Overview of Genetically Engineered Mouse Models of Distinct Breast Cancer Subtypes

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Advances in the screening of new therapeutic options have significantly reduced the breast cancer death rate over the last decade. Despite these advances, breast cancer remains the second leading cause of cancer death among women. This is due in part to the complexity of the disease, which is characterized by multiple subtypes that are driven by different genetic mechanisms and that likely arise from different cell types of origin. Because these differences often drive treatment options and outcomes, it is important to select relevant preclinical model systems to study new therapeutic interventions and tumor biology. Described in this unit are the characteristics and applications of validated genetically engineered mouse models (GEMMs) of basal-like, luminal, and claudin-low human subtypes of breast cancer. These different subtypes have different clinical outcomes and require different treatment strategies. These GEMMs can be considered faithful surrogates of their human disease counterparts. They represent alternative preclinical tumor models to cell line and patient-derived xenografts for preclinical drug discovery and tumor biology studies. © 2016 by John Wiley & Sons, Inc.

Keywords: gene expression • breast cancer • mouse models • drug testing • efficacy prediction

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INTRODUCTION

Advances in early screening and detection, as well as new therapeutic options, have reduced the breast cancer death rate over the past decade (SEER, 2012). Despite these advances, breast cancer remains the second leading cause of cancer death among women (SEER, 2012). This is due in part to the complexity of the disease which consists of multiple subtypes that are driven by different genetic mechanisms and that likely arise from different cell types (Hoadley et al., 2014). The breast cancer disease subtype heterogeneity determines prognosis and treatment options (Lehmann and Pietenpol, 2015; Prat et al., 2015). In particular, the subtypes that encompass triple negative breast cancers (TNBCs; i.e., estrogen receptor [ER]-negative, progesterone receptor [PR]-negative, and HER2-negative) have some of the worst outcomes. These TNBCs are predominantly of the basal-like and claudin-low subtypes (Prat et al., 2010; Prat et al., 2013). These two subtypes represent approximately 15% to 20% of all breast cancer cases, with the basal-like subtype showing unique genetic features, many of which it shares with serous ovarian cancers and lung squamous cancers (Hoadley et al., 2014). As a consequence of this, TNBC heterogeneity, preclinical drug discovery, and testing requires that multiple breast cancer models be employed to faithfully recapitulate the spectrum of the human disease, even when studying only one clinical TNBC disease subtype.

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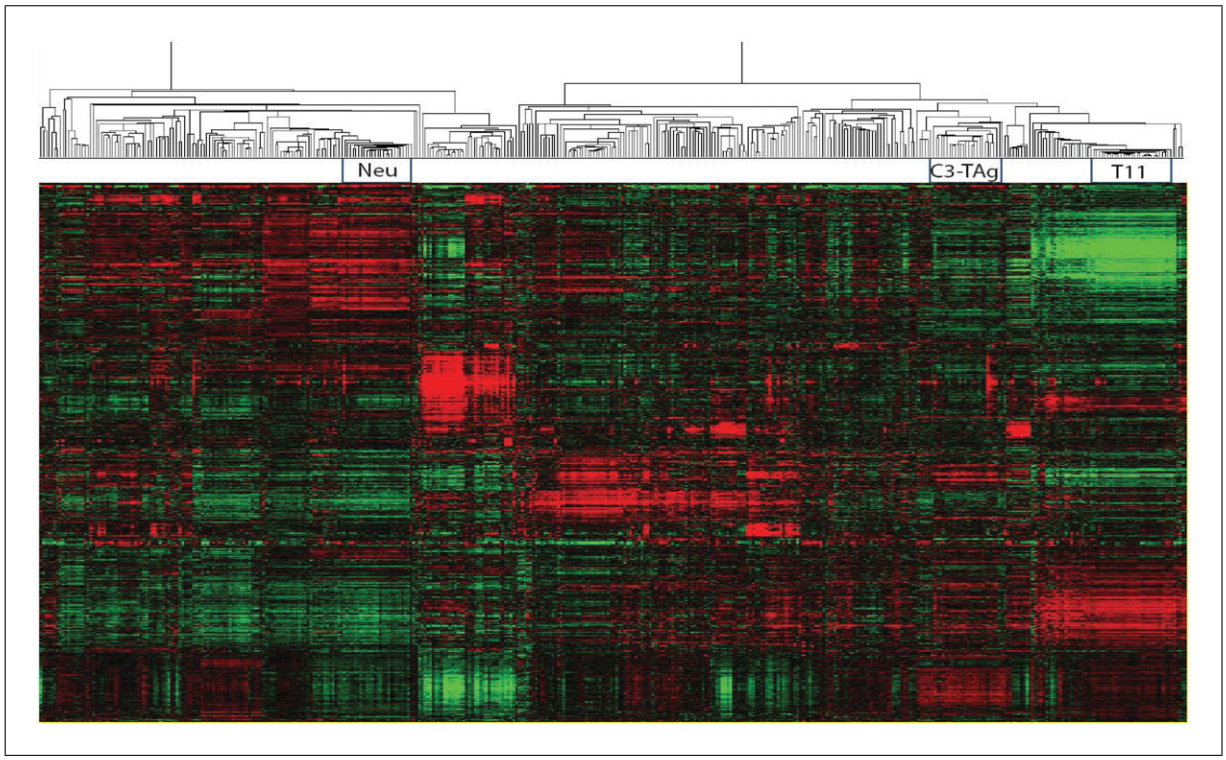

Figure 14.38.1 Hierarchical clustering of genetically engineered mouse models (GEMMs). Unsupervised clustering of mRNA expression levels reveals the stability of the GEMMs and orthotopic syngeneic transplant (OST) tumor murine models across time and across multiple microarray platforms. Each column indicates an individual microarray experiment of a single tumor, and each row represents an mRNA transcript with red indicating the highest, black indicating intermediate, and green indicating the lowest relative levels of expression. The Agilent arrays were done over a span of 12 years using 22,000-, 44,000-, and 180,000-feature array platforms. Microarrrays done on the three models described here are seen grouped together on the same dendrogram branches (identified), thus showing the robustness of the genomic analysis and of the biology of each GEMM model in question. Neu: luminal GEMM; C3-Tag: basal-like; T11: claudin-low subtype.

Several different in vivo models can be utilized to explore human breast tumors, including cell line-based xenografts (CDX), patient-derived xenografts (PDX), and genetically engineered mouse models (GEMMs) (Duncan et al., 2012; Howe et al., 2014; He et al., 2015). While CDX models have long been used in academic research and industry, they have many limitations. These include the strong selection process for the cell lines to grow first in vitro. While it is known that these cell lines maintain their cell type of origin signature, other features are altered including growth rates (Ross et al., 2000; Ross and Perou, 2001), and the clonal nature of the resulting CDX lines often lacks the heterogenic therapeutic response that is encountered in the clinic. The CDX models are usually established via subcutaneous inoculation of cells into the flank, which may limit normal tumor-stromal interactions. In addition, the use of immunocompromised mice eliminates the host immune system, which is becoming increasingly more important for cancer treatment with the advent of immune checkpoint inhibitors (Yao et al., 2014; Criscitiello and Curigliano, 2015; Karn et al., 2015). Due in part to these limitations, CDXs have not proven reliable as preclinical models for predicting responses in humans (Perrin, 2014). While PDX models may offer a more robust approach as the tumor cells have never been cultured in vitro, the development and maintenance of PDX models requires extensive infrastructure (Siolas and Hannon, 2013; Gao et al., 2015). The PDX models must also be grown in immunocompromised mice, yielding an altered microenvironment and limiting their utility in testing immunotherapies.

The GEMMs are robust models for testing hypotheses on tumor development, progression, interaction with the microenviroment, and potential therapeutic response. Multiple GEMMs have been engineered with conditional, often inducible and/or constitutively active mutant alleles (Pletnikov, 2009). As a result, many breast cancer GEMMs have been developed that reflect some of the diversity

of genetic lesions that are observed in the clinic (Herschkowitz et al., 2012; Pfefferle et al., 2013). However, it is often not obvious which human subtype a given GEMM most closely resembles, as common genetic alterations (e.g., TP53 loss) occur in multiple subtypes of human breast cancer. In an attempt to identify those models that most closely reflect human disease subtypes, we have profiled the gene expression of more than 27 different GEMMs of mammary carcinomas and compared the results to multiple human data sets (Herschkowitz et al., 2007; Herschkowitz et al., 2012; Pfefferle et al., 2013). Using this comparative genomics approach, we have identified murine gene expression counterparts of the basal-like and claudin-low subtypes as well as some GEMMs that display some tumor features of the human luminal subtype (Fig. 14.38.1).

GEMM CHARACTERIZATION

Based on genomic results and other parameters (e.g., histology and initiating transgene), three GEMMs were selected for multiple studies of therapeutic response (Table 14.38.1). Two of these are TNBC models, with the third being a model of the luminal tumor subtype. Representative histology of these models is displayed in Figure 14.38.2. Each model shows dense tumor cell morphology and general adenocarcinoma features, both of which are seen in high grade human breast tumors. Each GEMM faithfully recapitulates the gene expression characteristics of its human subtype counterpart, with the initiating transgene also being subtype consistent (Pfefferle et al., 2013).

C3-TAg

The FVB-Tg(C3-1-TAg)cJeg (C3-TAg) mouse from The Jackson Laboratory (strain 013591) contains a transgenic construct of the simian virus 40 (SV40) T-antigen under the promotion of the rat prostate binding protein (Maroulakou et al., 1994). This results in males developing prostate tumors at around 7 months of age. Female hemizygous transgenic mice develop mammary gland adenocarcinomas by 21 weeks of age with a penetrance of >90% and an average latency of palpable mass (~3 mm in diameter) of 19 weeks (Fig. 14.38.3). Untreated animals progress from initial tumor palpation to a terminal endpoint in an average of 30 days. The consistent high penetrance, short latency, and quick progression are positive attributes for work flow, colony management, and therapeutic testing.

The genetics driving tumorigenesis are highly relevant to the human basal-like disease (i.e., loss of TP53 and RB function). Using a cross-species genomics approach, murine expression profiles are compared to human breast cancers (see Herschkowitz et al., 2007; Pfefferle et al., 2013). The results of an unsupervised cluster analysis of the combined human and mouse gene expression profiling data set reveals gene signatures/modules with obvious shared features including a proliferation signature and patterns characteristic of each of the human subtypes (Pfefferle et al., 2013). The overwhelming majority (~90%) of C3-TAg strain tumors display characteristics of human basal-like breast cancer (BLBC; e.g., high proliferation and high expression of keratins 5 and 17 and P-Cadherin) that cluster together as a unique expression group (Fig. 14.38.1). Some 5% to 10% of C3-TAg tumors display the claudin-low subtype features and cluster with these tumors. Human BLBCs are RB1-deficient because of the loss of heterozygosity (LOH) of the RB1 loci, and BLBCs demonstrate the lowest average expression of RB1 mRNA (Herschkowitz et al., 2007). Studies of many cohorts have revealed that BLBCs exhibit a high frequency of p53 mutations or deletions (Sorlie et al., 2001; Carey et al., 2006; Troester et al., 2006). It is therefore not surprising that human BLBC is recapitulated by the mouse SV40 T-antigen

Table 14.38.1 Presented Genetically Engineered Mouse Models (GEMMs) of Breast Cancer

GEMM	Name	References	Median Latency	Source	Subtype
FVB-Tg(C3-1-TAg)	C3-TAg	Maroulakou et al., 1994	18.3 weeks	The Jackson Laboratory, strain 013591	basal
FVB/N-Tg(MMTVneu)	MMTV-Neu	Guy et al., 1992; Muller et al., 1996	variable, parous females 18 weeks	The Jackson Laboratory, strain 002376	luminal
OST *p53*$^{-/-}$	T11	Jerry et al., 2000; Herschkowitz et al., 2012	~10 days from inoculation	Baylor College of Medicine	claudin-low

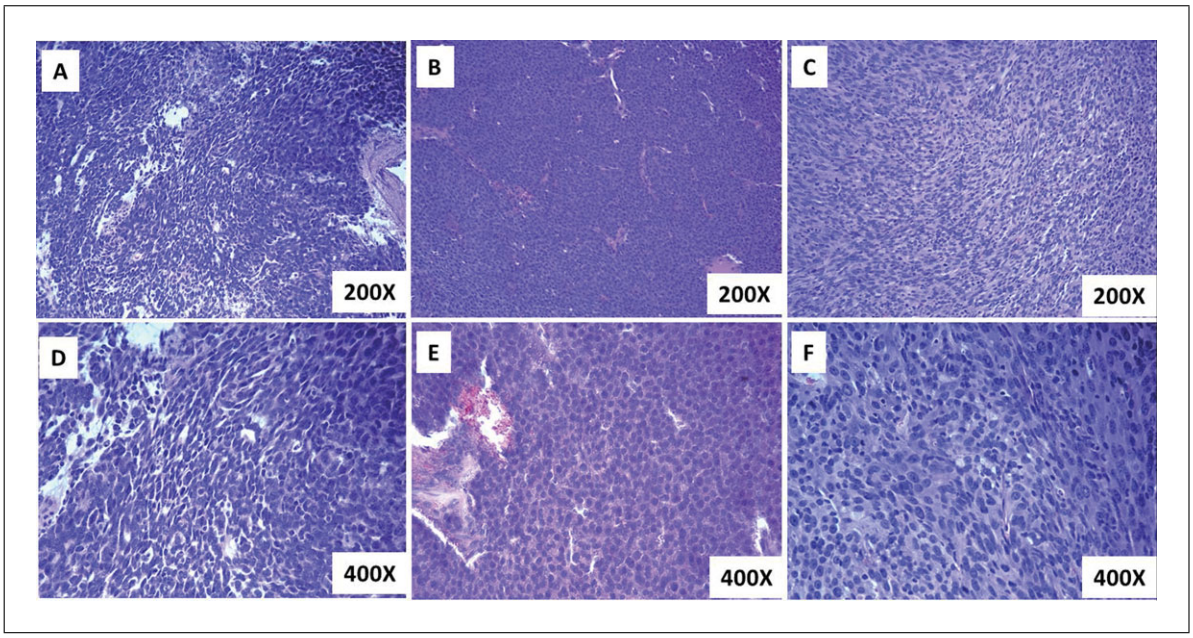

Figure 14.38.2 Murine tumor immunohistochemistry. Representative histology of H&E stained tissue sections from C3-TAg (**A** and **D**), MMTV-Neu (**B** and **E**), and T11 (**C** and **F**) murine tumors.

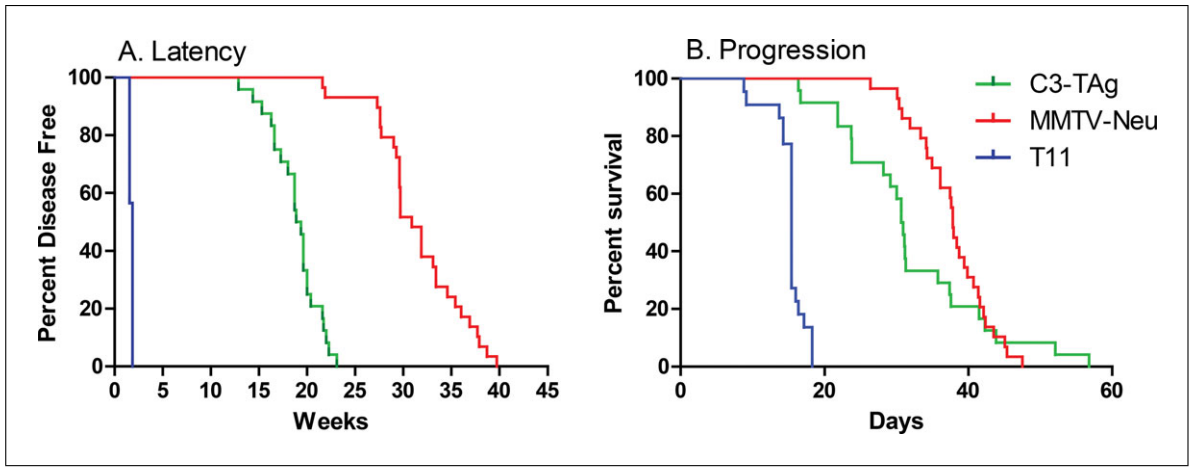

Figure 14.38.3 Latency and progression of three breast genetically engineered mouse models. (**A**) Latency is the time from birth, or inoculation in the case of the T11 model, to a palpable mass. T11 had the earliest latency with a mean of 12.6 days (range, 9 to 18 days). C3-TAg developed a mass on average at 19 weeks (range, 13 to 22 weeks). The MMTV-Neu, from a parous state, developed masses at 30 weeks (range, 16 to 52 weeks). (**B**) Progression is the time from palpable mass to terminal burden in days. The T11 was the most aggressive with a progression of 15 days, while the C3-TAg and MMTV-Neu grew more slowly with mean progressions of 30 and 37 days, respectively.

model C3-TAg, as this viral oncoprotein inactivates both RB1 and TP53. We observed a conserved DNA copy number gain in common between human BLBC and the C3-TAg model. Both human and mouse BLBCs show amplification and high expression of the K-RAS locus (Liu et al., 2001; Herschkowitz et al., 2007), as well as deletion of INPP4B (Herschkowitz et al., 2012) and amplification of nicastrin on human chromosome 1q21-23 (Silva et al., 2015). This unbiased credentialing has established the C3-TAg model as a faithful representation of human BLBC at both the gene expression and DNA somatic alteration levels.

MMTV-Neu

Mouse strain FVB/N-Tg(MMTVneu)202 Mul/J (MMTV-Neu; The Jackson Laboratory, strain 002376) expresses the c-NEU oncogene (the rat ortholog of human HER2) driven by the mouse mammary tumor virus (MMTV) promoter (Muller et al., 1996). Penetrance is >90% in females, with males being unaffected. The age at which disease presents depends on the breeding status of the animal.

Virgin females develop tumors later in life, while parous females have, on average, masses by 30 weeks of age. Similar to the C3-TAg, the MMTV-Neu model shows consistent colony management characteristics with consistent penetrance, latency times, and tumor growth characteristics. The MMTV-Neu allele has the additional benefit of having no deleterious effects if maintained in a homozygous state. Gene expression profiling has shown that this model represents a human luminal subtype, with a high expression of XBP1, a human luminal tumor-defining gene (Gruvberger et al., 2001). These tumors also express tight junction structural component genes, including occludin, tight junction proteins 2 and 3, and the luminal cell marker K8/18; however, they fail to express estrogen receptors and other estrogen responsive genes (Herschkowitz et al., 2007; Pfefferle et al., 2013). Nonetheless, its clear overall luminal features (Fig. 14.38.1), consistent latency, and easy colony management make it an ideal GEMM for repeated therapeutic testing.

p53 Null "T11"

This orthotopic syngeneic transplant (OST) model originates from a mouse with a germline homozygous deletion of p53 (Jerry et al., 2000). Animals deficient in p53 develop spontaneous thymic lymphomas in early life. However, when donor $p53^{-/-}$ mammary epithelium from young animals are transplanted into the cleared mammary fat pad of recipient syngeneic (*BALB/c)* females, tumors develop with a latency of 6 to 12 months (Jerry et al., 2000). These murine syngeneic transplants can be propagated through serial passage, with the individual tumor phenotypes being consistent over time in the large majority of tumors (Herschkowitz et al., 2012). Therefore, OSTs offer the convenience of a cell line, allowing the user to manipulate workflows as needed by inoculating only those animals required for a given experiment while leaving the animal fully immunocompetent. The particular OST line we have used the most is "T11" line. It is available for collaborative research through Baylor College of Medicine. This OST line displays a take rate of >99% and a 12-day latency from inoculation to palpable mass. A characteristic of this model is the aggressive nature of tumor progression, much like that seen in the human subtype it represents (i.e., claudin-low; Prat et al., 2010). Once a palpable mass is observed, the tumor progresses to a terminal burden within 18 days. The tumors develop rapidly and have a propensity to invade the peritoneal cavity.

While many of these $p53^{-/-}$ tumors were generated, 50 that display a variety of histological phenotypes and molecular subtypes were selected for further characterization. A subset of approximately 10% was identified as being the murine equivalent to human claudin-low tumors (Herschkowitz et al., 2012). These had low or absent CLDN3 and CDH1 expression and high expression of epithelial-mesenchymal transition (EMT) markers, such as SNAI1 and TWIST1, similar to the human claudin-low subtype (Prat et al., 2010). The tumor line chosen for subsequent research was Tumor 11 or "T11" (Fig. 14.38.2).

TRANSLATIONAL APPLICATION OF GEMMs

UNC Lineberger Mouse Phase 1 Unit and "Co-clinical" Trials

The Mouse Phase I Unit (MP1U) at the University of North Carolina was founded in 2005 to improve the predictive value of preclinical animal models. Initially relying on private and State of North Carolina funding, the MP1U capitalized on advances in murine genetics as well as changes in the intellectual property landscape (Hanahan et al., 2007) to begin medium-throughput efficacy testing of potential anticancer drugs in GEMMs. Work in the MP1U relies exclusively on faithful GEMM and OST lines as described above. The MP1U harnesses the capabilities of murine genetics to produce single and/or multi-allelic cancer models featuring autochthonous tumors with physiological tumor-stroma interface in immunocompetent hosts. Promising anticancer compounds are tested alone and in combination with other agents in these models using cohort sizes (10 to 20 animals per treatment arm) that provide for rigorous statistical analysis and reliable results. Tumor response and progression are assessed serially, including novel imaging approaches when needed, using criteria similar to human Response Evaluation Criteria in Solid Tumors (RECIST; Nishino et al., 2010). The median growth rate of C3-TAg and MMTV-NEU tumors over three consecutive time periods are presented as evidence of the consistency of these models (Fig. 14.38.4A and B). These data, which were acquired over many years and by multiple laboratory technicians, highlight the consistency of these models, which is critical for performing therapeutic studies over time when comparing multiple compounds against each other.

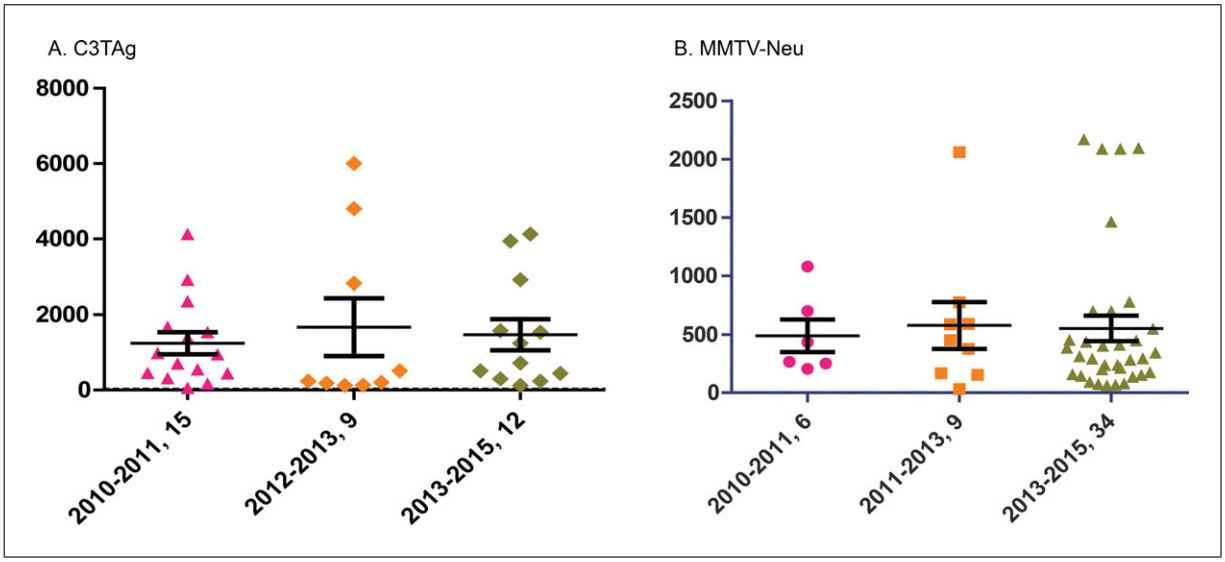

Figure 14.38.4 Model Fidelity. The percent increase in tumor size from initial time of detection to after 14 days for the (**A**) C3-TAg and (**B**) MMTV-Neu models. Data are compared between three time periods (2010-2011, 2011-2013, and 2013-2015), with each label showing the number of mice per group, which spans multiple technicians, and thus highlights the consistency of these models over time.

For our therapeutic testing paradigm, an agent is considered effective only if it induces tumor regression rather than only tumor growth inhibition. Compound dose, schedule, and exposure are monitored by pharmacokinetic and pharmacodynamic assessments. Although the MP1U performs only high-level toxicological analyses, testing in the MP1U is otherwise nearly identical to human Phase I/II trials, while also taking advantage of the powerful experimental features of murine tumor models. For each regimen tested, a rolling Phase I/II design is employed, including dose finding followed by a randomized Phase II study. With animal models, ideal control groups of either no treatment or treatment with the standard of care are included, as are precise measurements of changes in tumor size. Previous groups have identified the value of establishing "co-clinical" trials, where validated in vivo models are used to expedite drug discovery and to identify sensitive sub-populations of patients (Nardella et al., 2011; Chen et al., 2012; Lunardi et al., 2013). Outlined below are studies using these three models and the methodologies employed for addressing issues of clinical relevance.

Individual Drug and Combinatory Drug Testing

Although cyclin dependent kinase (CDK) inhibitors benefit many patients, there are many kinases associated with the cell cycle pathway, each of which may affect a different point in the cell cycle (Dickson and Schwartz, 2009). While patients administered CDK1 and CDK2 inhibitors experience adverse events (Sausville et al., 2014), CDK4/6 inhibitors are well-tolerated by a select population of patients who respond well to these therapies (Turner et al., 2015). For example, the CDK4/6 inhibitor PD-0332991 (palbociclib) has displayed efficacy in ER+ breast cancer patients receiving an aromatase inhibitor (Mayer, 2015). This agent has received conditional approval from the FDA as a treatment for metastatic breast cancer (Beaver et al., 2015).

Using GEMMs, we have shown minimal efficacy for palbociclib in $p16^{INK4a}$-null melanoma (Roberts et al., 2012b) and the basal-like breast model C3-TAg, but significant effectiveness in the luminal breast cancer model MMTV-Neu (Roberts et al., 2012a). Based on the known genetics of these models, this lack of efficacy in the C3-TAg and $p16^{INK4a}$-null melanoma GEMM was predicted (Roberts et al., 2012b). However, there is significant inhibition of the anti-tumor activity of palbociclib when it is administered concurrently with carboplatin in the MMTV-Neu model. This inhibition of activity was not observed in the C3-TAg model when carboplatin was administered with palbociclib. In the C3-TAg model, the concurrent administration reduced the myelosuppression activity of carboplatin significantly. Given these and other findings, it appears likely that CDK4/6 inhibitors have anticancer potential and might also be useful as preventative therapy for adverse bone marrow effects inasmuch as the

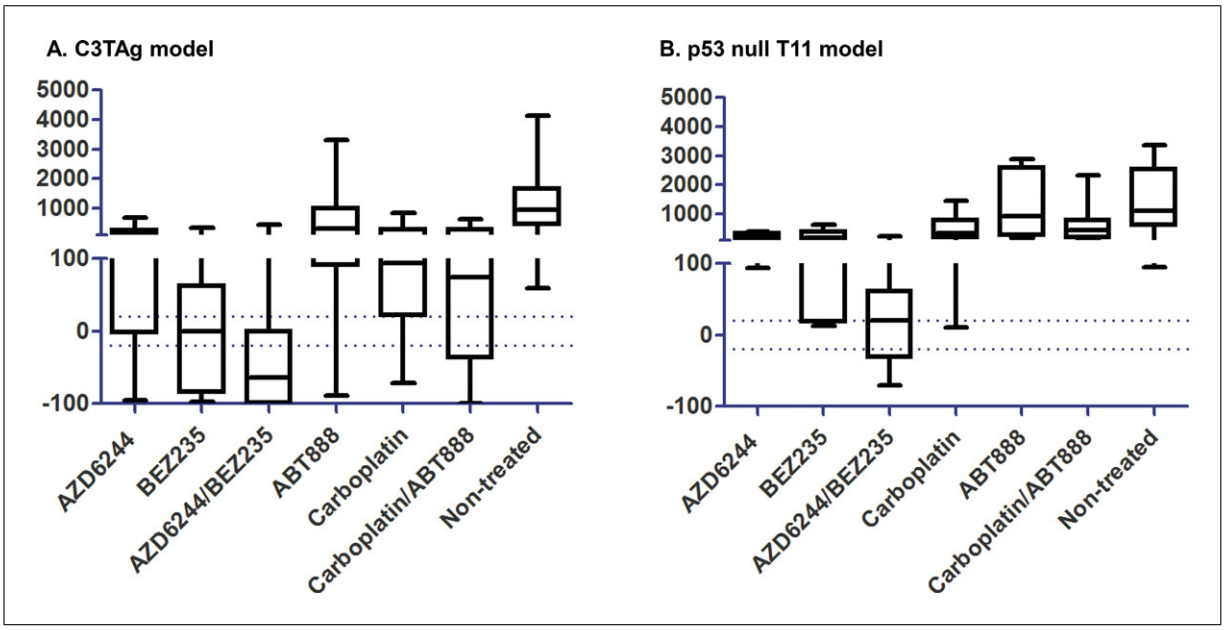

Figure 14.38.5 Treatment Response. Fourteen-day tumor response of triple negative breast genetically engineered mouse models. (**A**) Dotted lines show stable disease cutoff points. Of 155 C3-TAg mice treated, AZD6244/BEZ235 achieved an objective response (stable + regressive disease) in 17 of 20 animals, which was the highest percentage of any treatment assessed. (**B**) Of 99 T11 mice treated, 7 of 13 achieved an objective response when treated with AZD6244/BEZ235.

CDK4/6 inhibitors cause hematopoietic stem cell dormancy which is immediately reversed upon cessation of therapy (Roberts et al., 2012a).

Combinatorial treatment with novel agents can also be tested in GEMMs to determine broad anticancer properties. Combinatorial therapies are particularly challenging to test in the clinic as there are often unpredictable and toxic side effects associated with multiple drug treatments. As the GEMM setting provides an in vivo environment with all normal host tissues present, the potential toxic effects of drug candidates or candidate combinations can be identified and possibly addressed by changes in dosing regimens. In a recent study (Usary et al., 2013), we analyzed more than 600 breast cancer GEMM tumors for response to a range of therapeutics focused on MEK, mTOR, and PIK3CA/mTOR inhibitors. Therapy assessment was performed in more than 300 C3-TAg mice (Fig. 14.38.5), a number of subjects that is difficult to achieve using PDX models. Of the regimens assessed, the combination of an MEK (AZD6244) and a PI3K/mTOR (BEZ235) inhibitor resulted in tumor regression in a significant fraction of animals, more than doubling overall survival (from 4.5 weeks to 8 weeks). Likewise, this combination was equally effective in the claudin-low T11 model, again doubling overall survival (2 weeks to 4.5 weeks) and providing a significant improvement in overall response rates. It was noted, however, that this combination is associated with toxic side effects (weight loss). Several months of testing of different dosing regimens were needed to determine the optimum dose and dosing interval.

Another advantage of GEMMs is the ability to study, and potentially reverse, acquired resistance. To this end, we first studied the response of MMTV-Neu models to lapatinib, a compound to which this model is exquisitely sensitive (Roberts et al., 2012b). All tumors in this model initially responded to lapatinib, a potent HER2 inhibitor, with a few eventually progressing after 100 to 200 days on therapy. Tumors were harvested from mice with acquired lapatinib resistance and transplanted into mammary fat pads of syngeneic recipients. Mice bearing these tumor grafts were then re-challenged with lapatinib or other agents, such as AZD6244 or BEZ235. Tumors transplanted in this way remained refractory to lapatinib re-challenge, but were highly responsive to the MEK/PI3K/mTOR inhibitor combination (Fig. 14.38.6). The overall survival of treated mice went from 4 weeks to 16 weeks. Thus, through the use of serial transplantation on syngeneic backgrounds, resistant tumors can be selected for, further propagated, and then tested with new agents to identify compounds that are active against tumors resistant to common treatments.

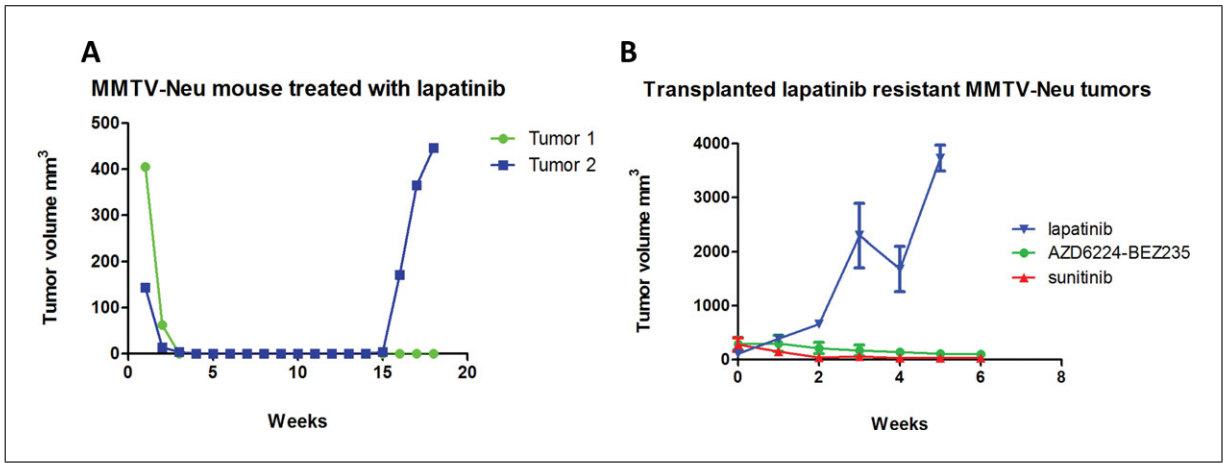

Figure 14.38.6 Development and re-challenge of MMTV-Neu lapatinib-resistant tumors. (**A**) Treatment of an MMTV-Neu mouse with lapatinib (220 mg/kg) resulted in tumors that shrank to a size nearly undetectable. After extended treatment, one of the tumors developed a resistant phenotype and rapidly regrew. (**B**) When the resistant tumor was serially transplanted into multiple syngeneic background (FVB) mice, the tumors remained resistant to lapatinib but were susceptible to other tyrosine kinase inhibitors.

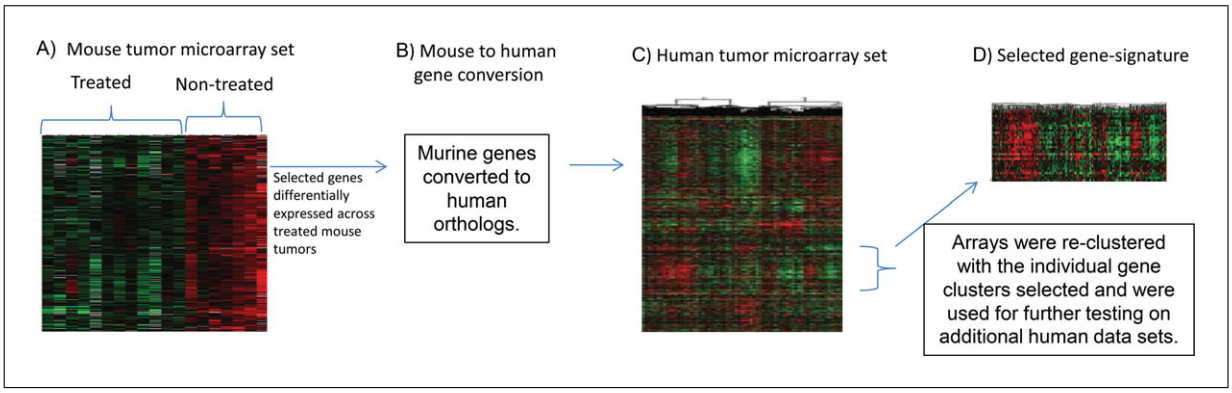

Figure 14.38.7 Predicting drug efficacy in human patients. (**A**) Differentially expressed murine genes were identified and (**B**) were converted their human orthologs. (**C**) These gene sets were used to cluster microarrays of human patient tumors, and (**D**) gene signatures were derived from sets of the genes that were most homologously expressed. The resulting gene signatures were tested against patient response data to find the ones that provided predictive power.

Predicting Compound Efficacy

Success has also been attained using GEMMs and OSTs to develop gene expression-based predictors of response to anticancer drugs in human patient tumors (Usary et al., 2013). After testing a number of therapeutic treatments, a model and regimen were selected that displayed the strongest heterogeneity of response to a single treatment. The C3-TAg model demonstrated a strongly heterogeneous response to treatment with carboplatin and paclitaxel, with one-third of tumors showing regression and two-thirds showing tumor growth (Usary et al., 2013). Microarray analysis revealed a set of genes that were differentially regulated in the untreated mice relative to the treated mice (Fig. 14.38.7). The murine genes were converted to their human orthologs which were then used to cluster a human breast cancer microarray set. This mouse to human "filtering" process is required because homogenous gene lists derived from murine tumors or cell line sources typically fragment into smaller gene sets when analyzed on human tumors. Individual dendrogram node gene sets were then selected and tested. These contained highly homogenously expressed gene sets for predictive value in a set of patients that had received taxane- and anthracycline-containing neoadjuvant chemotherapy. One of these gene sets predicted a pathological complete response (pCR) across all tumor types and even within the basal-like subtype and the triple-negative clinical grouping. Multivariate analysis revealed that the originally murine-derived gene signature provided predictive information for pCR beyond the commonly used clinical variables and breast tumor subtype. These results indicate that murine-derived gene signatures can

predict response in human patients after accounting for other clinical variables and suggest that these GEMMs may be useful in identifying biomarkers for human breast cancer.

SUMMARY

Drug discovery in oncology has historically been predicated on in vitro cell line models and/or cell lines grown in immunocomprised mice. However, these models are burdened by a number of limitations including limited genetic complexity, artificial tumor-host stroma interaction, and lack of a host immune response. GEMMs are useful alternative models that overcome these shortcomings and, in conjunction with CDXs and PDXs, provide the strong preclinical validation needed to move drug candidates into the clinic and to provide information on which subsets of patients may benefit from targeted therapies. Previous work in the UNC MP1U has shown that genomically selected GEMMs faithfully recapitulate specific human disease counterparts that represent important breast cancer subtypes. Furthermore, the phenotype (tumor growth, latency, and breeding) of these GEMMs allows for easy colony management and reduces the infrastructure demands that a similarly sized PDX colony requires. As the host immune system becomes increasingly important in mediating the therapeutic response to cancer chemotherapeutics, GEMMs will provide the models of choice for identifying and characterizing new drug candidates.

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Conflict of Interest Statement

C.M.P is an equity stock holder and Board of Director Member of BioClassifier, LLC and GeneCentric Diagnostics. C.M.P is also listed as an inventor on patent applications on the Breast PAM50 and is a consultant for G1 Therapeutics.

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