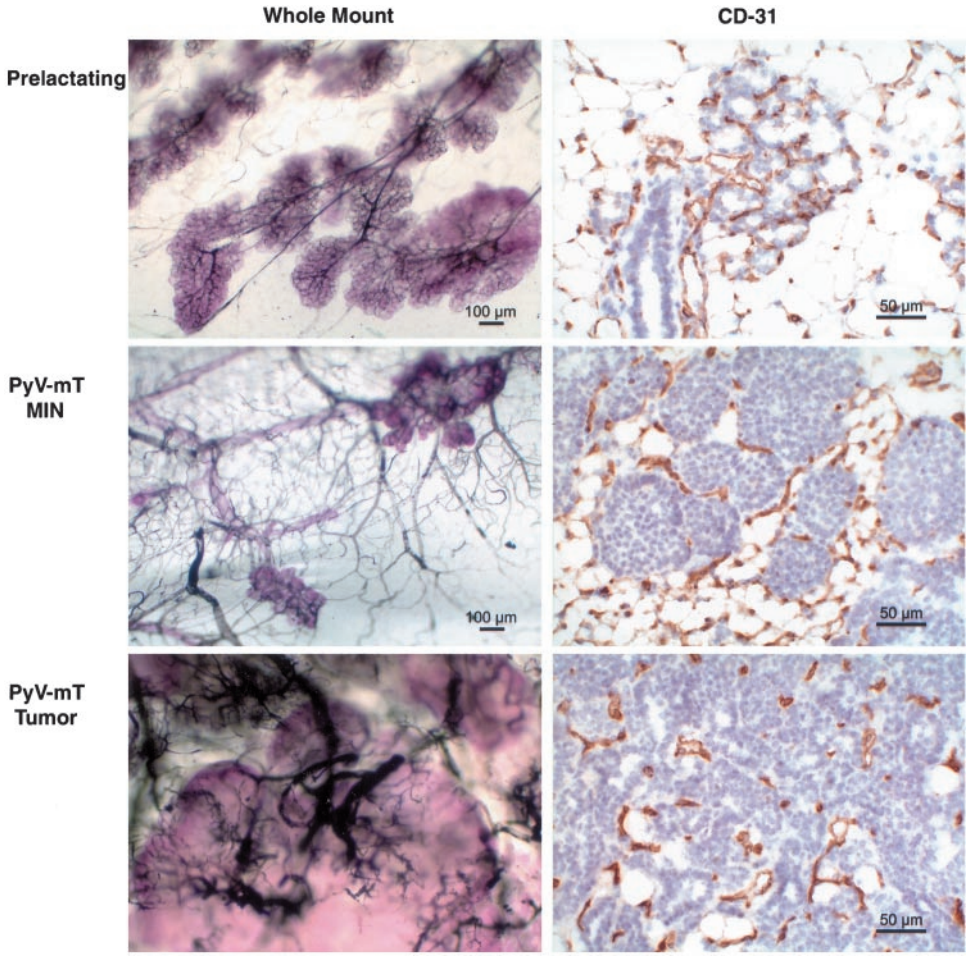

Fig. 4. Microvasculature of PyV-mT MIN/tumors. Photo images of India ink-injected whole mounts (*column 1*) and comparable frozen sections stained for CD31 (*column 2*) illustrating the three- and two-dimensional distribution of vessels in prelactating FVB/N mammary gland, PyV-mT MIN, and PyV-mT mammary tumor. Note the relative increases in vessel diameter and increased irregular distribution of the vessels in the whole-mount preparations (*column 1*). When seen in the two-dimensional sections (*column 2*), the number of vessels decreases relative to the epithelial area in the MIN and the tumors.

Vascularity. Blood vessels associated with atypical foci had larger diameters and displayed more irregular, tortuous courses (Fig. 4). In contrast, pregnancy-induced epithelial hyperplasia in nontransgenic mice is characterized by an evenly distributed network of fine vasculature (Fig. 4). The microvascular density expressed as the number of vessels/0.45 mm^2 microscopic field in nontransgenic prelactating mammary glands (89.8 $\pm$ 8.2) was higher than that of PyV-mT hyperplastic lesions (47.1 $\pm$ 6.4) or tumors (55.2 $\pm$ 1.6). Vessels associated with tumors were also larger in diameter and more tortuous than either prelactating or PyV-mT-induced hyperplasia.

Each group analyzed also differed in the average amount of epithelium filling the microscopic field. Interestingly, when normalized per 0.45 mm^2 of epithelium, the adjusted microvascular density of prelactating mammary glands (256.8 $\pm$ 21.8) was greater than that of hyperplasias (102.5 $\pm$ 12.8) or tumors (63.0 $\pm$ 2.0). These data suggest that despite evidence of angiogenesis, the atypical hyperplasias and especially the tumors are not adequately perfused. This interpretation was supported by the presence of necrotic areas within some tumors.

ER/PgR and p53 Expression. ER and PgR expression are important prognostic and predictive factors in human breast disease (31–33), although they have not been systematically documented in mouse models. Both receptors were expressed in nontransgenic mammary tissue (Table 1). Atypical lesions had low levels of detectable ER (Fig. 2, Table 1). There were, however, some patches of ER-negative cells within the lesions. PgR was detected in the ducts and acini of prelactating tissue as well as in the ducts and terminal end buds of normal virgin mammary tissue (Table 1). However, no PgR-positive cells were found within atypical lesions or tumors. IHC screening for the tumor suppressor *p53* is routinely assessed in humans because mutations in *p53* are a strong indicator of prognosis (38). Many *p53* mutations result in a nonfunctional but more stable protein that accumulates to levels detectable by immunohistochemical methods. Thus, measuring p53 protein expression by IHC is a relatively accurate surrogate method for detecting *p53* mutations (26). p53 protein was not detected in nontransgenic mammary epithelium (Table 1). No evidence of p53 staining was observed in the PyV-mT-induced hyperplasias, and it was detected in only 1 of 20 tumors.

Characteristics of Tumors. Tumors in PyV-mT mammary glands were poorly differentiated, as described previously (13), and they were frequently solid masses. Ki-67-positive proliferative cells were scattered throughout the mass (Fig. 2). Some tumor cells expressed high levels of PyV-mT, but the stain intensity was quite variable (Fig. 2 and Table 1). Similar to the atypical hyperplastic foci described above, tumors lacked organized myoepithelium (Fig. 3). Furthermore, OPN-positive cells were frequently scattered throughout the mass (Fig. 3), demonstrating that some cells retained secretory potential. In contrast to the atypical hyperplastic foci, tumors lacked detectable WAP (Fig. 3). Tumors also lacked organized basement membrane, suggesting invasive behavior.

Transplantation Studies. To determine whether the focal atypical hyperplasias described above were malignant, we subjected them to "test-by-transplantation" experiments. These experiments are the standard for testing the biological potential of putative precursor lesions in murine mammary tumor models (6). Malignant tissue will grow when transplanted to a location ectopic to the mammary fat pad.

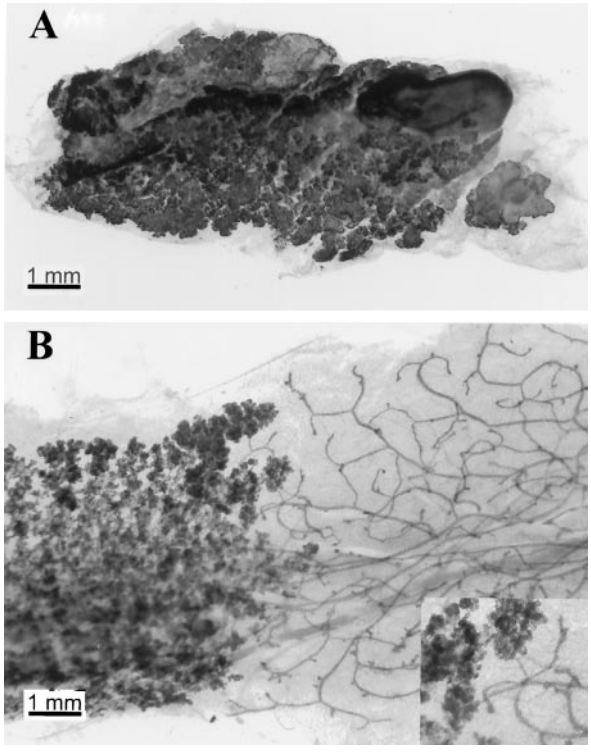

Fig. 5. Transplanted hyperplastic growth in an intact and cleared mammary fat pad. *A*, primary outgrowth of premalignant tissue. Hyperplastic foci isolated from zones 3 and 4 of transgenic mammary glands 8 weeks of age were transplanted into gland-cleared fat pads of syngeneic nontransgenic mice. After 10–17 weeks, noninvasive HPOs were observed in 64% (18 of 24) of recipient fat pads. Microscopic foci of solid, invasive tumors arose in continuity with the hyperplastic tissue (*arrow*), suggesting that these were premalignant tissues. *B*, primary outgrowth in intact fat pad. A fragment of PyV-mT hyperplastic mammary gland tissue was transplanted into zone 4 of an intact FVB female fat pad. When the PyV-mT mammary tissue grew to meet the FVB mammary gland, both tissues stopped growing, and the normal gland turned away from the transplant (*inset*). This experiment illustrates that the PyV-mT hyperplastic tissue has not gained total autonomy but still responds to normal tissue-growth inhibition.

In contrast, nonmalignant mammary tissue will not grow outside of the mammary fat pad (5).

Properties of Primary Outgrowths. Because palpable tumors were frequently located beneath the nipple, we reasoned that it might be easier to identify, isolate, and test putative premalignant tissue from zones distal to the nipple. Hence, foci isolated from zones 3 and 4 of transgenic mammary glands 8 weeks of age were transplanted both s.c. (ectopic) and into gland-cleared fat pads (orthotopic) of syngeneic nontransgenic mice. Whole-mount analysis revealed noninvasive HPOs in 64% (18 of 24) of transplant-recipient fat pads (Fig. 5). In contrast, no (0 of 14) tissues transplanted s.c. formed palpable tumors within a 10–17 week posttransplant window. These findings suggest that the tissues isolated and transplanted from zones 3 and 4 were hyperplastic but not malignant. Fifteen primary PyV-mT outgrowths were examined microscopically. They consisted primarily of cystic masses lined by multilayered, atypical epithelium with large, pleomorphic nuclei. However, some regions contained microscopic foci of solid, invasive tumors. These tumors arose in direct continuity with hyperplastic tissue and seemed to be emerging subsets of the original transplant population. The presence of focal tumors within some outgrowths suggested that the transplanted cells were, indeed, premalignant.

Growth Regulation. The growth properties of these premalignant foci were tested further by isolating and transplanting zone 4 foci into intact (noncleared), inguinal glands of mice 4 weeks of age. After 10 weeks, the growing ducts and the transplants converged near the lymph node (Fig. 5). The ducts and transplanted tissues stopped growing without overlapping. The transplanted transgenic mammary outgrowths displayed the expected, noninvasive, hyperplastic morphology. The host, nontransgenic epithelium had no subgross or microscopic evidence of hyperplasia. These results indicate that the transplanted hyperplasias obey normal tissue regulation and that transgene expression does not exert a paracrine influence over the normal gland (Fig. 5). Failure of transplanted foci to grow s.c. or overgrow the host mammary gland confirms that their growth was restricted to the mammary fat pad and that the cells were not malignant.

HPO Lines. The biology of zone 4 hyperplastic lesions was more thoroughly studied by establishing five different HPO lines (Table 2). These lines are currently in the eighth transplant generation. Their growth, without regression, documents that they were immortalized (7). Their malignant potential was tested by s.c. transplanting HPO tissue into syngeneic recipient animals. These ectopic (s.c.) transplants did not progress to tumors, providing strong evidence they were not malignant. However, all lines gave rise to areas of atypical hyperplasia and eventually developed tumors within the recipient fad pad, indicating that the HPO lines represent premalignant tissues. The tumors that developed within these HPOs grew in ectopic sites and metastasized, confirming that they were malignant.

Heterogenetiy of HPO Lines. Although all isolated HPO lines expressed PyV-mT protein (data not shown), their potential for malignant progression as well as the subgross morphology of the outgrowth lines were quite heterogeneous. Two lines had a high incidence of malignant transformation after 14 weeks, and the other three had a low incidence (Table 2). Additionally, pulmonary metastases were found in the lungs of mice bearing tumors arising from four of five transplant lines (data not shown). The difference in tumor latencies implies heterogeneity among the premalignant lesions that arise within the PyV-mT mammary gland.

DISCUSSION

Genetically engineered mouse models have become popular in the field of cancer research because they give researchers the unique opportunity to investigate the multiple aspects of cancer within an intact organism. Analysis of these mice has already provided insights into mechanisms of oncogenesis and metastasis (15, 39, 40). However, basic studies of the biology and pathology of premalignant lesions have generally lacked mammary transplantation experiments that provide important information about their growth potential (5, 6). Because early detection and intervention in human breast disease are critical to cancer prevention, the attributes of putative precursor lesions in model systems need to be carefully characterized and vali-

Table 2 *Tumor incidence and morphology of hyperplastic outgrowth lines*

Zone-4 lesions from mice 9 weeks of age were serially transplanted to gland-cleared recipients 4 weeks of age. After four passages, the tumor incidence for each of the five indicated HPO Lines (A–E) was measured at 14 and 21 weeks and is reported as the number of fat pads with tumor(s)/total number of fat pads with transplants. HPO morphological characteristics are reported as cystic or LA (lobuloalveolar), or both.

HPO line	Tumor incidence: Fat pads with tu/transplanted fat pads (%)	Time (wk)	Morphological characteristics
A	14/20 (70)	14	cystic/LA
B	0/17 (0)	14	LA/cystic
	11/17 (64.7)	21	
C	3/20 (15)	14	cystic/LA
	11/18 (61.1)	21	
D	0/18 (0)	14	LA
	13/18 (72.2)	21	
E	14/20 (70)	14	LA
	18/20 (90)	21	

dated by direct comparison with human lesions. Here we provide a comprehensive characterization of premalignant PyV-mT mammary lesions, including mammary transplantation experiments and immunohistochemical stains commonly used in both mouse and human histopathology. Our studies are intended to provide a basis for direct comparisons with other models and with human breast disease (6).

The similarities between PyV-mT premalignant lesions and many types of human atypical hyperplasias emphasize the value of this model system. Like their human counterparts, they were morphologically heterogeneous, with highly proliferative focal areas containing atypical nuclei. They had abnormal microvasculature, lacked an organized myoepithelium, and remained within an intact basement membrane. Furthermore, they expressed differentiation markers that were not expressed in tumors. Although they commonly expressed ER, they never expressed detectable PgR. The accumulation of p53 was seldom detected. It is noteworthy that PyV-mT and Ki-67 expression were concentrated at the periphery of MIN lesions, suggesting that transgene expression stimulated growth.

Transgenic tumors were distinct from MIN lesions in several respects. The tumors were less differentiated than MIN lesions; they had larger, more pleomorphic nuclei; they lacked a basement membrane; they had increasingly abnormal vasculature; and they were frequently metastatic. PyV-mT expression was variable in the tumors, suggesting that transgene expression is important for initiation but not maintenance of the malignant phenotype.

The precursor lesions and tumors would be considered high grade as scored by the morphological criteria used by the Scarff-Bloom-Richardson (SBR) or Elston-Ellis grading systems. Their gene expression pattern determined by IHC suggests high-grade, undifferentiated lesions when compared with the common forms of MIN and breast cancer of no specific type found in humans. However, detailed comparisons will require the completion of our projected detailed analysis of the various HPO lines. The data presented will permit us to compare the biological, morphological, and molecular attributes of the individual lines with those of the human disease. The results of our test-by-transplantation experiments underscore the value of these experiments in the characterization of MIN in mouse models. The early onset of palpable mammary masses supported the hypothesis that expression of the *PyV-mT* transgene alone might be sufficient for tumorigenesis, and therefore these mice represented a single-step model of transformation (13). The biological studies described herein have proven tumorigenesis is, indeed, a multistep process in this model. A similar literature controversy exists regarding whether transgenic MMTV/activated *neu* mice are a model for single step or multistep neoplastic progression (14, 41). Biological studies similar to those described here would likely resolve this issue.

The HPO lines produced in this study represent a new tool for the study of intermediate stages of PyV-mT-induced malignant progression. Several characteristics of these lines make them ideal for the detailed studies required to define the molecular pathways necessary to develop a malignant phenotype. The HPO lines produced in this study have stable and documented predictable growth behavior. The difference in the tumor latencies of the lines implies heterogeneity among the PyV-mT-induced premalignant hyperplasias. Likewise, human breast hyperplasias are associated with variable malignancy risk. In human tumor progression studies, it is not possible to definitively determine from which hyperplasia a given tumor developed. Thus, comparisons of gene expression patterns in human premalignant and malignant counterparts are difficult. The HPO lines make it possible to isolate and compare premalignant tissue and the tumors arising within that same tissue. These lines will facilitate investigations that focus on specific molecular changes related to malignancy, such as signaling-pathway alterations that occur during malignant progression. The *PyV-mT* transgene activates the Erb-B2/PI3 kinase pathway that is frequently dysregulated in human breast cancers (42). The HPO lines thereby enhance the utility of the *PyV-mT* transgenic model by making accessible the full range of events occurring in multistep mammary tumorigenesis, from the inception of the lesion to the development of pulmonary metastasis.

The predictable tumor latencies of the HPO lines will also facilitate preclinical studies testing therapeutic agents designed to arrest or reverse tumor progression at the premalignant stage. The transplantable nature of the HPO lines facilitates experiments testing the effects of stromal components on tumor progression. The HPO lines can be transplanted into the cleared mammary fat pads of genetically engineered mice lacking or overexpressing factors external to the mammary epithelium.

Meaningful information needed to accurately predict the biological outcome of hyperplastic lesions in humans will be gained by integrating molecular data with pathobiological findings, which, together, provide insight into the behavior of premalignant tissues as well as their response to therapeutics (5). Because investigations of the biological potential of human atypical hyperplasias are limited to retrospective epidemiological analysis, mouse models of MIN are particularly valuable because they are amenable to prospective test-by-transplantation. More attention to the biological growth properties of premalignant lesions in these systems will provide essential clues for unraveling the mysteries of tumor progression.

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