Accelerated Preclinical Testing Using Transplanted Tumors from Genetically Engineered Mouse Breast Cancer Models

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Abstract

Purpose: The use of genetically engineered mouse (GEM) models for preclinical testing of anticancer therapies is hampered by variable tumor latency, incomplete penetrance, and complicated breeding schemes. Here, we describe and validate a transplantation strategy that circumvents some of these difficulties.

Experimental Design: Tumor fragments from tumor-bearing MMTV-PyMT or cell suspensions from MMTV-PyMT, -Her2/neu, -wnt1, -wnt1/p53+/−, BRCA1/p53+/−, and C3(1) T-Ag mice were transplanted into the mammary fat pad or s.c. into naive syngeneic or immunosuppressed mice. Tumor development was monitored and tissues were processed for histopathology and gene expression profiling. Metastasis was scored 60 days after the removal of transplanted tumors.

Results: PyMT tumor fragments and cell suspensions from anterior glands grew faster than posterior tumors in serial passages regardless of the site of implantation. Microarray analysis revealed genetic differences between these tumors. The transplantation was reproducible using anterior tumors from multiple GEM, and tumor growth rate correlated with the number of transplanted cells. Similar morphologic appearances were observed in original and transplanted tumors. Metastasis developed in >90% of mice transplanted with PyMT, 40% with BRCA1/p53+/− and wnt1/p53+/−, and 15% with Her2/neu tumors. Expansion of PyMT and wnt1 tumors by serial transplantation for two passages did not lead to significant changes in gene expression. PyMT-transplanted tumors and anterior tumors of transgenic mice showed similar sensitivities to cyclophosphamide and paclitaxel.

Conclusions: Transplantation of GEM tumors can provide a large cohort of mice bearing mammary tumors at the same stage of tumor development and with defined frequency of metastasis in a well-characterized molecular and genetic background.

Preclinical animal models are a major tool for the selection and development of anticancer agents. The standard practice of xenografting tumors into immunocompromised mice generates reproducible tumors, but has only moderate predictive value when the same drugs are tested in phase II clinical trials (1–4). Hollow fiber assays, which are a combination of in vivo and in vitro models (5), provide a better correlation with human disease as does the passage of human tumors directly in mice without exposure to tissue culture (6–8).

It is likely that better guidance from preclinical studies would reduce the failure rate of drugs in phase II clinical trials. Preclinical models fail to correlate with clinical efficacy, in part because drug efficacy studies in mice frequently do not address differences in drug metabolism, pharmacokinetics, and pharmacodynamics (8, 9), and whether molecularly targeted drugs succeed in reaching the appropriate target. In addition, the use of immunocompromised mice for preclinical testing makes it difficult to predict the role of the immune system in response to therapies. Many chemotherapeutic agents have originated from screening drugs in syngeneic mouse models for which tumors were passaged directly from mouse to mouse (10). Genetically engineered mouse (GEM) models of human cancers have potential advantages for testing antitumor
therapies (8, 11, 12). In these models, the development of malignancy is causally related to a specific genetic event, and offers a “proof of principle” for testing whether molecularly targeted drugs affect an expected target. Unfortunately, GEM models have been rarely used for the development of therapies for human diseases, such as breast cancer, in which a correlation with current preclinical models is poor, information on causative genetic events is extensive, and multiple GEM models are available. Thus, the value of GEM mammary tumor models for predicting the efficacy of drug therapies in clinical trials has yet to be systematically addressed.

Many obstacles exist in taking GEM into the mainstream of preclinical testing. The limited use of GEM is hampered by variable tumor latency, incomplete penetrance, and complicated breeding schemes, all of which translate into high costs and protracted experimental designs. Most importantly, there is significant variability, measured in weeks to months, in the time of progression to a predetermined tumor size, the source and the time needed for development of metastatic disease. Although this variability may recapitulate the development and progression of human disease, differences in animal age and tumor burden/stage at any given time in the same mouse colony makes it nearly impossible to properly assess drug efficacy or translate the results into the design of a correlative clinical trial. The multifocal nature of transgenic tumors, developing at random in 10 mouse mammary glands, introduces an additional level of complexity in evaluating the therapeutic response. To be useful for preclinical testing, mouse models should be cost-effective and generate tumors in a relatively short period of time. Only few GEM models, such as the mammary PyMT and the pancreatic RIP-TAG model, develop tumors over a narrow time span (2-3 months from birth; refs. 13, 14) with relatively uniform appearance, histology, and molecular markers (15, 16).

An alternative modality for generating mice with GEM tumors is transplantation. A recent study showed that s.c. transplantation of mammary tissue from young MMTV-PyMT mice into syngeneic nude recipients can generate tumors, although it requires multiple passages in vivo to generate large amounts of donor tumor tissue (17). A similar approach has been used for allografting prostate cancer tumor fragments from 12T10 transgenic mice (18). However, generating multiple small similar-sized fragments limits the number of secondary recipients and introduces variability due to regional heterogeneity within the tumor. Cell suspensions prepared with or without enzymatic digestion of extracellular matrix have been successfully used for studying mouse and human hematopoietic tumors and, more recently, for characterizing the tumor stem cell compartment (6, 7, 18). The Developmental Therapeutics Program at the National Cancer Institute has also used cell suspensions for drug screening in human and murine tumors.5 To our knowledge, transplantation of cell suspensions without exposing these cells to tissue culture conditions has not been previously done using mouse mammary tumors derived from GEM.

We systematically addressed the transplantation of mammary tumors from multiple GEM models into naïve recipients. Tumors derived from anterior (axillary) or posterior (inguinal) mammary glands of PyMT mice transplanted as tumor fragments or as cell suspensions reliably generated tumors in syngeneic, scid, and nu/nu naïve recipient mice. We optimized the protocol for implanting PyMT cell suspensions into orthotopic or s.c. locations. We also validated this strategy using cell suspensions derived from multiple breast cancer mouse mammary models.

Materials and Methods

Mice. All studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility in compliance with the Public Health Service Guidelines for the Care and Use of Animals in Research. naïve 6- to 8-week old female athymic nu/nu/NCr, scid/NCr (BALB/c background), and FVB/NCr mice from the National Cancer Institute Animal Production Program (Frederick, MD) were used as transplant recipients. Autoclaved feed and hyperchlorinated water were provided ad libitum. FVB/N TgN (MMTV-PyMT)634-Mul from The Jackson Laboratory, Bar Harbor, ME (13) were bred with FVB/NCr from the National Cancer Institute Animal Production Program. FVB/N TgN (MMTV-Her2/neu; ref. 19), MMTV-wnt1/p53+/- in C57Bl/6/129Sv background (20), were also from The Jackson Laboratory, and MMTV-wnt1 were backcrossed to F10 in C57Bl/6. BRCA1/p53+/- tumors were provided by Chuxia Deng (21), and FVB/N TgN [C3(1)T-Ag] tumors were provided by Jeff E. Green (22).

Drugs. All compounds used in the efficacy studies were from the DTP Drug Repository (Rockville, MD). Paclitaxel (NSC 125973) was diluted in 10% ethanol/10% cremaphor, in 80% saline. Cyclophosphamide (NSC 262791) was diluted in saline. All drugs were injected using a standard injection volume of 0.1 mL/10 g body weight.

Isolation of tumors and preparation of cell suspension. Tumors were collected aseptically from donor mice using blunt dissection, trimmed of extraneous tissues, mechanically dissociated by mincing, passaged through a 40-μm mesh sterile screen, and suspended in serum-free RPMI 1640 (Quality Biological, Gaithersburg, MD). Cells were further dissociated by serial passage through a syringe with 18- to 25-gauge needles. The cell suspension was washed twice, resuspended in serum-free RPMI 1640, and viable cell counts were determined using 0.4% trypan blue. Cells were diluted to the desired concentration for immediate transplantation or suspended at 1 × 107 cells/mL in cell-freezing medium containing 10% DMSO, and cryopreserved using stepped rate freezing.

Tumor implants. Fragments (2 mm3) from matched tumors (anterior from an axillary and posterior from inguinal gland tumors from the same donor) were implanted s.c. on the lateral body wall in the axillary region caudal to the foreleg of 10 female athymic nude mice. For s.c. transplantation of cell suspensions, the cells were washed, suspended in 100 μL of serum-free RPMI 1640, and inoculated as above. For mammary fat pad (MFP) transplantation of tumor cell suspensions, the no. 4 MFP was visualized through a small skin incision just anterior to the rear leg, and cells were injected in 50 μL of RPMI 1640 with visual confirmation of MFP engorgement. The incision was closed with a sterile wound clip (removed in 7 days).

Tumor monitoring. Tumor size was determined biweekly using caliper measurements (in millimeters) in two perpendicular dimensions (length and width). Tumor weights (in milligrams) were calculated using the formula for a prolate ellipsoid and assuming a specific gravity of 1.0 g/cm3 (23). Tumors were collected at a predetermined time following implantation or when they reached a maximum size of ~1 g wet weight as indicated in the text. Tumors were divided into the following fragments: half was used to prepare a cell suspension, the remaining half was divided into three pieces, snap-frozen, fixed in 10% neutral-buffered formalin, or embedded in optimum cutting carbaryl transferase. In studies in which metastases were analyzed 60 days after resection of the primary tumors, the lungs were resected, inflated and fixed in 10% neutral-buffered formalin, and

5 http://www.dtp.nci.nih.gov/branches/btb/availabledocuments.html
metastases were counted. For histologic examination, the lung lobes were spread and metastatic lesions enumerated on 20-µm step sections.

RNA isolation, microarray hybridization, data analysis, and validation by quantitative reverse transcription-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, followed by DNeasy treatment and RNA clean-up (RNeasy mini kit; Qiagen, Valencia, CA). RNA integrity was determined using the RNA 6000 Nano LabChip Kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA from PyMT and wnt1 tumors was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Arrays, scanned with an Affymetrix GeneChip scanner 3000, and analyzed using Microarray Suite 5.0 software (MASS; Affymetrix Inc., Santa Clara, CA). Mean signal intensity was scaled to a target value of 500 using all probe sets. Normalization, gene filtering, comparative gene expression, and hierarchical clustering analysis were done using BRB Array Tools software developed by the Biometric Research Branch of the National Cancer Institute.6 The genes for all analysis were filtered by requiring the spot intensity to be >10. Each array was normalized using the median over the entire array. Only genes that reported values in at least 75% of the samples were included for further analysis. The Class Comparison Tool, using Univariate Two-Sample t test with random variance model and permutations of the class labels was applied to all probes that passed the signal intensity and missing-value filters. In addition, only the most variable genes, with a minimum 2-fold expression change in either direction from the median value of the gene in at least 20% of the samples were included for unsupervised analysis by hierarchical clustering or multidimensional scaling.

For data validation by quantitative reverse transcription-PCR, total RNA isolated from individual tumors was reverse-transcribed and analyzed in Science Applications International Corporation Frederic using premade TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). The following genes were analyzed: Fgfr3, Bid, Bag1, Trnfsf2, Cldn4, Tbc1, Eef1d, Lyst, Dnaic3, and Thbs1. Expression values were derived by the comparative Ct method as suggested by the manufacturer.

Statistical analysis of drug response. Tumor responses were calculated as net log cell kill = 0.301 × [(T – C) – duration of treatment] / Td, where T and C are the median times in days for treated and control groups, respectively, to attain the specified tumor size and Td is time to doubling (23). The significance of the comparisons between different treatment groups was determined by Student’s t test or by actuarial analysis of tumor growth rates to a predetermined size, when appropriate.

Results

Selection of mammary tumor tissues for transplantation. Mammary tumors were noted to appear earlier in the anterior mammary glands of PyMT mice as well as in other breast cancer mouse models. These observations were consistent with anterior-to-posterior differences in tumor growth rate described in spontaneous mammary cancer models (24). To select a reproducible source of mammary tumors, we compared the growth of paired tumor fragments arising from anterior (axillary) mammary glands with posterior (inguinal) mammary glands obtained from the same PyMT mice. Paired axillary and inguinal tumor fragments from nine individual transgenic PyMT animals were transplanted s.c. into naive athymic nude mice. These tumors were subsequently transplanted using tumor fragments into another cohort of 10 mice for six additional passages. All axillary-derived tumors grew faster than inguinal tumors, as shown for one example of paired tumors in Fig. 1A. Although higher growth rates were observed during subsequent passages, axillary tumors retained a growth advantage over the inguinal tumors. These data suggested that axillary and inguinal tumors have inherently different growth properties.

To determine whether cell suspensions recapitulate differences in growth rates observed for tumor fragments, we implanted 1 million PyMT cells from anterior and posterior tumors into the anterior (gland no. 2) or posterior (gland no. 4) MFP of 10 syngeneic hosts. Tumor cells from anterior mammary glands retained a growth advantage when implanted into either the anterior or posterior MFP (data not shown). These data indicate that cell suspensions preserve the biological functions of the original tumors.

To examine the molecular nature of differences observed in growth rates, total RNA extracted from anterior and posterior PyMT tumors were analyzed by expression profiling as described in Materials and Methods. A distinct subset of genes differentially expressed between anterior and posterior tumors was identified (Fig. 1B) from a scatterplot of averaged log-intensity values. Unpaired class comparison showed that 224 probes discriminated the five anterior tumors from the five posterior tumors with statistical significance (P ≤ 0.001; Fig. 1C; Supplemental Table S1). Hierarchical clustering of the samples based on these 224 probes showed tight clustering of anterior and posterior tumors within each group (Fig. 1C). Although there were no apparent microvessel density or overall morphology differences between anterior and posterior tumors (data not shown), Cxcl2 (GROb/MIP-2a), a growth-related chemokine, was highly expressed in anterior tumors. This molecule is active in picomolar concentrations and has been implicated in the recruitment of inflammatory cells, angiogenesis, migration, tumor invasion, and metastasis (25). Other genes of interest highly expressed in anterior tumors were aldehyde dehydrogenase (Aldh1a3), a stem cell marker (26), which opens an intriguing question of enrichment in stem cell population, and genes related to milk production (caseins-α, -γ, and lactotransferrin, Ltf), which may reflect luminal differentiation previously associated with PyMT tumors (16). Of interest, higher expression of an apoptosis-related gene, BID, and a group of cell adhesion genes were observed in the posterior tumors.

To validate the gene expression data, we did a quantitative reverse transcription-PCR using 10 genes selected for differences in their expression and biological interest. Their expressions, as detected by microarray and reverse transcription-PCR, were highly correlated with r² = 0.8982 (Fig. 1D). Based on these observations, we limited all subsequent studies to tumors derived from anterior axillary glands unless indicated otherwise.

Thus, our studies uncovered novel biological and molecular differences that distinguished PyMT tumors by their anatomic location. Because of these findings, only anterior mammary gland tumors were used for all subsequent studies.

Transplantation of cell suspensions from PyMT tumors. To determine the optimal schedule for generation of tumors, tumor cell suspensions were transplanted into MFP no. 4 of naive hosts. We measured time to progression (applicable for clinical development) to a predetermined tumor size of 700 to 900 mg of wet weight. Implantation of an increasing number of

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Fig. 1. Growth rates and molecular profiling of transplanted anterior and posterior PyMT tumors. A, growth rates of anterior (dashed line) and posterior (solid line) PyMT tumor fragments derived from a single transgenic mouse (representative of nine independent experiments) implanted s.c. into the lateral body wall of an athymic nu/nu mice (n = 10 mice/group). Tumor weight is calculated as described in Materials and Methods. B, scatterplot of averaged log-intensity values. Red dots, probes reporting at least a 2-fold difference in expression ratios in anterior versus posterior tumors from a total of 25,281 probes that passed the signal intensity and missing-value filters. C, hierarchical clustering of five anterior (Ant1-5) and five posterior tumors (Pst1-5) based on 224 genes derived from class comparison. In the vertical axis, the 224 genes were clustered according to similarities in relative expression values. The color of each cell in the matrix represents the relative expression level of each gene (bottom). D, expression values for Trnasf22, Chln4, Tbc1d, Bnd, Eef1d, Bag1, Fgfr3lyst, Dnaic3, and Thbs1 were derived by the comparative Ct method. The anterior/posterior (A/P) ratios were calculated and plotted against those obtained from microarray analyses. Expression ratios of these genes by both methods was highly correlated with $r^2 = 0.8982$. 

Transplantation of GEM Tumors

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tumor cells shortened the time to progression (Fig. 2A). Tumors from $1 \times 10^6$ PyMT-transplanted cells grew consistently in 30 to 32 days in syngeneic recipients (Fig. 2B), and in 42 days, in scid mice (Table 1). As few as $1 \times 10^5$ cells were sufficient to generate tumors in 80 days in syngeneic mice, but 10-fold more ($5 \times 10^5$) cells were required to generate tumors in athymic nude mice over the same time period, whereas $1 \times 10^6$ cells resulted in smaller (<500 mg) tumors in nude mice in >90 days with more intragroup variability (data not shown).

To determine whether storing and freezing cells affects the growth rate of transplanted tumors, freshly prepared cell suspensions and cells prepared and frozen from the same PyMT

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**Table 1.** Tumor growth rates and frequency of metastasis for each mouse model

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Syngeneic</th>
<th>MFP</th>
<th>scid</th>
<th>Metastasis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyMT</td>
<td>30 ± 4</td>
<td>81 ± 17</td>
<td>48 ± 6</td>
<td>91</td>
</tr>
<tr>
<td>Her2/neu</td>
<td>Rejected</td>
<td>&gt;80</td>
<td>40 ± 8</td>
<td>15</td>
</tr>
<tr>
<td>Wnt1/p53+/-</td>
<td>Not tested</td>
<td>30 ± 3</td>
<td>26 ± 6</td>
<td>40</td>
</tr>
<tr>
<td>BRCA1/p53+/-</td>
<td>Not tested</td>
<td>Not tested</td>
<td>30 ± 4</td>
<td>40</td>
</tr>
<tr>
<td>FVBf[N(C3(1)/T-Ag)]</td>
<td>Not tested</td>
<td>30 ± 2</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

NOTE: The background of the recipient group of mice is indicated above. The numbers indicate time to progression to a predetermined tumor size of 7 to 900 mg (in days ± SD). One million cells from each GEM tumor was transplanted into the MFP of naive mice ($n = 10-12$). The frequency of metastasis was scored for tumors growing in scid mice 60 d after removal of the primary tumor.
tumor were implanted into naïve recipients (six mice in each group). There were no significant differences in time to progression between these tumors. Therefore, stored frozen cell suspensions were used for all subsequent experiments.

We also tested cell transplantation into a heterotopic (s.c.) location in syngeneic mice. One million cells transplanted s.c. grew slower (40 days) than the tumors growing in MFP. Thus, cell suspensions from transgenic tumors have a biological behavior similar to that described in allografts and xenografts that are reported to grow faster in orthotopic locations.

**Validation of transplantation protocol using tumors from other GEM tumors.** To validate the transplantation strategy for other breast cancer mouse models, we harvested and froze cells from C3(1)T-Ag, BRCA1/p53+/−, MMTV-Her2/neu, -wnt1/p53+/−, and -wnt1 tumors. Cells were transplanted into syngeneic and, when appropriate, scid and athymic nude mice using 0.1 × 10⁶/MFP, 1 × 10⁶/MFP, and 2 × 10⁶ cells/MFP. Figures 2C and D show the growth rates of 1 × 10⁶ and 1 × 10⁵ cells from Her2/neu and BRCA1/p53+/− tumors implanted into the MFP of scid mice. As previously reported, C3(1)T-Ag tumors were rejected when implanted into naïve syngeneic recipients (27). Therefore, C3(1)T-Ag, Wnt1/p53+/−, and BRCA1/p53+/− tumors, derived in mixed genetic backgrounds, as well as Her2/neu (rejected in syngeneic recipients because Her2/neu in this model is a rat protein), were tested in immunosuppressed hosts. The background of each model and its corresponding human disease are described in Supplemental Table S2. In all cases, reducing the numbers of transplanted cells below 1 million cells resulted in higher intragroup variability and slower growth rates. Wnt1/p53+/−, BRCA1/p53+/−, and C3(1)T-Ag tumors transplanted into MFP of scid mice grew at similar rates, reaching a target size of 700 to 900 mg in 26 to 30 days, PyMT and Her2/neu tumors reached similar sizes in 48 and 40 days, respectively (Table 1).

The expression of MMTV-driven genes was thought to be hormonally dependent (28). A deficiency in estrogen and other glucocorticoids in athymic nude mice has been previously reported (28), although other studies using a different strain of athymic nude mice did not reach the same conclusion (29). We evaluated the level of 17β-estradiol in serum from different genetic backgrounds involved in our study (FVB, BALB/cAn/NCr, athymic nude, and scid mice). We found no significant differences in serum estradiol levels among any of these strains (data not shown).

**Transplanted tumors have similar histologies with the original GEM tumors.** MMTV-PyMT tumors rapidly progress from mammary intraepithelial neoplasia to carcinoma with a well-established pattern (15). Early carcinomas have glandular structures with cytomegaly, nuclear pleomorphism, and a disruption of architecture. More advanced carcinomas are composed of sheets of anaplastic cells accompanied by reactive stroma and leukocyte infiltration (15). The morphology of tumors arising in the transgenic mice was comparable to those that grew from cells transplanted into the MFP, except that the stroma was less prominent (Supplemental Fig. S1). In contrast, stromal encapsulation was more abundant in tumors implanted s.c. (data not shown).

By CD31 immunohistochemistry, microvessels followed the fibrous septa and extended into lobules of tumor cells in the original and transplanted PyMT tumors (Supplemental Fig. S1). Original and transplanted tumors also had a similar rate of cell proliferation as identified by Ki67 immunohistochemistry (Supplemental Fig. S2).

We also compared the histologic appearance of lung metastasis. The morphology of macrometastases or micrometastases arising from transplanted tumors was indistinguishable from metastases in transgenic mice. The proliferative rate (Ki67) and the degree of angiogenesis (CD31) seemed somewhat more prominent in metastases from the original tumors than in metastases from the transplanted tumors (Supplemental Fig. S3).

**Metastasis development in mice with transplanted tumors.** Most transgenic MMTV-PyMT mice develop lung metastases within 3 to 4 months (13). Lung metastases also occur in wnt1/p53+/− mice (30). To determine whether the transplanted tumors retain the ability to metastasize, we resected transplanted tumors at 700 to 900 mg wet weight and did a full necropsy 60 days later, a time selected based on the development of lung metastasis in transgenic PyMT mice (13). More than 90% of scid and syngeneic mice with PyMT transplants developed lung metastases, and most animals had multiple lesions. There was no metastatic disease detected in the liver or other organs, consistent with previous reports in the original transgene (13). Pulmonary metastases were found in 40% of wnt1/p53+/− tumors. Two out of 10 mice implanted with cell suspensions derived from BRCA1/p53+/− tumors developed lung metastases, and two additional mice had abdominal carcinomatosis. Fifteen percent of mice transplanted with 10⁶ Her2/neu tumors also developed pulmonary metastasis. Thus, the transplanted tumors recapitulate the metastatic rates described for each original GEM.

**Growth rates and molecular features of serially transplanted tumors.** In order to use GEM tumors for preclinical testing, serial transplantation will be necessary to expand these tumors to multiple recipients. As expected, serial passage in vivo of tumor fragments derived from the original PyMT passed serially in vivo had accelerated growth rates with each subsequent passage, as expected. This acceleration occurred regardless of the model or the host (Fig. 3; data not shown). Because the value of the proposed transplantation strategy depends on the ability of transplanted tumors to preserve molecular and biological features of the original GEM, gene expression profiling was done on original tumors and tumors derived from first and second passages from PyMT and wnt1 models as examples of luminal and basal tumor types, respectively. Global expression profiles were compared by multidimensional scaling based on the 3,692 most variable probes across all PyMT and wnt1 tumors (Fig. 4A). There was a clear separation of PyMT and wnt1 tumors into two groups, as expected. These differences were preserved after serial transplantation with the transplanted tumors positioned in proximity to their tumors of origin in three-dimensional space. These data support the key hypothesis that the specific oncogenic origin of each tumor is marked by a unique expression fingerprint and indicates that serial transplantation preserves the molecular characteristics of these tumors.
To identify genes and pathways that discriminate between PyMT and wnt1 tumors, class comparison was applied to 19,975 probes that passed the signal intensity and missing-value filters. As a group, PyMT and wnt1 tumors differed by 6,978 probes with statistical significance ($P < 0.001$). Relevant genes and gene ontology categories identified by this supervised analysis are presented as Supplemental Table S3. Interestingly, we identified multiple genes of the overall Wnt pathway in Fig. 4.

Serially passaged PyMT and wnt1 tumors do not diverge significantly from the original tumors. **A**, multidimensional scaling analysis of original and transplanted PyMT and wnt1 tumors using 3,692 most variable probes across experiments. A clear separation is observed between all PyMT (solid oval) and wnt1 (dashed oval) tumors, including original (●), first passage (○) and second passage (□) tumors. **B**, dendrogram showing the unsupervised hierarchical clustering of PyMT tumors samples based on 2,094 most variable probes. This analysis is unable to discriminate original (PyMT0) tumors from transplanted tumors obtained from first (PyMT1) and second (PyMT2) passage. **C**, unsupervised hierarchical clustering of wnt1 tumor samples based on 1,532 most variable probes does not distinguish wnt1 original (Wnt0), from first (Wnt1) or second (Wnt2) passage tumors.

Fig. 3. Growth rates of serially passed 1 million cells originating from PyMT (A), BRCA1/p53+/- (B), or C(3)/T-Ag GEM tumors (C) were shown as examples of growth rates of tumors in first and second passage. Tumor cells from original GEM were harvested at 700 to 900 mg of wet weight and transplanted into MFP of 10 naive recipients (●, first passage) as described in Materials and Methods. One million cells obtained from these tumors, harvested at similar tumor sizes were transplanted into the MFP of another group of 10 mice (●, second passage). Tumor growth rates were recorded biweekly as described in Materials and Methods. Bars represent SD.
wnt1-driven tumors when compared with PyMT tumors. High expression of Wnt5a was reported in transgenic wnt1/p53<sup>−/−</sup> tumors as compared with Her2/neu tumors (31). As shown in Supplemental Table S3, we found that >6-fold expression was evident for Wnt5b and many other genes in the Wnt signaling pathway, including Dkk3, Cmnb1 (β-catenin), Porec, Sostdc1, Hbp1, Syp2, Fbxw2, and Ccnd1 (cyclin D1). In addition, Id4, a negative regulator of BRCA1 and a marker for basal-like breast cancers (32) showed the highest (100-fold) expression in wnt1 relative to PyMT tumors. Two genes, characteristic of luminal differentiation, Wap and Lalba (lactoalbumin), showed a >30-fold expression in PyMT as compared with wnt1 tumors, in agreement with luminal differentiation previously assigned to PyMT tumors (16). Many of the genes identified by this analysis have not been previously linked directly to PyMT or wnt1 expression, and it would be of interest to confirm their role in tumor development.

To examine whether serially transplanted tumors significantly diverge from the tumors of origin within each model, unsupervised hierarchical clustering was done for each tumor model. Hierarchical clustering of PyMT tumors based on all 18,882 probes or the 2,094 most variable probes was unable to accurately discriminate between the original and serially transplanted tumors (Fig. 4B). Hierarchical clustering of wnt1 tumors based on either all 21,860 probes or the 1,532 most variable probes (Fig. 4C) was also unable to accurately discriminate between original and transplanted wnt1 tumors. It is possible that more samples are required to uncover some differences, but these data indicate that cell suspensions from PyMT and wnt1 tumors expanded by two serial passages preserve the overall molecular features associated with the original tumors.

Transgenic mammary tumors and tumors from PyMT-transplanted tumor show similar sensitivities to chemotherapeutic agents. To adapt the transplantation protocol for screening drugs in transplanted GEM tumors, the original tumors and tumors that arise from cells transplanted into naive mice should have similar sensitivities to conventional therapies. To that end, we compared the response of PyMT transgenic mouse tumors with tumors transplanted into syngeneic recipients to cyclophosphamide and paclitaxel. The growth rates and response to therapy scored on anterior and inguinal tumors separately showed that, although all tumors were sensitive to both agents, the kinetics of the growth rate and the response to therapies were significantly different (Fig. 5A and B). On day 92, all transgenic mice treated with cyclophosphamide had anterior tumors weighing 250 to 500 mg, whereas there were no detectable posterior tumors. In addition, when the posterior tumors were <500 mg, the mice were sacrificed because of the anterior tumors. The growth delay of anterior tumors in mice treated with 25 mg/kg of cyclophosphamide [calculated as % (T − C) / C] was 22 days, and 16 days for posterior tumors. The growth delay of anterior tumors to paclitaxel at 15 mg/kg was 22 days (Fig. 5C). Because the transplanted tumors grow to >1 g within 30 days, every week, therapies repeated three and reduced cumulative dose for cyclophosphamide were used (Fig. 5D). In spite of the dose reduction, the transplanted tumors were sensitive to the same drugs as the original PyMT transgenic tumors with a growth delay of 9 days for cyclophosphamide and 45 days for paclitaxel.

Discussion

We show here that it is possible to generate synchronously growing tumors by transplantation of tumor fragments and cell suspensions from several GEM mammary cancer models. In developing a transplantation strategy for generation of a large number of synchronous tumors from genetically engineered breast cancer mouse models, the following important questions were systematically addressed: the tumor source, method of harvesting and storing, site of implantation, and recipient’s background. We used mechanical disruption to limit stromal contamination and reduce ex vivo manipulation. Cell suspensions from an individual mouse mammary tumor provided sufficient viable cells for 100 or more recipients that substantially exceeded the capacity of fragments obtained from an individual tumor. Pooling cells from several tumors or expanding individual tumors in secondary recipients can potentially generate a virtually unlimited on-demand source of tumor cells for preclinical studies.

Transplantation of cells derived from GEM tumors harvested at the same stage of tumor development allowed us to circumvent many obstacles associated with tumor-bearing GEM. The advantages and limitations of original and transplanted GEM tumors for preclinical testing as compared with xenografts are outlined in Supplemental Table S4. The transplantation modality also has limitations, as it does not allow the study of tumor-initiating events and there may be specific advantages in using human rather than murine cells for specific drugs, although these issues have never been systematically addressed.

A major limitation in using the transplantation of GEM tumors is immune rejection. For example, tumors from Her2/neu mice were rejected in naive syngeneic recipients and PyMT and wnt1 mice grew faster in syngeneic immunocompetent and scid mice as compared with athymic nude mice. Higher natural killer cell activity was reported in athymic nude mice (33). Differences in the growth rates of normal tissues (34) and inconsistencies in the response to therapies of tumors grown in athymic nude and scid mice have been previously reported (35). Differences in gene expression profiles of tumors grown in athymic nude and scid mouse confirm the importance of host immune responses (36). The characterization of specific immune responses by transplantation of tumors into the 6- to 9-week-old transgenic Her2/neu mice (before they develop the mammary tumors) could be useful in exploring the role of immune response, tumor tolerance, and immunization strategies with potential application to human clinical trials. This strategy could be expanded to all other GEM models that develop tumors later than 6 months of age. Transplanting some tumors, such as wnt1 in pure C57Bl/6 background into syngeneic mice with well-defined molecular backgrounds, such as COX-2<sup>−/−</sup>, RAG1<sup>−/−</sup> or others, offers an opportunity to test the molecular basis of response to specific drugs, and has a further advantage that cross-species barriers to the establishment of proper tumor-stroma and tumor-host interactions are not an issue.

We also observed that PyMT tumor cells grew significantly faster in the MFP than s.c., regardless of the host. Few studies have systematically addressed the importance of orthotopic implantation in predicting response to therapies (37), although there are indications that the implantation site influences tumor progression, drug availability and response (38).
A major shortcoming in testing novel anticancer drugs for breast cancer is a paucity of preclinical models with metastatic disease. Unlike the original GEM, in which tumors arise in multiple mammary glands, making it impossible to establish the exact source of the metastatic lesion, transplanted tumors generate metastasis from a defined site. In this case, the primary lesion can be resected at any stage of tumor development or therapy, thereby allowing studies of metastasis prevention and drug resistance. Only few GEM models, such as PyMT, develop lung metastasis within 3 to 4 months of age (15). In our study, all transplanted GEM tumors developed metastasis with similar morphologic features of lesions in the original GEM. Thus, these valuable models can be used for testing mechanisms of metastatic spread and drug development.

Our studies using transplanted tumors also uncovered intriguing biological and molecular differences between anterior and posterior tumors in the PyMT mouse model. Anterior mammary gland tumors grew faster in serial passages than did the fragments or cell suspensions from posterior glands regardless of the site of implantation. Posterior tumors in the original GEM were also more sensitive to chemotherapeutic agents. Although there were no significant morphologic differences between the anterior and posterior tumors, there were clear differences in molecular signatures that distinguished these tumors. Differences in gene expression in individual PyMT tumors have been described (16), and tumors derived from mammary explants also revealed significant molecular heterogeneity (17). These differences were also reported for other transgenic and spontaneous mouse models and may be partly due to mixed analysis of tumors arising in different anatomic locations. Thus, we propose that tumors evolved from anterior and posterior glands maintain their biological differences.
differences and should not be mixed for studies of mammary tumor biology and preclinical testing. In spite of the faster growth rate of tumors expanded in two subsequent passages in vivo, gene expression analysis of PyMT and Wnt1 models did not show significant differences between the original and transplanted tumors. Our data suggests that serial transplantation of these tumors does not lead to significant divergence at the molecular level, and that transplanted tumors preserve most molecular features associated with their original genotype. Whether there are differences in gene expression after serial transplantation of other GEM tumors, some of which may have a more unstable genome, such as BRCA1/p53+/-, remains to be established.

It is clear that no single experimental model will satisfy all aspects of preclinical testing. Tumors from GEM can be particularly useful in testing and guiding the design of clinical trials for molecularly targeted therapies and provide valuable models for metastatic disease. Transplantation allows selection and expansion in vivo of individual mouse tumors based on their similarity with human tumors as established by gene expression profiling and other characteristics. Thus, the transplantation strategy can be viewed as a complementary approach in preclinical testing and understanding tumor-host interactions, tumor progression, and development of metastasis.

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