Polyomavirus middle T–induced mammary intraepithelial neoplasia outgrowths: Single origin, divergent evolution, and multiple outcomes

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Abstract

The development of models to investigate the pathobiology of premalignant breast lesions is a critical prerequisite for development of breast cancer prevention and early intervention strategies. Using tissue transplantation techniques, we modified the widely used polyomavirus middle T (PyV-mT) transgenic mouse model of breast cancer to study the premalignant stages of tumorigenesis. Premalignant atypical lesions were isolated from PyV-mT transgenic mice and used to generate two sets of three mammary intraepithelial neoplasia (MIN) outgrowth lines. Investigation of these six unique lines, each of which fulfills the criteria for MIN, has provided new information regarding the biology of PyV-mT-induced neoplasia. Although expression of the PyV-mT transgene was the primary initiating event for all lines, they exhibited different tumor latencies, metastatic potentials, and morphologies. Six distinguishable morphologic patterns of differentiation were identified within the premalignant outgrowths that are likely to represent several tumorigenic pathways. Further, several tumor phenotypes developed from each line and the tumors developing from the six lines had different metastatic potentials. These observations are consistent with the hypothesis that distinct pathways of PyV-mT-initiated neoplastic progression lead to different outcomes with respect to latency and metastasis. The MIN outgrowth lines share several characteristics with precursors of human breast cancer including the observation that gene expression profiles of tumors are more similar to those of the MIN outgrowth line outgrowth from which they developed than to other tumors. These lines provide an opportunity to study the full range of events occurring secondary to PyV-mT expression in the mammary gland. [Mol Cancer Ther 2004;3(8):941–53]

Introduction

Breast cancer remains a major cause of cancer-related mortality for women. Improvements in breast cancer screening protocols and diagnostic technologies have resulted in early detection of abnormalities associated with breast cancer. In the wake of these advances, characterizing preinvasive lesions and understanding their complex pathobiology have become critically important goals. However, our present state of knowledge provides little guidance in connecting the profiles of abnormal mammary foci to their ultimate fate. There is a paucity of molecular markers that are generally useful in grading premalignant breast lesions, and the molecular changes that enable progression from one stage to the next have not been elucidated. Animal models of premalignant disease, specifically designed to mimic early and intermediate steps of tumor progression, are needed to complement the molecular profiling data that have been collected.

The foci of atypical epithelial cells observed in the mammary glands of many genetically engineered mice share characteristics of human premalignant breast lesions (1). However, morphologic resemblance alone does not satisfy the classic operational definition of premalignancy (2). In the mouse mammary gland, premalignant tissue must meet certain criteria: (a) the tissue must be immortalized (‘‘protoneoplasia’’) as shown by the capacity to be serially transplanted into gland-cleared mammary fat pads, (b) the tissue should not grow when transplanted s.c. (3), and (c) the outgrowth resulting from transplantation of premalignant tissue into a gland-cleared fat pad should have a high probability of developing focal tumors. In contrast, nonimmortalized tissues cannot be transplanted indefinitely (4), and malignant tissues will grow outside the mammary fat pad microenvironment when transplanted s.c.

Serial transplantation of hyperplastic mouse mammary tissue is useful for generating lines of outgrowth tissue that become stable and predictable in terms of tumor latency and metastatic capability. Such outgrowth lines, previously called hyperplastic outgrowth lines, enable investigation of...

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both the biological behavior (latency and metastasis) and the molecular properties of premalignant tissues (5–10). Premalignant lesions in genetically engineered mouse models of breast cancer are termed mammary intraepithelial neoplasia (MIN; ref. 2). Hence, hyperplastic outgrowth lines of premalignant tissues can be more accurately termed MIN outgrowth lines. To create the MIN outgrowth serial transplant lines described here, MIN from transgenic mice expressing the mammary targeted polyomavirus middle T (PyV-mT) transgene (11) were identified in situ, selected, and transplanted into the gland-cleared mammary fat pad of syngeneic recipient mice. As the transplanted tissue grew out to fill the gland-cleared fat pad, tissue from the resulting outgrowth was again selected and transplanted into the gland-cleared mammary fat pad of another recipient mouse.

Like cell lines, MIN outgrowth lines are easily expandable and amenable to experimental manipulation. Whereas in vitro cell lines and three-dimensional tissue culture systems have played important roles in dissecting molecular pathways in breast cancer (12–19), the in vivo mammary fat pad provides an ideal microenvironment in which to study the complexities of tumor progression. The biological potential of MIN outgrowth line tissues can be characterized in a more rigorous manner than is possible for human lesions using standard transplantation tests ("test-by-transplantation") that distinguish malignant and premalignant lesions from one another (2, 5).

FVB mice expressing PyV-mT under control of the mouse mammary tumor virus (MMTV) long terminal repeat promoter are of particular interest, because PyV-mT activates the phosphatidylinositol 3-kinase signaling pathway (20–22). The expression and activity of phosphatidylinositol 3-kinase are frequently up-regulated in human breast cancer tissues (23, 24). This and other pathways activated by PyV-mT are also activated by erbB2 (Her2/neu), which is overexpressed in up to 30% of human breast cancers (25). Further, MMTV/PyV-mT-induced mammary tumors have similar gene expression profiles to tumors from MMTV/neu and MMTV-myc transgenic mice (26). The mice eventually develop multiple tumors and metastases with 100% frequency in the FVB background (11, 27). For these reasons, the PyV-mT model is considered a powerful surrogate for Her2/neu-mediated tumorigenesis.

PyV-mT transgenic mice develop several distinct types of MIN lesions in the FVB background (28). Two sets of three PyV-mT MIN outgrowth lines were generated from a variety of PyV-mT-induced MIN lesions. One set of three lines (8w-A, 8w-B, and 8w-D), developed from those partially described in the previous publication, was derived from lesions isolated from the periphery of mammary glands (far from the nipple; ref. 28). The second set of three lines (4w-4, 4w-6, and 4w-11) was developed subsequently from lesions near the nipple where most tumors first appeared.

Here, we provide the first detailed study of these MIN outgrowth lines including morphology, histology, tumor latency, metastasis, and gene expression data. Although neoplastic progression in all lines was initiated as a result of expression of the same promoter and transgene in an identical genetic background, we report here that PyV-mT MIN outgrowth lines are divergent both morphologically and with respect to their potential for both tumor formation and metastasis. Furthermore, the morphologic and biological behaviors associated with each line are consistent and predictable. Secondary molecular changes are associated with the progression of the premalignant tissues to tumors. Together, these findings indicate that the PyV-mT MIN outgrowth lines provide a valuable biological resource that can be used to determine the nature of secondary molecular changes involved in PyV-mT-initiated tumorigenesis.

Materials and Methods

Mice
Recipient FVB mice weighing 12 to 14 g were purchased directly from either Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Davis, CA). Hyperplastic mammary tissue used to initiate the MIN outgrowth lines was isolated from FVB mice expressing the MMTV long terminal repeat/PyV-mT. These mice, originally provided by Prof. William J. Muller (McGill University, Montreal, Quebec, Canada), were bred and maintained at the University of California-San Diego (La Jolla, CA). All mice were maintained in University of California vivariums (La Jolla, CA or Davis, CA) according to NIH guidelines.

Generation of MIN Outgrowth Lines
Specific mammary gland lesions near the nipples of nos. 3 and 4 mammary glands of 4-week-old transgenic female mice (MIN outgrowth lines 4w-4, 4w-6, and 4w-11) or in the periphery of a single tumor-bearing no. 3 mammary gland of an 8-week-old female mouse (MIN outgrowth lines 8w-A, 8w-B, and 8w-D) were visualized through a dissecting scope. Tissue segments (1 mm³) were surgically isolated and transplanted into cleared mammary fat pads as described (29). Recipient mice were palpated weekly for tumors. At 5- to 15-week intervals, nonmalignant outgrowth tissue was selected and retransplanted into cleared mammary fat pads of 2 to 12 mice.

Test-by-Transplantation
At intervals (between 3 and 10 transplant generations), samples of MIN outgrowths tissue were transplanted s.c. to verify that MIN outgrowth tissue was not malignant. Thirty-five tumors emerging from MIN outgrowth tissues were s.c. transplanted to confirm malignant status of the tumors.

Tumor Latency
Tumor latency of MIN outgrowth lines was monitored each transplant generation in 2 to 12 recipient mice. The mice were monitored every 7 to 10 days for palpable tumors. Tumor latency reported is the average time to development of the first tumor in each mouse. Because tumor latencies became more predictable after generation 8, generations after the eighth generation have been reported here.
**Metastasis**

To determine the metastatic rate of tumors developing from each line, mice with MIN outgrowth line tissue transplanted into gland-cleared fat pads were monitored weekly for tumor development. Tumors were allowed to grow until they reached 2 cm in greatest diameter. Whole mounts of lungs were prepared as described below and analyzed under a dissecting microscope. After metastases were recorded, whole mounts were sectioned and metastases were confirmed histologically.

**Mammary Whole Mounts and Immunohistochemistry**

Mammary whole mounts were prepared as described (30). For microscopic analyses, glands fixed overnight in 4% buffered formalin or paraformaldehyde were paraffin embedded and sectioned to 4 μm. Routine H&E staining and appropriate immunohistochemistry were done. Because a baseline for this comparison was already established with the characterization of primary PyV-mT-induced MIN (28), a similar immunohistochemical panel was used. Cytokeratin-8 (CK-8) (31) was used to identify epithelial cells; smooth muscle actin was used to identify differentiated myoepithelial cells (32); whey acidic protein (33) and osteopontin (34) were used to identify differentiated secretory mammary epithelial cells; and Ki-67 was used to identify proliferating cells (35, 36). Several proteins commonly used as markers for diagnosis, grade, and prognosis in human breast cancer were also included [estrogen receptor (ER), progesterone receptor, and erbB2].

Antigen retrieval was accomplished by microwaving in citrate buffer. Immunohistochemical studies used anti-CK-8 (PH19211.xs; binding site: I400), anti–smooth muscle actin (A-2547; 1:1,000; Sigma Chemical Co., St. Louis, MO), anti-ER (L-299; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-osteopontin (AF808; 1:800; R&D Systems, Minneapolis, MN), anti–whey acidic protein (1:5,000; Dr. Lothar Hennighausen, NIH, Bethesda, MD; ref. 37), anti-erbB2 (C811; 1:40; Novo Castra, Newcastle, United Kingdom), anti-Ki-67 (C811; 1:1,800; Novo Castra), anti-PyV-mT (B4Rat7; 1:50; Dr. Gernot Walter, University of California-San Diego), and anti–progesterone receptor (A0098; 1:500; DAKO, Carpinteria, CA; ref. 38). Anti–smooth muscle actin was amplified and detected using ARK (DAKO). All other antibodies were amplified and detected using the Vector ABC Kit. Images of slides were captured using 20× and 40× objectives on a Carl Zeiss (Thornwood, NY) Axioscope microscope with Axioscam camera and processed using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA) software.

**Lung Whole Mounts**

Mice were sacrificed by anesthetic overdose. Lungs were perfused with 4% buffered formalin via cannulation of the trachea and forcible inflation and were removed. Lobes were separated, fixed for 48 hours in 4% buffered formalin, dehydrated with alcohol, cleared with xylene, and stored in methyl salicylate.

**Tissue Sampling for Transgene Expression and Micro-array Analysis**

Anesthetized mice were surgically opened to reveal the transplanted fat pads. Hyperplastic and tumor tissues were identified under a dissecting microscope, and biopsies of tissues were taken. Tissues were immediately frozen on dry ice or in liquid nitrogen. H&E-stained sections were prepared from the remaining tissue as described above, and examination of slides corresponding to each sample was done to verify histologic pattern.

**Quantification of Transgene Expression**

Transgene expression was determined for three independent MIN outgrowth and tumor pairs from each line (18 total pairs). DNA isolated from a nontransgenic pre-lactating mammary gland provided a negative control. Frozen tissues were homogenized using the Kinematica Polytron PT 1200 (Brinkman, New Haven, CT), and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. All RNA samples were treated twice with DNase I (DNA-free, Ambion, Austin, TX), and their concentrations were measured with the SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). An aliquot from each RNA sample was run on a 0.8% agarose gel with ethidium bromide to determine quality. Total RNA (5 μg) was reverse transcribed using SuperScript first-strand synthesis system for RT-PCR (Invitrogen).

Real-time RT-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The amount of mammary epithelium varied between samples. For comparison of transgene expression between lines relative to mammary epithelium, PyV-mT expression was normalized to expression of CK-8, which is expressed in MIN outgrowth lines (Fig. 4, left column). The following primers for PyV-mT and CK-8 were designed using Primer Express version 1.5 software (Applied Biosystems): PyV-mT (forward) 5' -GAAGACGAATTCCTTTGTGGCTGCT' and 5' -TCTTGGTCGCTTTCTGGATAC-3', PyV-mT (reverse) 5' -TCTTTGTGCCCTTCTGGGATAC-3', CK-8 (forward) 5' -GAACCCCCCTAAAGGCCAGAG-3' and CK-8 (reverse) 5' -TGGTCTGGGCGATCCCTTAATG-3'. PCR assay optimization assays were done for each primer pair as recommended by the manufacturer (Applied Biosystems). All primers were used at their optimal concentrations of 200 nmol/L, and sample template (1 nmol) was added to each reaction. Samples were denatured at 94°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were generated for each gene-specific pair on each plate using cDNA template from a polyoma expressing MIN outgrowth line 8w-B tumor at 0.05, 0.5, 5, and 50 ng per well. Nontemplate control reactions were done in duplicate for each gene-specific reaction mixture. Sequence Detection Software version 1.7 was used for analysis of the collected data. Dissociation curve analysis was done using ABI Dissociation Curves version 1.0 software (Applied Biosystems) to confirm the specificity of the melting point of the final PCR product as well as the lack of primer-dimer formations in the absence of template. Quantitation was done twice on the samples with similar results.
Oligonucleotide Microarrays

Frozen outgrowth tissues and tumors from MIN outgrowth lines 4w-4, 4w-6, 4w-11, 8w-B, and 8w-D; intact fat pads from virgin nontransgenic adult MIN outgrowth line recipients; intact mammary fat pads from 16-day prelactating FVB females; and mammary tumors from two transgenic MMTV/PyV-mT animals were also collected as described above.

Total RNA from the frozen tissues (20–50 mg) was isolated with TRIzol reagent according to the manufacturer’s protocol followed by additional extraction with phenol/chloroform with isoamyl alcohol (pH 4.3; Fisher, Pittsburgh, PA). The isolated total RNA was cleaned further with the RNeasy RNA isolation kit (Qiagen, Valencia, CA), and the quantity and quality of final total RNA were examined spectrophotometrically as well as with RNA 6000 Nano Labchip on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Purified total RNA (10 μg) was used for double-stranded cDNA synthesis followed by biotin-labeled cRNA synthesis and cRNA fragmentation according to the GeneChip expression analysis protocol (Affymetrix, Santa Clara, CA). The quantity of in vitro transcription and fragmentation products was assessed using the Agilent 2100 Bioanalyzer. Labeled and fragmented cRNA (15 μg) was used for hybridization on Affymetrix oligonucleotide Murine Genome Array U74Av2 according to the Affymetrix expression analysis protocol. The hybridized arrays were scanned with Agilent GeneArray Scanner. The images were processed using Microarray Analysis Suite 5.0 (Affymetrix).

The processed images were normalized and analyzed with dChip version 1.2 (39). Hierarchical clustering analysis was done with 1,812 probes, which had a present call in at least 20% of 32 samples and coefficient of variation (SD)/(mean) across the 32 samples of >0.3. The gene list is available as supplementary material on the Internet.

Statistical Analyses

Tumor latency data are expressed as mean time to development of first palpable tumor in each mouse. Error bars in tumor latency graph depict SE. An ANOVA was done to test whether the average tumor latency was different across the six MIN outgrowth lines, and the multiple comparisons procedure (Tukey’s simultaneous confidence interval) was used to further identify which lines were significantly different from one another. To determine whether tumors had a higher level of transgene expression compared with the MIN outgrowth tissues from which they emerged, a one-sided paired Wilcoxon test was done. Cox regression model was used to study the association of PyV-mT to tumor latency.

Results

MIN Outgrowth Lines

Multiple abnormal foci are observed, by examination of whole mounts, beneath the nipple of 4-week-old developing PyV-mT transgenic mammary glands (Fig. 1, top left). When whole mounts of mammary glands from 8-week-old sexually mature females are examined, expansile solid tumor masses are generally observed in the area beneath the nipple, and multiple small atypical foci are observed in the periphery of the gland (Fig. 1, top right). When early dysplastic lesions located close to the nipple of a 4-week-old female mammary gland were transplanted s.c., three of four grew into tumors within 6 weeks, indicating that they contained malignant cells. In contrast, as reported previously, no (0/14) transplants of abnormal primary foci taken from the non-tumor-bearing periphery of an 8-week-old mammary gland grew under the skin, indicating that they did not contain malignant cells (28).
Together, these observations suggest that early lesions located under the nipple of a 4-week-old mammary gland are more likely to contain malignant cells than lesions in the periphery of an 8-week-old mammary gland.

To study a range of PyV-mT-induced MIN, two sets of MIN outgrowth lines were established (Fig. 1). Several small abnormal lesions were identified near the nipples of 4-week-old mammary glands and transplanted into gland-cleared fat pads of recipient mice (MIN outgrowth lines 4w-4, 4w-6, and 4w-11). Small abnormal lesions were also identified in the periphery of a tumor-bearing mammary gland in an 8-week-old mouse and transplanted into gland-cleared fat pads of recipient mice (MIN outgrowth lines 8w-A, 8w-B, and 8w-D). Subsequently, the two sets of outgrowths were serially transplanted to establish the MIN outgrowth 4w (Fig. 1, left column) and 8w lines (Fig. 1, right column).

At each transplant generation, donor tissue was surgically excised from the growing margin for the transplant and was transplanted into gland-cleared mammary fat pads of recipient mice. The outgrowths were allowed to grow in the fat pad for at least 5 weeks before the next samples were selected for serial transplantation. Outgrowths typically filled between 40% and 57% of the cleared mammary fat pad after 5 to 6 weeks of growth. After several rounds of serial transplantation, MIN outgrowth lines that had different characteristics (morphology and tumor latency) were chosen from each set for further serial transplantation. Selection and serial transplantation of outgrowth tissue for 14 to 18 transplant generations resulted in the six different MIN outgrowth lines described here.

**Biological Tests for MIN**

The PyV-mT MIN outgrowth lines were subjected to "test-by-transplantation" to determine whether they met the criteria for MIN (1, 2). Every 2nd to 10th transplant generation, fragments of selected MIN outgrowth tissue from each line were subdivided and transplanted into both gland-cleared mammary fat pads and s.c. tissues. Subcutaneous transplants could not be detected by palpation within the first 20 weeks after transplantation, indicating that the tissues selected from the MIN outgrowth lines were not "malignant." However, all of the MIN outgrowth lines have undergone at least 14 mammary fat pad serial transplantations, indicating that they are immortalized lines (3).

MIN outgrowth tissue, confined to growth within the host cleared mammary fat pad, grew into the approximate shape of the fat pad. Tumors emerged from the central regions of the outgrowths to form palpable oval masses. In contrast to outgrowth tissue, tumors grew as raised foci of denser tissue with defined borders that could be distinguished grossly from surrounding MIN outgrowth tissue. Tumors developed as subpopulations in all six MIN outgrowth lines. The defined borders of tumors enabled the sampling of outgrowths and tumors from the same fat pad (pairs) for molecular comparisons.

To verify the malignant phenotype of the tumors, tumors from each outgrowth line (n = 35) were transplanted to s.c. locations. All s.c. transplants of suspected tumors grew to form palpable masses within 6 weeks of observation. The attribute of immortal growth combined with high risk of developing malignant neoplasms fulfills the test-by-transplantation criteria, demonstrating that the MIN outgrowth tissues are premalignant (2).

**Tumor Latency**

The six PyV-mT MIN outgrowth lines have different tumor latencies ranging from 11 to 22 weeks (Fig. 2). Average tumor latencies varied for the first five to eight generations. However, latencies became more predictable after the eighth generation. Figure 2 shows the tumor latency for each line over multiple generations (after generation 8). In this study, tumors eventually developed in all MIN outgrowths. However, the average tumor latency of the six MIN outgrowth lines differed (P < 0.001). When compared pair-wise, the following significant differences were found: (1) 8w-A was different from lines 4w-6, 8w-B, and 4w-4; (2) line 4w-11 was different from lines 8w-B and 4w-4; and (3) 8w-D was different from lines 4w-6, 8w-B, and 4w-4. However, the following lines with similar average latencies did not differ from each other significantly: (1) lines 8w-A, 8w-D, and 4w-11; (2) lines 4w-6, 8w-B, and 4w-4; and (3) lines 4w-11 and 4w-6. The data are suggestive of at least two risk categories. MIN outgrowth lines with comparable latency were developed from both 4-week-old and 8-week-old mammary glands, indicating that the age of the donor mouse at the first transplant did not significantly impact the latency associated with the premalignant cells within the lesion.

**Metastasis**

To determine whether the MIN outgrowth lines progressed to metastatic disease, tumors were allowed to grow until they reached the maximum of 2 cm diameter.
Hematoxylin-stained lung whole mounts were examined for evidence of metastases. One to five metastases were observed in several lungs from mice bearing tumors from lines 4w-11, 8w-A, and 8w-D (Fig. 3 and Table 1). Metastases were not observed in mice with transplants arising from line 4w-4, 4w-6, or 8w-B (Table 1). Again, the metastatic frequency was not related to the age of the donor mouse, implying that the anatomic location of the original atypical lesion does not determine metastatic potential. Several metastases from each line were confirmed histologically.

**General Morphologic Characteristics**

Extensive histologic and immunohistochemical analyses were done to compare the lines with each other and with primary MIN lesions from PyV-mT transgenic mice. The outgrowths grew, as does normal mammary tissue, by extension of the mammary tree. Analysis of whole-mounted outgrowths revealed dense bulbous masses at the peripheral growing margins, which resembled aberrant end buds histologically. However, they were larger and more closely packed together than normal. These structures did not have the typical organization of the terminal end bud and frequently had multiple branches. The peripheral margins typically had a higher proportion of Ki-67-positive (proliferating) cells compared with the center of the outgrowth.

The central regions of the MIN outgrowths were typically distinct from the growing margins with better differentiated structures and less mitotic activity. Three general patterns of differentiation were observed within the hypercellular central epithelial zone: cystic, solid, and microacinar. Cystic and solid patterns were described previously in the PyV-mT mammary dysplasias (28). However, microacinar patterns have not been recognized previously. Further, several distinct patterns of both cystic differentiation and microacinar differentiation were observed. In total, six major patterns of growth were observed including two distinct types of cysts, solid cords/nests, and three distinct types of microacini (Figs. 4 and 5). Different combinations of the six major growth patterns were observed in each line (Table 2). The patterns are described in detail below.

**Cystic Differentiation Patterns**

Two distinct types of cysts were frequently observed in the MIN outgrowth lines. Well-differentiated cysts ("organized cysts") were typically lined with one to three layers of luminal epithelial cells, defined by CK-8 expression, and were often associated with smooth muscle actin-positive myoepithelial cells (Figs. 4 and 5, arrow O). However, the myoepithelium was frequently disorganized and fragmented. These cysts contained abundant fluid that frequently stained for the secretory product osteopontin. They varied in size, and especially large cysts, frequently noted in line 8w-A, were often palpable.

In contrast, poorly differentiated cysts ("dysplastic cysts") were dysplastic, characterized by a highly irregular, frequently discontinuous, luminal epithelium (Figs. 4 and 5, arrow D). Sloughed cells were often observed within the cyst lumen. The luminal cell nuclei were large, hyperchromatic, and pleomorphic and had dense heterochromatin. The cytoplasm tended to be bluish, attenuated, and loosely adhesive.

**Solid Differentiation Pattern**

The solid pattern consisted of solid cords and nests of cells with little or no glandular differentiation (Figs. 4 and 5, arrow S). The nuclei were pleomorphic and the nuclear-cytoplasmic ratio was inverted. This pattern resembled the solid lesions observed in the founder PyV-mT transgenic mice.
Microacinar Differentiation Patterns

The microacinar areas were composed of small clusters of columnar cells oriented around small central lumens (Figs. 4 and 5, arrow M). Three different types of microacinar structures could be distinguished based on immunohistochemical staining. The most abundant microacinar population was not lined by an organized myoepithelium. More highly organized microacini, lined by an organized myoepithelial layer, were also observed. These microacini closely resemble those found in MMTV-induced hyperplastic nodules. Interestingly, a third identifiable subset (Figs. 4 and 5, arrow EM) of microacini was identified in MIN outgrowth line outgrowths. These structures had unique, well-differentiated cells with apical eosinophilic cytoplasm. The “eosinophilic” microacini were particularly interesting because they usually had ER-positive nuclei (Fig. 6, arrow EM) and did not express PyV-mT (data not shown). To our knowledge, microacinar patterns of growth have not been observed in PyV-mT transgenic mice.

Comparison of MIN Outgrowth Lines

Each of the patterns of differentiation described above were frequently observed in multiple MIN outgrowth lines. However, each MIN outgrowth line retained an individual and characteristic mixture of the patterns that distinguished one from the other (Fig. 4 and Table 2). For example, dysplastic cysts were almost exclusively observed in line 8w-B together with better differentiated microacinar structures. This line also had abundant vasculature. Lines 8w-A and 4w-11, the most cystic lines, were easily distinguishable because larger cysts and more fibrosis were

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**Figure 4.** MIN outgrowth line differentiation patterns. Several distinct differentiation patterns were observed including organized cysts (O), dysplastic cysts (D), solid nests of cells (S), microacini (M), and eosinophilic microacini (EM). One or more of each differentiation pattern were typically observed in each line. The images show growth patterns that are commonly observed in each line.
observed in line 8w-A. Line 8w-D generally had the most prominent and distinct microacinar patterns, particularly eosinophilic microacini. Line 4w-4 was almost exclusively composed of solid chords and nests. Finally, line 4w-6 was the most heterogeneous line with many different types of structures frequently observed.

**Figure 5.** Mammary epithelial cell differentiation and transgene expression in MIN outgrowths. Various immunohistochemical stains were done to determine the differentiation status of the cells in the MIN outgrowths. All MIN outgrowth lines contained CK-8-positive mammary epithelial cells. Smooth muscle actin–positive myoepithelium (middle column) was infrequently observed; however, organized and continuous myoepithelium was associated with some microacini. All MIN outgrowths stained positively for PyV-mT protein (left column). Abbreviations: O, organized cysts; D, dysplastic cysts; S, solid differentiation patterns; M, microacini; and EM, eosinophilic microacini.

**Tumors**
Two histologically distinct tumor phenotypes developed from the MIN outgrowth lines. Solid, poorly differentiated tumors with high mitotic rates and varying amounts of necrosis were the most common. These tumors were generally CK-8 positive with scattered osteopontin-positive cells but
did not typically express smooth muscle actin or whey acidic protein. The solid tumors arising from the MIN outgrowth lines closely resembled the tumor phenotype associated with the PyV-mT transgenic mouse (11, 28). A second tumor phenotype, microacinar, was also observed (Fig. 7). Organized myoepithelium was often associated with tumor microacini. To our knowledge, the microacinar tumor phenotype has not been observed previously in PyV-mT-induced mammary tumors. Both tumor phenotypes were observed in all MIN outgrowth lines.

**Transgene Expression**

Two hypotheses regarding transgene expression were considered: (1) progression to malignancy is caused by increased levels of expression of PyV-mT and (2) differences in latency and metastases correlate with the amount of transgene expressed in the MIN outgrowth tissue. Transgene expression was compared in pairs of outgrowth and corresponding tumor from the same fat pad. Two different techniques were used to analyze expression of the transgene. Patterns of PyV-mT protein expression were determined by immunohistochemical stain of outgrowth and tumor sections (Fig. 5, right column). PyV-mT protein expression was observed in all of the MIN outgrowth lines and tumors. MIN outgrowth tissue was compared with tumors, and outgrowths of different lines were compared with each other. Although PyV-mT protein expression seemed to be variable in some outgrowths and tumors, no differences in the number of cells expressing PyV-mT or the pattern of transgene expression were obvious in this analysis.

**Table 2. A combination of differentiation patterns characterizes each MIN outgrowth line**

<table>
<thead>
<tr>
<th>Line</th>
<th>Cysts, Organized</th>
<th>Cysts, Dysplastic</th>
<th>Solid, Chords/Nests</th>
<th>Microacini, No Myoepithelium</th>
<th>Microacini, Myoepithelium</th>
<th>Microacini, Eosinophilic</th>
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<tbody>
<tr>
<td>8w-A</td>
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NOTE: Characteristic growth patterns observed in MIN outgrowth lines. H&E-stained sections of outgrowths from each line (15 – 20 per line) were examined for the presence of distinct morphologic differentiation patterns. One or more growth patterns were observed in each of the MIN outgrowth lines. Differentiation patterns that were particularly abundant in the line are indicated by a double plus (++); these patterns occupied more than half of the outgrowth space in most of the slide samples (≥80%) as estimated by the examiner. Other growth patterns observed are indicated by a single plus (+).

**Figure 6.** ER-positive cell populations. ER-positive cells were observed in several MIN outgrowth lines and tumors. Frequently, ER-positive cells were observed in small clusters within an ER-negative outgrowth or tumor (top row). Eosinophilic microacini (EM) consistently stained positive for ER, whereas other microacinar populations (M) did not.
To examine transgene expression using a more quantitative technique, mRNA expression levels were determined using real-time RT-PCR. In many (9/18) MIN outgrowth and tumor pairs, amount of transgene expressed in tumors was similar or lower than the amount of transgene expressed in the corresponding MIN outgrowths. Further, the amount of transgene expressed in the MIN outgrowth did not correlate with the tumor latency period as assessed by Cox regression ($P = 0.22$).

Expression Microarray Patterns

Global gene expression of outgrowth tissues and tumors from several MIN outgrowth lines (MIN outgrowth lines 4w-4, 4w-6, 4w-11, 8w-B, and 8w-D) was assessed using oligonucleotide microarrays. Pairs of outgrowth and tumor tissue isolated from the same fat pad were used. Ten pairs of outgrowth and tumor tissue, isolated from the same fat pad, were used including three pairs from 4w-4, four pairs from line 8w-B, two pairs from line 4w-6, three pairs from 4w-11, and one pair from line 8w-D. Mammary tumors from PyV-mT transgenic mice, nontransgenic virgin mammary glands, and nontransgenic prelactating mammary glands were also included in this analysis. To focus the analysis on genes that are differentially expressed in these samples, genes that showed little difference in expression across the samples and genes that were not expressed in at least 20% of these samples were filtered out. This filtering strategy resulted in a subset of 1,812 probes. The samples were subjected to hierarchical clustering analysis based on the expression values of these probes.

MIN outgrowth tissues and their corresponding tumors were highly similar with respect to gene expression (Fig. 8A). Many MIN outgrowth tumors clustered more closely to the corresponding MIN outgrowth tissue than to other tumors and tissues from the same line tended to cluster together. In contrast, the outgrowth and tumor sample pair designated as 8w-D_5309L did not cluster closely together. A whole slide image of the fat pad containing the outgrowth and tumor is shown (Fig. 8B). This particular outgrowth was unique in that it was composed mainly of eosinophilic microacini (Fig. 8B and C). A complete analysis of this large and complex data set will be the subject of a subsequent publications.

Discussion

Recent gene expression studies of human breast cancer progression support the hypothesis that critical molecular events, which have a profound influence on outcome, occur at an early stage. Despite significant morphologic differences between the different stages, expression profiles of early lesions are highly similar to the more advanced, invasive lesions (40). The stable, yet unique, phenotypes of the six MIN outgrowth lines described here, combined with the similarity of gene expression profiles among samples from the same line and outgrowth and tumors from the same fat pad, similarly suggest that early molecular events have significant influence on the biological outcome.

The PyV-mT MIN outgrowth lines were created from lesions initiated in the same genetic background (FVB), by the same molecular event (PyV-mT expression), driven by the same promoter (MMTV long terminal repeat). Although two donors were used to initiate one set of MIN outgrowth lines (4w series), and the process began in a single mammary gland in the other set (8w series), the premalignancies represented by the six PyV-mT-induced MIN outgrowth lines have distinct characteristics in terms of morphology, tumor latency, and metastatic potential. The divergent phenotypic characteristics are reproducibly related to the line of origin, indicating that they are transmitted from one transplant generation to the next.
The divergent biological behavior of the MIN outgrowth lines may have been caused by different levels of PyV-mT expression. However, the amount of PyV-mT RNA expressed in MIN outgrowth samples did not correlate with tumor latency of that outgrowth, suggesting that secondary molecular changes, rather than the level of transgene expression itself, are responsible for the progression of the MIN to malignant tumors. Clearly, the different biological potentials are associated with different expression patterns. It is not clear whether the differences in expression patterns are simply associated with the distinct biological behaviors or are causal. Either interpretation raises the possibility that undefined molecular differences are predictive.

The possibilities that the diverging characteristics of latency and metastatic potential are related to the topographical origin of the original transgenic lesion (nipple proximal versus peripheral) and the age of the original transgenic donor were also considered. However, no difference was observed in the tumor latencies of the two groups of MIN outgrowth lines based on the origin of the original donor tissue: (1) near the nipple of a 4-week-old PyV-mT transgenic donor (4w-4, 4w-6, and 4w-11) and (2) periphery of an 8-week-old mammary gland (8w-A, 8w-B, and 8w-D). Similarly, metastases were observed in mice with tumors from lines from both 4w and 8w sets (4w-11, 8w-A, and 8w-D) but not from 8w-B, 4w-4, or 4w-6. Because outgrowths
were allowed to grow for 6 weeks after donor tissue transplantation before selection of tissue for serial transplantation, we may have inadvertently selected for low-risk lesions.

The generation of outgrowth lines with diverging biological properties from premalignant tissues in which progression was initiated by the same molecular event has been reported previously in studies of hyperplastic outgrowth lines generated from MMTV-induced premalignant lesions. Diverging lines with differing biological properties, generated when a single lesion or single outgrowth was subdivided, maintained the same genotype and restriction genotype as the original parental line (41, 42). In MMTV-induced premalignancies, patterns of viral integration proved the tissue was clonally derived. Thus far, there is no direct proof of clonality in the MIN outgrowth lines. However, the lines are generated by successive selection and transplantation of relatively undifferentiated tissue from the growing margin of the MIN outgrowth. Hence, the cells at the growing margins have an indefinite capacity for self-renewal and for generation of populations of more differentiated cells. These observations are indicative of an encoded stem cell and presumably clonal population.

PyV-mT-induced MIN were categorized previously into three groups based on distinct differentiation patterns as cystic, solid, and mixed (28). However, at least six distinct differentiation patterns were observed in the MIN outgrowth lines. These morphologic patterns might have some predictive value, because lines with longer tumor latencies, 8w-A, 8w-D, and 4w-11, generally consisted mainly of more differentiated cell populations such as microacini and the well-differentiated cysts. In contrast, lines with shorter tumor latencies, 4w-4 and 8w-B, were frequently associated with more dysplastic differentiation patterns such as solid nests and dysplastic cysts, respectively.

Whereas the more morphologically atypical (dysplastic) differentiation patterns correlated with tumor latency, the converse was true of metastatic potential. The MIN outgrowths with less frequent dysplastic differentiation patterns and longer tumor latencies, such as 4w-11, 8w-A, and 8w-D, were more frequently associated with metastatic tumors. While perplexing, this seeming disparity between structure and outcome has been observed previously in high and low metastatic variants of PyV-mT tumors (43). Taken together, these observations are consistent with the hypothesis that several different molecular pathways may be represented in the MIN outgrowth lines.

The PyV-mT MIN outgrowth lines share several characteristics with precursors of human breast cancer such as ductal carcinoma in situ. Their growth is noninvasive, confined to the mammary fat pad, and inhibited by normal mammary tissues (28, 44). The breast lesions of both species are composed of populations of atypical cells that are associated with varying risks for tumor development. Further, less organized and more dysplastic differentiation patterns are associated with higher risk of earlier tumor development. The experiments discussed here show that MIN from murine model systems are as heterogeneous as the human precursor lesions and that different classes of premalignant cells can consistently be identified.

The MIN outgrowth lines provide the opportunity to study the full range of events that may occur after the initiation of tumorigenesis by the PyV-mT transgene. Using this system, more complex analyses of gene expression data may be combined with in vivo experiments designed to test the importance of various molecular changes for neoplastic progression. Our studies reinforce the concept that neoplastic progression in mouse models and humans share similar molecular, morphologic, and biological attributes. Thus, mouse model systems, such as the PyV-mT MIN outgrowth lines, may provide insight into the origin, evolution, and clinical outcome of human disease. The full potential of this system awaits further exploration.

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References


Polyomavirus middle T–induced mammary intraepithelial neoplasia outgrowths: Single origin, divergent evolution, and multiple outcomes
