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Genetically Engineered Mice as Experimental Tools to Dissect the Critical Events in Breast Cancer

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Abstract

Elucidating the mechanism of pathogenesis of breast cancer has greatly benefited from breakthrough advances in both genetically engineered mouse (GEM) models and xenograft transplantation technologies. The vast array of breast cancer mouse models currently available is testimony to the complexity of mammary tumorigenesis and attempts by investigators to accurately portray the heterogeneity and intricacies of this disease. Distinct molecular changes that drive various aspects of tumorigenesis, such as alterations in tumor cell proliferation and apoptosis, invasion and metastasis, angiogenesis, and drug resistance have been evaluated using the currently available GEM breast cancer models. GEM breast cancer models are also being exploited to evaluate and validate the efficacy of novel therapeutics, vaccines, and imaging modalities for potential use in the clinic. This review provides a synopsis of the various GEM models that are expanding our knowledge of the nuances of breast cancer development and progression and can be instrumental in the development of novel prevention and therapeutic approaches for this disease.

1. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among American women, excluding skin cancers, and is the second leading cause of cancer-related deaths among women. It is estimated that one in eight women in the United States will develop invasive breast cancer in their lifetime (<http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-key-statistics>). Understanding the molecular basis of the initiation and progression of breast cancer is essential to developing a cure for this disease as well as for producing vaccines or other approaches to prevent the disease. Although significant progress has been made with

respect to understanding breast cancer progression and spread, treatment options are still limited once metastasis has occurred.

Animal models have been used for decades to help determine causes, and provide platforms for testing therapies and potential cures for human diseases. The first genetically engineered mouse (GEM) model, a transgenic mouse engineered to express HSV (herpes simplex virus) thymidine kinase, was developed in 1981 (Brinster et al., 1981), and multiple GEM models of breast cancer were generated shortly thereafter (e.g., Muller, Sinn, Pattengale, Wallace, & Leder, 1988; Sinn et al., 1987; Stewart, Pattengale, & Leder, 1984; Tsukamoto, Grosschedl, Guzman, Parslow, & Varmus, 1988). As our understanding of the various genetic alterations involved in cancer has progressed, GEM models, including both transgenic and knockout models, have become essential tools for defining the role of these genetic alterations in cancer initiation, progression, and metastasis. These models allow not only for determining the consequences of alterations in individual genes of potential relevance but also for determining the mechanisms of cooperation between multiple genes in tumorigenesis. Interestingly, many of the genes that are altered in human breast cancer are also found to promote the development of mammary tumors in GEMs (Cardiff, 2003), allowing for the creation of genetically accurate models of this disease. Such models are useful both for understanding the pathogenesis of breast cancer, as well as for evaluating novel therapeutic approaches that may target specific genetic alterations or pathways and strategies for the prevention of breast cancer.

Breast cancer is not a single disease, but is a heterogeneous family of diseases with diverse genetic and histopathologic characteristics and varying clinical outcomes. It would therefore be naïve to expect a single animal model to fully recapitulate all aspects of breast cancer. In fact, a wide variety of approaches have been utilized to model breast cancer in the mouse, including the use of spontaneous and carcinogen- or virally-induced models, xenograft models, and most recently GEMs (Cardiff & Kinney, 2007). The use of chemical carcinogens to induce mammary tumors in rodents has proven useful in the genetic analysis of initiation and progression of mammary tumors (Sukumar, McKenzie, & Chen, 1995) and for prevention studies, and xenograft models have been widely used for studying various aspects of disease progression and for evaluating therapeutic approaches. However, these approaches present drawbacks that can be addressed, at least in part, by the use of GEM models (Borowsky, 2011; Vargo-Gogola & Rosen, 2007). The development of GEMs provided a new avenue for understanding the events involved in cancer initiation and progression, and for creating genetically accurate models of human breast cancer. Vast arrays of GEM breast cancer models are currently available that represent different facets of this heterogeneous disease. Constitutive tissue-specific expression of oncogenes and global or tissue-specific knockout of tumor suppressor genes has produced tumor-prone mice that are useful for delineating the underlying molecular mechanisms of breast cancer, as well as for aiding in the design and investigation of treatment options for this disease. GEM models are also useful in the development of novel imaging modalities to detect tumors, particularly, during early stages, as well as metastases. Given the number of GEM models available today, it is important to select a model appropriate for the question being addressed or the breast cancer subtype one is endeavoring to understand. An informative set of reviews on the mammary gland as an experimental model (Bissell, Polyak, & Rosen, 2011) and a

thorough history of the many experimental approaches to studying mammary tumor biology in mice (Cardiff & Kinney, 2007) have been published. Additional reviews have described and categorized many of the current transgenic models of breast cancer (e.g., Borowsky, 2011; Cardiff et al., 2000; Ottewill, Coleman, & Holen, 2006; Vargo-Gogola & Rosen, 2007). In this review, we will examine some of the prominent transgenic models that are proving invaluable to our understanding of the molecular processes of breast cancer, how these models have advanced the evaluation of experimental therapies, as well as current approaches in the application of these models in the development of small molecule inhibitors and other approaches for the treatment and prevention of breast cancer.

1.1. Development of the mouse mammary gland and human breast

The mouse mammary gland undergoes three distinct phases of proliferation: embryonic, at puberty and during pregnancy-lactation. Mouse mammary gland development begins shortly after mid-gestation. The milk line or the bilateral epidermal thickening, that runs from the neck to the inguinal regions, forms the base on which five placodes develop. These placodes later invaginate to form the nipples. As the mouse develops, limited branching occurs in these early structures to form the rudimentary ductal tree (Figure 8.1) (Hens & Wysolmerski, 2005; Robinson, 2007). The mammary glands become more extensively branched at puberty when hormone-dependent events activate the formation of terminal end buds and more primary ducts in a radial pattern. Alveolar growth and differentiation occurs during pregnancy and lactation, and glandular regression follows lactation (Watson & Khaled, 2008). In contrast, humans have two breasts that develop from the milk buds on the anterior chest wall. Details of the human breast development from fetal to adult life have recently been reviewed (Gusterson & Stein, 2012; Russo & Russo, 2004). While the complexity of branching and molecular details of development is different in humans and mice, both species have mammary glands that grow mainly from the terminal end buds. These buds contain undifferentiated progenitor cells or stem cells and eventually give rise to milk-producing lobuloalveolar (LA) structures in mice, or the terminal ductal lobuloalveolar units (TDLU) in humans (Cardiff & Wellings, 1999). In most cases, breast cancer originates from these lobules or ducts of the mammary glands (Dimri, Band, & Band, 2005).

The mouse mammary gland offers several distinct advantages for modeling human breast cancer, despite many of the above-mentioned differences in their development. The mouse mammary gland has been extensively studied in the context of normal development, lactation changes, and in development and characteristics of spontaneous tumors. The mouse mammary gland is also transplantable into syngeneic or immunodeficient athymic mice, providing an extra platform to separately study individual tumors formed from the multifocal neoplasia in mice (Caligiuri, Rizzolio, Boffo, Giordano, & Toffoli, 2012). Additionally, there are several mammary-specific gene promoters available that assist in engineering mammary-specific target gene expression.

1.2. Genetically engineering mouse models of breast cancer

Several approaches have been used to genetically engineer mouse models of breast cancer (Borowsky, 2003). The first approach is to create transgenic mice in which a gene of interest (e.g., an oncogene or reporter gene) is introduced into the germline of the mouse genome *via*

random insertion, by directly injecting DNA into one of the two pronuclei of a fertilized egg (Cho, Haruyama, & Kulkarni, 2009; Voncken, 2011). This approach has been used to create transgenic mice expressing a wide range of oncogenes under the control of various mammary-specific or -selective promoters (discussed in Section 1.3), resulting in lines of mice that develop mammary tumors with varying latencies and properties. The second approach is to create knockout mice in which a gene of interest has been inactivated, an approach that is, particularly, suited for investigating the role of tumor suppressor genes in breast and other cancers. Knockout mice are generated by introducing a targeting vector encoding a modified, nonfunctional version of the gene of interest into mouse embryonic stem (ES) cells and selecting clones in which the targeting vector has integrated into the genome by homologous recombination, thereby replacing one of the wild-type alleles. The resulting ES cells are then used to generate chimeric mice, and eventually mice carrying the targeted allele in the germline in either a heterozygous or homozygous state (Hughes & Saunders, 2011). Global inactivation of tumor suppressor genes can in some cases model specific human hereditary tumor syndromes, for example, Li-Fraumeni syndrome, in which patients have inherited a mutant p53 allele and develop a range of tumor types, including breast cancer (Donehower et al., 1992; Malkin, 2011). However, most tumor-associated mutations arise somatically and are restricted to only the tumor cells in the patient. Further, global inactivation of some genes in mice results in embryonic lethality, thereby precluding analysis of the effects on tumorigenesis. Thus, in some cases it is preferable to create conditional knockout mice in which the gene of interest is modified in the germline, but becomes inactivated only in somatic cells in a fashion that can be both tissue specific and temporally regulated. The most well-established approach for generating tissue-specific conditional knockouts employs the Cre/loxP recombinase system (Lewandoski, 2001). LoxP sites are 34 bp elements from the P1 bacteriophage that are specifically recognized by the phage Cre recombinase enzyme. Using the gene targeting approach described above, “knock-in” mice can be generated in which two loxP sites have been inserted into intronic (or promoter) regions flanking critical exons in the gene of interest, creating a “floxed” allele, which remains functionally wild-type despite the presence of the two loxP sites. However, when these mice are crossed with transgenic mice expressing Cre recombinase under the transcriptional control of a tissue-specific promoter, Cre-mediated recombination results in deletion of the sequences between the two loxP sites, creating a knockout allele, but only in the tissues in which Cre is expressed. Tissue-specific inducible conditional knockout mice can be obtained by crossing mice carrying a floxed gene of interest with mice expressing Cre-ER, a fusion protein in which Cre is linked to a mutant estrogen receptor ligand binding domain. In these models, spatial control again comes from the promoter driving Cre-ER expression, while temporal control is mediated by administration of Tamoxifen, since the fusion protein can only translocate to the nucleus when bound to Tamoxifen (Friedel, Wurst, Wefers, & Kuhn, 2011). Similarly the tetracycline-inducible (Tet-on/Tet-off) systems can be utilized for inducible expression or silencing of a gene of interest (Fantozzi & Christofori, 2006; Saunders, 2011). More detailed information of various strategies for generating GEM models can be found elsewhere (Hofker & van Deursen, 2011; Pease & Saunders, 2011).

1.3. Mammary-specific promoters

Significant progress in the development of transgenic mouse models to investigate mammary tumorigenesis has been enabled by the identification of mammary-specific or -selective promoter elements (Table 8.1). The most widely used regulatory element for inducing mammary-selective transgene expression is the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). Several strains of *Mus musculus* (BR6, C3H, GR, RIII) develop spontaneous mammary tumors due to a high incidence of MMTV in the mice. MMTV is a nonacute transforming retrovirus that induces tumors by insertional mutagenesis in the DNA of the host genome, causing deregulated expression of adjacent cellular genes. This highly infectious virus is transmitted congenitally through the milk to the offspring of the mice of these strains (Asch, 1996). The MMTV LTR contains response elements for glucocorticoids, androgens, and progesterone, which are regulated by the estrous cycle in virgin mammary glands. The expression of genes adjacent to the LTR is highly upregulated during pregnancy, and peak during lactation. Transcription from the MMTV LTR also results in lower level of transgene expression in a wide range of other tissues, including the salivary glands and the male accessory glands (Wagner et al., 2001).

The promoter of the gene encoding the milk serum protein, whey acidic protein (WAP), is also commonly used for directing expression of transgenes to the mammary gland. The WAP promoter is hormonally regulated by prolactin, hydrocortisone, estrogen and insulin, and strongly activates mammary-specific expression of genes during mid-pregnancy and lactation, although it also directs low levels of expression in other tissues as well (Wen, Kawamata, Tojo, Tanaka, & Tachi, 1995).

The ovine BLG (beta-lactoglobulin) promoter is another mammary-specific promoter that has been used to generate transgenic mice (Webster, Wallace, Clark, & Whitelaw, 1995). BLG is hormonally regulated by prolactin, estrogen, hydrocortisone and insulin, and transgenes under the transcriptional regulation of the BLG promoter are induced during pregnancy and lactation earlier than transgenes expressed from the WAP promoter. However, transcription from the BLG promoter is not very tightly regulated, as a low level transgene expression is observed in virgin animals.

Additional promoters that have been used to direct oncogene expression in mammary tumor models include the 5'-flanking region of the C3(1) subunit of the rat prostatic steroid-binding protein, and the mouse metallothionein 1 (MT) promoter. The C3(1) promoter has been shown to target transgene expression to the epithelium of both the mammary and prostate glands (Green et al., 2000). Unlike the promoters described above, expression from this promoter is not influenced by pregnancy, and thus some of the artificial effects of pregnancy on MMTV- and WAP-driven transgenic models may be avoided with this promoter (Green et al., 2000; Yoshidome, Shibata, Couldrey, Korach, & Green, 2000). The mouse MT promoter directs expression to most epithelial cells, including those in the mammary gland, and is activated by heavy metals such as zinc and cadmium (Palmiter, Sandgren, Koeller, & Brinster, 1993).

Transgenic mouse tumor models in which oncogene expression is driven by the MMTV, WAP, or other pregnancy/lactation-dependent promoters are sometimes referred to as

“artificial” models, and the extent to which they mimic the tumor biology of human breast cancer has been the center of considerable scientific debate (Borowsky, 2011; Vargo-Gogola & Rosen, 2007). In humans, pregnancy is often associated with a reduced risk of breast cancer (Britt, Ashworth, & Smalley, 2007; Russo, Moral, Balogh, Mailo, & Russo, 2005). In contrast, pregnancy generally promotes tumorigenesis in transgenic mouse models in which expression of an oncogene is under the control of the MMTV LTR or WAP promoter, due to direct activation of the promoters by pregnancy-associated hormones, resulting in increased expression of the oncogene. These models are thus not generally well suited for studies of the mechanisms mediating hormonal effects on breast tumor initiation or progression. Additional caveats with transgenic mouse models driven by any of the promoters mentioned above include potential differences in the specific subpopulations of mammary epithelial cells targeted by these promoters, as compared to the tumor-initiating cell types in human breast cancer, and artificially high levels of oncogene expression that may be driven by these promoters. “Knock-in” models, in which an oncogenic version of a gene replaces the wild-type protooncogene in its normal genomic locus and under the transcriptional regulation of its natural promoter, can potentially alleviate some of the concerns of MMTV- or WAP-driven breast tumor models (Andrechek et al., 2000). However, despite the potential artifacts and caveats of the conventional transgenic models, they have contributed substantially to a better understanding of the genetics and biology of breast cancer and provide powerful tools for the evaluation of targeted therapeutic approaches for the treatment of breast cancer.

2. MODELING VARIOUS ASPECTS OF HUMAN BREAST CANCER INITIATION AND PROGRESSION IN MICE

Breast cancer is a multifactorial disease (Figure 8.2). The hallmarks of cancer as defined by Hanahan and Weinberg describe the various biological traits acquired during the multistep development of tumors (Hanahan & Weinberg, 2000, 2011). Cancer cells gain attributes that allow them to sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, gain replicative immortality, induce angiogenesis, resist cell death, avoid immune destruction, and deregulate cellular energetics. Insight into the mechanisms that allow cancer cells to acquire such abilities can be teased out using GEM models.

Several genes have previously been identified as tumor suppressors or oncogenes in breast cancer. The cancer genome atlas network recently analyzed comprehensive molecular changes occurring in human breast tumors (Koboldt et al., 2012). Mutations in a number of genes such as *PIK3CA*, *PTEN*, *AKT1*, *TP53*, *GATA3*, *CDH1*, *RBI*, *MLL3*, *MAPK1*, *CDKN1B*, *RUNX1*, and *NF1* have been implicated to play a role in breast cancer. A number of germline variants such as *ATM*, *BRCA1*, *BRCA2*, and *PTEN* also predispose humans to breast cancer. Also contributing to the development of many breast cancers is the enhanced expression of protooncogenes such as human epidermal growth factor receptor (EGFR) family members (HER2 or NEU). Such altered gene expression results in an abnormal cellular signaling and protein signature of the tumor. In theory, any gene that might play a role in breast cancer can be evaluated using mammary-specific transgenic or knockout animals. As such, many of the current mouse models aim to target key molecular events that are critical mediators of the overall cancer phenotypes such as cell growth, cell cycle

progression, survival, apoptosis, migration, invasion, metastasis, angiogenesis, and drug resistance. GEM models that target a single transgene such as an oncogene, tumor suppressor, growth factor and/or receptor, or combinations of the above, have been developed to gain a better understanding of the role of the transgene/s in breast tumorigenesis. Although the number of breast cancer GEM models continues to expand, no single model is currently able to completely replicate the complexity of the genetic and molecular deregulation of human breast cancer.

2.1. Modeling effects of oncogenes on cell proliferation, survival, and apoptosis in breast cancer

Cancer cells overexpress/downregulate molecules that regulate cell proliferation by functioning at multiple levels, including transcriptional, translational, and/or posttranslational, to activate the cascade of cyclins and cyclin-dependent kinases and entry into the cell cycle. The tumorigenic potential of cancer cells is frequently enhanced through the synchronized expression of molecules that promote survival and permit evasion from apoptotic death. The following transgenic models will be discussed in this section: the protooncogenes *c-myc* and *v-Ha-Ras*, the cell cycle regulator cyclin D1, the growth factor TGF α , and the viral SV40 T antigen.

2.1.1 c-Myc transgenic models—The protooncogene *c-myc* encodes a 62-kDa multifunctional, nuclear phosphoprotein, and is located on chromosome 8q24.21 in humans. *c-Myc* functions as a transcription factor that activates expression of a number of genes required for cell cycle progression from G1 to S phase. Paradoxically, *c-Myc* expression can also strongly promote apoptotic cell death. *c-Myc* expression increases during pregnancy-related proliferation in the normal mammary gland. However, it is absent in differentiated alveolar cells during lactation, but increases again during the normal apoptotic mammary involution process (Liao & Dickson, 2000). In human tumors, deregulation of *c-myc* expression may occur *via* multiple mechanisms. The *c-myc* gene is amplified in about 16% and rearranged in about 5% of human breast cancers, while the *c-Myc* protein is overexpressed in 45–70% of human breast cancers (Blackshear, 2001; Deming, Nass, Dickson, & Trock, 2000; Spandidos, Field, Agnantis, Evan, & Moore, 1989). Amplification of *c-myc* correlates with poor patient prognosis (Deming et al., 2000).

Activation of *c-myc* has been implicated as a critical step in the development of mammary carcinoma in both humans and mice. The Leder laboratory developed MMTV-*c-myc* transgenic mice that express *c-myc* specifically in the mammary glands at high levels in female mice, and in the salivary glands of both males and females at lower levels (Stewart et al., 1984). These mice develop mammary adenocarcinomas in multiparous animals with a 100% incidence. Given the hormone responsiveness of the MMTV promoter, it is not surprising that tumor incidence and latency depends on the number of pregnancies. In multiparous females, adenocarcinomas normally develop at 5- to 6-months of age and correspond with the second or third pregnancy. Metastatic tumors are rare, but have been observed in the lung. No tumors are observed in male mice (Stewart et al., 1984).

Transgenic mice expressing c-myc under the control of the murine WAP promoter have also been generated by two-independent groups (Sandgren et al., 1995; Schoenenberger et al., 1988). The frequency of tumors in WAP-c-myc mice was much higher than that of their MMTV-driven counterpart. WAP-c-myc female mice begin to develop mammary tumors within 2-months of initiation of transgene expression (pregnancy). Approximately 80% of females develop palpable tumors after undergoing two rounds of pregnancy, and individual mice often develop multiple tumors affecting a single or multiple glands (Schoenenberger et al., 1988). Virgin mice do not develop tumors (monitored up to 14 months). WAP-c-myc mice form mammary tumors that are well-differentiated and acquire constitutive expression and secretion of β -casein and transgenic myc.

While these studies validate the potential of c-myc to drive mammary tumorigenesis, further observations revealed the requirement for additional genetic events in MMTV-c-myc and WAP-c-myc derived mammary carcinoma development. For example, tumors arose focally, even though c-Myc expression was observed throughout the normal mammary epithelium of both MMTV-c-myc and WAP-c-myc female mice during the latter part of pregnancy and throughout lactation. In addition, although random chromosomal integration of c-myc in MMTV-c-myc mice caused the expression of the transgene in multiple tissues, c-myc only elicited neoplasms in mammary tissue. Additionally, the long latency of tumors and dependence on pregnancy suggests that c-myc itself might not be sufficient for the induction of mammary tumors.

The observation that c-myc on its own was insufficient to drive tumorigenesis in the entire mammary glands instructed follow-up studies using double transgenic (or bitransgenic) mouse models. Double transgenic mice carrying both the c-myc and another gene, such as v-Ha-ras (Sinn et al., 1987), TGF- α (transforming growth factor α) (Amundadottir, Johnson, Merlino, Smith, & Dickson, 1995), bcl-2, or neu, develop mammary tumors at higher frequencies and with shorter latency. MMTV-c-myc/MMTV-v-Ha-ras and MMTV-c-myc/MMTV-TGF α double transgenic mice helped elucidate that c-myc could synergize with v-Ha-ras and TGF α to decrease tumor latency and eliminate the requirement for pregnancy for tumor initiation. In particular, the double transgenic MMTV-c-myc/MMTV-TGF α mice do not develop normal mammary tissue, and mammary tissue from 3-week-old mice can form tumors when transplanted into athymic mice. This indicates that c-myc and TGF α can synergize in transforming mammary epithelium, requiring few or no additional genetic alterations (Amundadottir et al., 1995; Amundadottir, Nass, Berchem, Johnson, & Dickson, 1996).

The Chodosh laboratory developed a transgenic mouse model that allowed for temporal control of mammary-specific expression of c-myc upon treatment with tetracycline derivatives (Boxer, Jang, Sintasath, & Chodosh, 2004). In this model, the MMTV promoter drives expression of reverse tetracycline-dependent transcriptional activator, rtTA, and in the presence of doxycycline, induces expression of c-myc fused to a tetracycline-dependent promoter (MMTV-rtTA/TetO-MYC transgenic mice). Chronic induction of c-myc in these transgenic mice results in solitary mammary adenocarcinomas after about 22-weeks. This model is of importance as it allows for the assessment of tumor dependence upon continued c-myc expression once the tumor has formed, or “oncogene dependence.” Once mammary

tumors were observed, the mice were taken off doxycycline to assess whether the tumors would regress. Interestingly, only about half of the tumors regressed after inhibition of c-myc expression by removal of doxycycline. Thus, c-myc inactivation did not lead to a reversal of tumors and the tumors were able to grow in the absence of c-myc overexpression. Among the tumors that failed to regress following c-myc inactivation, nearly all the tumors resumed growth even in the absence of c-myc expression. This suggests that the majority of primary mammary tumors that arose from c-myc activation already acquired the ability to grow in the absence of c-myc overexpression. Among the tumors that had regressed upon absence of c-myc, a majority of the tumors spontaneously recurred at the initial site within 1 year following doxycycline withdrawal. Mammary tumors were found to recur due to the presence of residual neoplastic cells that persisted in the mammary glands of nearly all animals with fully regressed tumors. Reactivation of c-myc in the residual neoplastic cells resulted in a rapid regrowth of tumors. Additionally, successive cycles of c-myc expression resulted in progression of nearly all tumors to a doxycycline-independent state.

2.1.2 v-Ha-ras transgenic models—The protooncogene *Ha-ras* (Harvey rat sarcoma viral oncogene homolog) is a small, guanine nucleotide binding protein located on chromosome 11p15.5 in humans. *Ha-ras*, one of three *ras* oncogenes (the others being *K-ras* and *N-ras*), encodes a 21-kDa protein. These evolutionarily conserved GTPases play pivotal roles in many cellular processes. In response to growth factor stimulation, GTP-bound or activated Ras interacts with multiple effector molecules, particularly, the well-studied Ras/MEK/ERK or MAPK (mitogen-activated protein kinase) signaling cascade that ultimately promotes cell proliferation. In many human tumors, one or another of the *ras* genes is found to have acquired a point mutation that renders it constitutively activated, such that downstream signaling is activated even in the absence of growth factors. Although *ras* mutations have been observed in breast cancer patients (Bos, 1989), they do not appear to play a pivotal role in the etiology of breast cancer (Spandidos, 1987). Elevated levels of Ras, both at the transcript and protein level, have also been documented in human breast cancer patients (DeBortoli, Abou-Issa, Haley, & Cho-Chung, 1985; Hand et al., 1984; Slamon, deKernion, Verma, & Cline, 1984), although amplification and rearrangement of *ras* genes were not observed in breast cancer patients (Schondorf et al., 1999). Interestingly, in studies using the chemical carcinogen *N*-nitroso-*N*-methylurea to induce mammary tumors in rodents, activation of *Ha-Ras* was identified as the earliest genetic event observed in the mammary gland (Sukumar et al., 1995).

Transgenic mice expressing v-Ha-ras (a virally derived *Ha-ras* gene carrying an activating point mutation) under the control of the MMTV promoter were developed by the Leder laboratory (Sinn et al., 1987) and the Jolicoeur laboratory (Tremblay et al., 1989). Mice from multiple-independent lines from both groups were found to express the transgene in the mammary and salivary glands, the Harderian gland (an intraorbital lacrimal gland present in rodents), and at lower levels, the spleen, thymus, and lung. Expression in seminal vesicles of male mice was also reported by Sinn et al. Further, mice from both groups developed mammary and salivary adenocarcinomas and Harderian gland hyperplasias, as well as occasional lung tumors, and lymphomas (Sinn et al., 1987) or splenomegaly (Tremblay et

al., 1989). Although Sinn et al. reported detecting mammary adenocarcinomas as early as 32 days of age in two females, the mean age for detection of mammary adenocarcinomas in females was 168 days of age in their best-characterized model. Similarly, Tremblay et al. reported mammary adenocarcinomas arising between 4 and 10 months of age in females from multiple lines. In both cases, mammary tumors were less common in males than females. Histologically, the mammary tumors in these mice were locally invasive with some metastasis to the liver and/or lung. Transgenic mice expressing v-Ha-ras under the control of the WAP promoter were developed by the Gerlinger laboratory (Andres et al., 1987). In this model, less than 1% of transgenic mice developed mammary tumors (and/or salivary gland tumors) with a tumor latency of about 1-year.

Double transgenic mice expressing MMTV-c-myc and MMTV-v-Ha-ras were also developed by the Leder laboratory (Sinn et al., 1987). The two oncogenes interacted synergistically, leading to markedly accelerated tumor kinetics in both female and male mice. However, uniform malignant transformation of the mammary gland did not occur even upon expression of both oncogenes, as evidenced by the development of focal, though multiple, stochastic mammary tumors. Similarly, double transgenic mice expressing WAP-c-myc and WAP-v-Ha-ras showed a synergistic effect on mammary tumorigenesis, although the tumors that arose were still focal in nature (Andres et al., 1988). Thus, it could be concluded from these studies that even two “hits” are insufficient to induce mammary tumorigenesis.

The tumor growth characteristics of MMTV-c-myc (Stewart et al., 1984), MMTV-v-Ha-ras (Sinn et al., 1987), and the double transgenic tumors were evaluated to better understand the potential cooperative role of the myc and ras oncogenes in mammary tumorigenesis (Hundley et al., 1997). MMTV-c-myc tumors showed the highest level of spontaneous apoptosis, followed by MMTV-c-myc/MMTV-v-Ha-ras, while MMTV-v-Ha-ras tumors had very low levels of spontaneous apoptosis. The cell cycle characteristics of tumors obtained from the three transgenic mice were also significantly different, with MMTV-c-myc tumors showing the highest rates of proliferation (higher S-phase fractions), MMTV-v-Ha-ras tumors having very low rates of proliferation, and MMTV-c-myc/MMTV-v-Ha-ras having intermediate levels. Despite these differences in rates of apoptosis and proliferation, the tumor growth rates in these three transgenic mouse models were similar, indicating the importance of the balance between cell cycle regulation and cell death in determining kinetics of tumor growth and the fact that different oncogenes had a profound influence on that balance (Hundley, Koester, Troyer, Hilsenbeck, Barrington, et al., 1997).

The influence of another cell cycle regulator, a cell cycle-stimulating phosphatase CDC25A, on tumorigenesis in MMTV-v-Ha-ras, MMTV-neu, and MMTV-c-myc mice was also evaluated (Ray et al., 2007). CDC25A is known to be significantly overexpressed in human cancers including breast tumors. CDC25A might contribute to some of the tumorigenic effects mediated by Ha-ras and neu oncogenes. Cdc25A^{+/-} mice develop normally. Mating Cdc25A^{+/-} mice with MMTV-Ha-ras or MMTV-neu transgenic mice caused development of mammary and salivary tumors after about 18-weeks, however Cdc25A^{+/-}/MMTV-Ha-ras mice develop tumors after about 60-weeks. In fact, about 10% of Cdc25A^{+/-}/MMTV-Ha-ras mice did not develop tumors even at 2-years of age. Similar results were obtained with

Cdc25A^{+/-}/MMTV-neu mice. Cdc25A^{+/-}/MMTV-neu mice developed tumors at about 32-weeks of age compared to Cdc25A^{+/+}/MMTV-neu mice, which formed tumors at about 23-weeks of age. Thus, hemizygous loss of CDC25A delays or inhibits ras- and neu-induced tumorigenicity. In contrast to ras- and neu-induced tumorigenesis, tumor latency was not statistically different between Cdc25A^{+/-}/MMTV-c-myc and Cdc25A^{+/+}/MMTV-c-myc mice (Ray et al., 2007). Thus, CDC25A might be a critical factor in neu- and ras-mediated tumorigenesis. These studies also suggest that both c-myc and ras oncogenes have profound influence on the necessary balance between cell cycle regulation and cell death in determining kinetics of tumor growth.

2.1.3 Cyclin D1 transgenic and knockout models—Cyclin *D1* is a 36-kDa protein located on chromosome 11q13, a region commonly amplified in breast cancer (Fantl, Smith, Brookes, Dickson, & Peters, 1993). Cyclin D1 functions in regulation of progression of the cell cycle through the G1 to S phase and in cellular metabolism (Fu, Wang, Li, Sakamaki, & Pestell, 2004). Cyclin D1 plays an essential role in mammary gland development, where it is required for normal lobuloalveolar development. In fact, cyclin D1 null mice have impaired mammary development with undeveloped alveoli during pregnancy and lactation does not occur (Fantl, Stamp, Andrews, Rosewell, & Dickson, 1995; Sicinski et al., 1995). Cyclin *D1* is amplified in 15–20% of human breast cancers (Lammie & Peters, 1991) and is associated with poor prognosis of estrogen receptor positive patients. Cyclin D1 is overexpressed in about 45–50% of breast carcinomas (Bartkova et al., 1994; Buckley et al., 1993; Gillett et al., 1994). Overexpression of cyclin D1 was observed in all histological types of breast cancer (Bartkova et al., 1994), although a majority of human breast tumors that overexpress cyclin D1 tend to be well differentiated and estrogen receptor positive (Barnes & Gillett, 1998). Cyclin D1 can be detected at the various stages of breast cancer from ductal carcinoma in situ all the way to metastatic lesions (Bartkova et al., 1994; Gillett et al., 1996).

The Schmidt laboratory developed MMTV-driven cyclin D1 transgenic mice (MMTV-cyclin D1) (Wang et al., 1994). MMTV-cyclin D1 virgin mice show increased proliferation and precocious alveolar development in the mammary gland, mimicking pregnancy. MMTV-cyclin D1 mice developed proliferative abnormalities in the mammary gland that increased with age, number of pregnancies, and level of cyclin D1 expression (Wang et al., 1994). Female transgenic mice develop focal mammary adenocarcinomas after a latency of more than 1-year (with mean tumor onset age of 551±26 days). Male mice also develop tumors. Random nonspecific testicular and uterine wall tumors with liver metastasis were observed in one strain. Given the long latency and focal nature of the tumors, cyclin D1 appears to promote mammary tumorigenesis, however, additional genetic changes are necessary for development of mammary tumors.

Another transgenic mouse that expressed constitutively nuclear and nondegradable cyclin D1 (phosphorylation-deficient cyclin D1 T286A) under the transcriptional control of the MMTV promoter was generated by the Diehl laboratory (Lin et al., 2008). The mutated cyclin D1 transgenic mice developed multifocal adenocarcinomas at 18-months of age while the normal cyclin D1 transgenic took 22-months to develop adenocarcinomas with less than 100% incidence, in both cases (Lin et al., 2008). As seen in human tumors, tumors from both the cyclin D1 transgenic mice were estrogen receptor positive and exhibited estrogen-

dependent growth. This suggests that temporal control of cyclin D1 localization and proteolysis are essential for maintaining mammary epithelium homeostasis.

The Law laboratory generated transgenic mice with a cyclin D1-Cdk2 fusion protein under the transcriptional control of the MMTV promoter (MMTV-cyclin D1-Cdk2) that showed resemblance to human basal-like subtype of breast cancer (also known as triple negative), which is highly invasive, has a high postsurgical recurrence rate and poor prognosis (Corsino et al., 2008). Previous mouse models of basal-like breast cancer involved deletion of BRCA1 and p53. However BRCA1-inactivated tumors express progesterone receptor and overexpress HER2 and are not representative of the human basal-like breast cancer. The MMTV-cyclin D1-Cdk2 mice develop mammary fibrosis and hyperplasia that ultimately leads to mammary tumors with desmoplasia. This is a useful model for testing CDK2 inhibitors as well as for the development and testing of novel therapeutic agents targeting tumor cell/fibroblast paracrine loops.

To determine the role of p53 in MMTV-cyclin D1-derived mammary tumors, MMTV-cyclin D1 mice were crossed with p53 null mice (p53^{-/-}) (Hosokawa et al., 2001). Mammary tumors were not observed in MMTV-cyclin D1/p53^{+/-} and MMTV-cyclin D1/p53^{-/-} mice suggesting that p53 inactivation did not cooperate with cyclin D1 in mammary tumorigenesis.

The Sicinski lab, that had previously developed cyclin D1 null mice (Sicinski et al., 1995), crossed these mice to four mammary tumor-prone transgenic mice to further elucidate the role of cyclin D1 in breast cancer. Cyclin D1 null mice (cyclin D1^{-/-}) were crossed with MMTV-c-myc, MMTV-v-Ha-ras, MMTV-c-neu, and MMTV-Wnt-1 transgenic mice (Yu, Geng, & Sicinski, 2001). Loss of cyclin D1 expression inhibited mammary tumor formation in MMTV-c-neu and MMTV-v-Ha-ras mice but not in MMTV-c-myc and MMTV-Wnt-1 mice. Mammary tumor inhibition in MMTV-v-Ha-ras appears to be cyclin D1 specific, as mice generated by crossing cyclin D2 and cyclin D3 null mice with MMTV-v-Ha-ras mice developed mammary tumors (Yu et al., 2001). Additionally mice lacking the interacting partner of cyclin D1, CDK4, are resistant to mammary tumors in MMTV-c-neu mice (Yu et al., 2006). Cyclin D1 was not only able to inhibit mammary tumors arising in MMTV-c-neu mice, but also suppressed tumorigenesis in mice carrying a mutant constitutively active c-neu oncogene (Bowe, Kenney, Adereth, & Maroulakou, 2002). Interestingly, compensation by cyclin E1 resulted in recovered tumor potential in 35% of MMTV- mutant c-neu/cyclin D1^{-/-} mice.

2.1.4 TGF α transgenic models—Transforming growth factor α (TGF α) is a member of the epidermal growth factor (EGF) family of cytokines. TGF α encodes a 17-kDa protein and is located on chromosome 2p13. It was originally observed in culture media of certain retrovirus transformed fibroblasts. TGF α binds to the EGFR and activates its endogenous tyrosine kinase activity and further EGFR downstream signaling pathways. TGF α is overexpressed in breast carcinomas and stimulates epithelial cell migration and promotes angiogenesis (Schreiber, Winkler, & Derynck, 1986). TGF α has been directly implicated in modulation of tumor transformation.

The Coffey laboratory developed MMTV-TGF α transgenic mice (human TGF α being expressed under the transcriptional control of the MMTV promoter/enhancer) (Matsui, Halter, Holt, Hogan, & Coffey, 1990). High expression of TGF α was observed in the normal mammary glands in both virgin and pregnant, multiparous females, and in neoplastic mammary tissue. While the TGF α transgene was expressed solely in the mammary glands in the females, transgene expression was also detected in the testes, seminal vesicles, salivary glands, and lungs in the males. Significant alveolar hyperplasia was evident in virgin transgenic females at approximately 100-days of age but not at 4-weeks of age, suggesting a role of sexual maturity and hormonal influences for hyperplastic changes. Among the multiparous females, hyperplastic alveolar nodules and cysts were observed, with an increase in hyperplastic and dysplastic characteristics upon increasing number of pregnancies. Only one multiparous female developed adenocarcinoma but no metastases were observed. Interestingly, no histological abnormalities were observed in the males up to 6-months of age despite expression of TGF α . A follow-up study by the same group evaluated the generation of hyperplasia and tumors in greater detail (Halter et al., 1992). By 12-months of age, hyperplasia was observed in 65% of multiparous and 45% of virgin females. By 16-months of age, 40% of multiparous and 30% of virgin females developed adenocarcinoma.

Another TGF α transgenic mouse was generated by the Lee laboratory in which transgenic mice expressed rat TGF α under the transcriptional control of the WAP promoter (Sandgren et al., 1995). Expression of TGF α was not detected in virgin females and these mice remained tumor free. TGF α was expressed at high levels during pregnancy and lactation, and parous WAP-TGF α females developed tumors with a 100% incidence but variable tumor latency. Tumor incidence and tumor latency was increased in the WAP-TGF α mice as compared to the MMTV-TGF α model. The majority of the tumors observed were well-differentiated adenomas and adenocarcinomas (53%) and fibroadenomas (35%) with characteristics of late pregnant mammary epithelium. Most of the tumors that formed were transplantable. Some of the high TGF α expressing females were unable to lactate. Following cessation of the lactogenic stimulus, TGF α overexpression also delayed or inhibited normal mammary involution. This delay in involution might predispose the proliferating epithelial cells to transformation, further aiding in the malignant transformation of the mammary gland. Cyclin D1 expression was found to be elevated in the WAP-TGF α induced tumors, suggesting cyclin D1 might act in concert with TGF α during mammary tumorigenesis. Mating of WAP-c-myc mice with WAP-TGF α resulted in double transgenic WAP-c-myc/WAP-TGF α mice that had further decreased tumor latency, increased tumor growth (more than five large mammary tumors in females) and also induced mammary tumors in virgin female and male mice. Double transgenic females had an average life span of less than 4-months as compared to WAP-c-myc mice (about 8-months) and WAP-TGF α mice (about 9.5-months). This suggests that the two transgenes cooperate during mammary tumorigenesis.

2.1.5 SV40 T antigen transgenic models—Simian virus 40 (SV40) is a papovavirus that causes sarcomas when injected into newborn hamsters. Monkeys are the natural host for the SV40 virus. Although the SV40 T antigen is not known to initiate human breast cancer,

it deregulates cellular pathways that are known to be important in human breast cancer. Most importantly, SV40 T antigen binds and functionally inactivates both the p53 and Retinoblastoma (Rb) tumor suppressor proteins (Ahuja, Saenz-Robles, & Pipas, 2005).

Transgenic mice expressing SV40 T antigen under the transcriptional control of the WAP promoter were developed by the Graessmann laboratory (Tzeng, Guhl, Graessmann, & Graessmann, 1993). Eight-independent WAP-SV40 T antigen transgenic mouse lines were generated. Female mice from three of the eight lines developed mammary tumors with high frequency, usually observed during lactation. Transgenic females were not capable of producing milk, as T antigen expression induced apoptosis during late pregnancy, causing premature mammary gland involution before birth of the litters. Despite the mammary gland apoptosis, female transgenic mice developed mammary tumors following the first lactation period (Tzeng, Gottlob, Santarelli, & Graessmann, 1996). Interestingly, 5–10% of the tumors showed a reduction in expression of T antigen during tumor progression. Both T antigen positive and negative tumor cells maintained expression of estrogen and progesterone receptors at comparable levels, indicating that T antigen expression was independent of hormone receptor level. Approximately 70% of virgin mice developed T antigen positive breast tumors. However, ovariectomized animals had reduced tumor rates, indicating a critical role of ovary-associated hormones in breast cancer formation (Santarelli, Tzeng, Zimmermann, Guhl, & Graessmann, 1996).

The Furth laboratory performed further studies using one of the eight previously generated WAP-SV40 T antigen mice to understand the molecular changes induced by expression of SV40 T antigen in the mammary gland (Li, Lewis, Capuco, Laucirica, & Furth, 2000). Mammary adenocarcinomas, ranging from well- to poorly-differentiated, formed in the female mice with a 100% tumor incidence by 8- to 9-months of age. On average, palpable tumors were observed in these mice at 6-months of age following at least a single pregnancy (Li, Lewis, Capuco, Laucirica, & Furth, 2000). The mechanisms underlying the inability of the transgenic females to produce milk were investigated. With the onset of the normal mammary involution process, there is a cessation in milk protein gene expression and alveolar cells undergo apoptosis. Both p53-dependent and p53-independent mechanisms of apoptosis have been identified *in vitro* using mammary epithelial cells. To determine whether p53 was involved in apoptosis during mammary involution, involution was examined in the presence and absence of functional p53. Mammary involution and remodeling was determined to be independent of p53. The mammary cells that escaped apoptosis continued to differentiate; however milk production, processing, and secretion were impaired and hence lactation failed (Li, Hu, Heermeier, Hennighausen, & Furth, 1996a, 1996b).

The tumorigenic process in these mice appears to be influenced by competition between proliferation and apoptosis. Expression of T antigen during the first pregnancy triggers both proliferation as well as p53-independent apoptosis. While cell proliferation was maintained throughout tumorigenesis, apoptosis was decreased as cells transitioned to hyperplasia and adenocarcinoma (Li, Lewis, Capuco, Laucirica, & Furth, 2000). As mammary cells transitioned from normal to adenocarcinoma, the expression of several cell survival and cell cycle control proteins were elevated. For example, proteins involved in cell cycle regulation

such as cyclin D1, CDK2 and E2F-1, and cell survival proteins Bcl-xl, Bax, Bag-1, and Bad were increased in the adenocarcinomas. About 10% of the tumors also showed *K-Ras* amplification. Hormonal changes also appeared to play a role in tumor progression, with estrogen mainly acting during the early stages of tumor progression. Additionally, transient exposure to glucocorticoids resulted in tetraploidy, premature appearance of irreversible hyperplasia and early tumor development. Overexpression of the cell survival protein, Bcl-2 caused acceleration of tumor appearance. Bcl-2 reduced apoptosis and decreased cell proliferation rate. However, Bcl-2 inhibition in cell proliferation was lost once the cells transition to adenocarcinoma (Li, Lewis, Capuco, Laucirica, & Furth, 2000). This WAP-SV40 T antigen model can be used to investigate the molecular mechanisms underlying cell proliferation, apoptosis, DNA mutation, and DNA repair as modulated by extrinsic and intrinsic signals during mammary tumorigenesis (Li, Lewis, Capuco, Laucirica, & Furth, 2000).

The Green laboratory developed transgenic mice that placed the SV40 large T antigen under the transcriptional control of the C3(1) promoter (Maroulakou, Anver, Garrett, & Green, 1994). Similar to the WAP-SV40 T antigen transgenic mice, the C3(1)-SV40 T antigen female transgenic mice developed mammary hyperplasia by 3-months of age and mammary adenocarcinomas developed by 6-months of age with a 100% tumor incidence, with occasional metastasis to the lung. As would be expected, onset of mammary tumors correlated with expression of T antigen. Of note, the male transgenic mice developed prostate adenomas or adenocarcinomas that were capable of metastasizing to the lung, and these mice serve as a model for understanding the mechanisms underlying development of prostate cancer. As observed in the WAP-SV40 T antigen model, apoptosis occurred by p53- and Bcl-2-independent pathways, and was suppressed during the transition from preneoplasia to carcinoma. Thus, suppression of apoptosis appears to play a role in the tumorigenic process (Shibata et al., 1996). Since Bax was one of the proteins elevated during the preneoplastic stages where apoptosis was increased, the role of Bax in tumor progression was assessed using C3(1)-SV40 T antigen crossed with Bax knockout mice. C3(1)-SV40 T antigen/Bax^{-/-} mice developed increased tumor numbers with larger tumor mass, accelerated tumor growth rates, and decreased survival as compared to mice with wild-type Bax. Loss of a single *Bax* allele was sufficient to accelerate tumorigenesis. Thus, Bax appears to be a critical suppressor of mammary tumor progression at the preneoplastic stage *via* upregulation of apoptosis, but this protective effect is lost during the transition from preneoplastic to invasive carcinoma (Shibata et al., 1999).

Double transgenic animals were developed to determine the significance of other genes in tumorigenesis in WAP-SV40-T antigen transgenic mice. The ability of Maspin, a unique serpin, in inhibiting tumor progression was evaluated. Transgenic animals expressing Maspin under the transcriptional control of WAP promoter were generated. These mice were then crossed with the WAP-SV40 T antigen mice to generate double transgenic WAP-SV40 T antigen/WAP-Maspin mice. Introduction of Maspin reduced tumor growth and pulmonary metastases. Maspin appears to slow tumor progression by increasing apoptosis, decreasing angiogenesis, and inhibiting tumor cell migration (Zhang, Shi, Magit, Furth, & Sager, 2000). Some of the mammary adenocarcinomas that developed in the WAP-SV40 T antigen

transgenic mice displayed constitutively active Stat5a/b and Stat3. The significance of Stat5a in WAP-SV40 T antigen tumorigenesis was therefore evaluated by crossing WAP-SV40 T antigen mice with mice carrying germ line deletion of Stat5a (Ren, Cai, Li, & Furth, 2002). Reducing Stat5a expression in the WAP-SV40 T antigen mice resulted in delayed tumor onset, smaller tumor size, and increased apoptosis, indicating that Stat5a plays a role in WAP-SV40 T antigen-mediated tumorigenesis.

2.2. Modeling breast tumor metastasis

Metastasis is a complex, multistep process that involves local invasion and dissemination of tumor cells from the primary tumor site, intravasation into the vasculature, survival, and extravasation from the vasculature, followed by colonization at a distant organ in the body (Figure 8.2b) (Chambers, Groom, & MacDonald, 2002; Nguyen, Bos, & Massague, 2009). Understanding the molecular events that regulate tumor cell migration and metastasis from the primary tumor site to distant organs will greatly aid in developing novel therapeutic targets to inhibit tumor metastasis. The following transgenic models have been demonstrated to display significant rates of metastasis and will be discussed in this section: the growth factor receptors ERBB2 and EGFR, the viral polyoma middle T antigen and the Wnt protooncogenes.

2.2.1 ERBB2/NEU transgenic models—An obvious strategy for tumor cells to gain growth advantage is by over-expression of one or more of its growth-related receptors, which results in constitutive receptor activation and subsequent aberrant downstream signaling. One such growth-related receptor is the protooncogene *ERBB2*. *ERBB2* (also known as HER2 and the rat homologue, Neu) belongs to the EGFR family and encodes a receptor tyrosine kinase. *ERBB2* is located on human chromosome 17q12. *ERBB2* signals downstream through the Ras/MAPK cascade, activating c-myc, c-jun and c-fos, and activated protein-1 (AP-1). By heterodimerization with *ERBB3*, *ERBB2* can also activate the phosphatidylinositol 3 kinase (PI3K)/AKT signaling pathway. Both the Ras/MAPK and the PI3K/AKT pathways drive cell proliferation, migration, transformation, and survival through the inhibition of apoptosis. Frequently amplified and overexpressed in breast cancer patients, *ERBB2* is associated with an aggressive tumor phenotype, with reduced responsiveness to hormonal therapy and poor patient outcome. Fifteen to twenty percent of human breast cancers are associated with *ERBB2* gene amplification, while 30% of breast cancers show increased expression of activated *ERBB2*. *ERBB2* overexpression is used as a prognostic marker of breast cancer in the clinic (Taneja et al., 2009). The role of *ERBB2* in mammary tumorigenesis was elucidated by several laboratories using transgenic mice that expressed ErbB2 under the transcriptional control of mammary-specific promoters. A review by Ursini-Siegel et al. provided a timeline of all ErbB2 mouse models developed up to 2005 (Ursini-Siegel, Schade, Cardiff, & Muller, 2007). The list has grown and continues to grow since the publication of that timeline.

Transgenic mice expressing an activated form of rat Neu under the control of the MMTV promoter were developed by both the Leder laboratory (Muller et al., 1988) and the Jolicoeur laboratory (Bouchard, Lamarre, Tremblay, & Jolicoeur, 1989) and helped elucidate the role of ErbB2 over-expression in tumor initiation in the mammary gland. In the

first MMTV-neu transgenic model, mice developed tumors in multiple mammary glands with a 3-month latency (Muller et al., 1988). The involvement of multiple mammary glands with a short tumor latency suggested that few additional genetic changes were necessary to transform the mammary epithelium and promote tumorigenesis when an activated form of Neu was overexpressed (Muller et al., 1988). Increased Src tyrosine kinase activity was observed in mammary tumors from the MMTV-neu mice, implying that Src may be activated by Neu. In fact, c-Src might be activated through direct interaction with activated Neu during mammary tumorigenesis (Muthuswamy, Siegel, Dankort, Webster, & Muller, 1994). In the second MMTV-neu transgenic model, however, mice developed focal, stochastic mammary tumors with longer latency (Bouchard et al., 1989) than the mice developed by the Leder laboratory. This discrepancy might have been due to differences in integration site and copy number of the transgene, resulting in differences in transgene expression levels achieved in the mammary epithelium. Subsequent studies with additional transgenic mice showed multifocal mammary tumors with short latencies (Guy, Cardiff, & Muller, 1996) suggesting that overexpression of activated Neu may be sufficient to efficiently transform mammary epithelial cells in transgenic mice (Ursini-Siegel et al., 2007).

Constitutive activation of Neu is observed in rats that have a valine to glutamic acid substitution (at amino acid 664) in the transmembrane domain. This mutation stabilizes dimerization of the receptor, resulting in constitutive tyrosine kinase activity. Of note, comparable activating mutations have not been detected in ERBB2 positive breast cancer in humans, where wild-type ERBB2 is overexpressed primarily through gene amplification. Thus, transgenic models that more closely mimic human breast cancer were generated using wild-type neu. MMTV-neu transgenic mice develop focal mammary tumors with an 8- to 12-month latency period and subsequent lung metastasis (Guy et al., 1992). However analysis of the tumors from these mice showed sporadic in-frame deletions, point mutations, or cysteine residue insertions within the NEU extracellular domain. These cysteine insertions lead to formation of intermolecular disulphide bridges that promote receptor dimerization, causing receptor activation, a process that is normally triggered by ligand binding (Guy et al., 1996; Siegel, Dankort, Hardy, & Muller, 1994). These studies suggest that activating mutations of NEU are essential for mammary tumor initiation in these transgenic mice. Interestingly, an alternatively spliced, constitutively active ERBB2 isoform with a 16-amino acid in-frame deletion has been detected in humans (Siegel, Ryan, Cardiff, & Muller, 1999). This ERBB2 splice variant has only been observed at a 5% level with the wild-type receptor, in both normal and cancer breast tissue, and hence further validation of the role of this ERBB2 splice variant is required to clearly understand its role in tumorigenesis.

The Chodosh laboratory generated mice that conditionally expressed activated Neu under the control of both the MMTV promoter and a tetra-cycline regulatory element (MMTV-rtTA/TetO-NeuNT mice) (Moody et al., 2002). Administration of doxycycline induced the expression of activated Neu in the mammary gland and resulted in multiple invasive and metastatic mammary carcinomas similar to tumors arising in MMTV-activated neu mice. Removal of doxycycline resulted in a loss of Neu expression and complete regression of mammary tumors and lung metastasis. However, many of the mice later developed Neu-

independent mammary tumors, which might have resulted from the reactivation of dormant tumor cells that persisted in the primary tumor. Of note, a recent study has carried out extensive proteome and transcriptome profiling of the Her2/Neu-driven transgenic mammary model (MMTV-rtTA/TetO-NeuNT), and all the data including biospecimens are available to the research community (Schoenherr et al., 2011).

Transgenic mice expressing ErbB2 under the transcriptional control of the MMTV or WAP promoters illustrated the importance of ErbB2 in induction of mammary tumors. However, the use of these strong promoters induced nonphysiologically elevated levels of ErbB2 expression. Therefore, to determine the role of Neu in mammary tumor induction in a model that was more relevant to that observed in humans, the Muller laboratory developed a knock-in transgenic mouse using the endogenous Neu promoter to drive transcription of activated Neu (Andrechek et al., 2000). In this modified locus, expression of activated Neu was transcriptionally silenced by an upstream loxP-flanked neomycin cassette. However, crossing these mice with MMTV-Cre transgenic mice resulted in the mammary-specific excision of the neo cassette and expression of activated Neu from the endogenous mouse ErbB2 promoter. Expression of activated Neu accelerated lobuloalveolar development, and focal mammary tumors were observed after a long latency. Interestingly, in all the mammary tumors, Neu expression was increased through gene amplification, and this therefore represents a model that more closely mimics human ERBB2 positive breast tumors (Andrechek et al., 2000).

As mentioned previously, abrogating CDK4 was able to suppress tumor growth in MMTV-Neu mice. CDK2 was also tested for its role in MMTV-Neu-mediated tumorigenesis (Ray, Terao, Christov, Kaldis, & Kiyokawa, 2011). Only 13% of MMTV-Neu/Cdk2^{-/-} mice developed mammary tumors with a longer latency than MMTV-Neu/Cdk2^{+/-} and MMTV-Neu/Cdk2^{+/+} mice. The rest of the mice remained tumor free up to 16-months, indicating a role of Cdk2 in ERBB2-mediated tumorigenesis.

2.2.2 EGFR transgenic models—*EGFR* is another member of the receptor tyrosine kinase family that is located on chromosome 7p12 in humans. EGFR plays a key role in normal mammary development and function and is present in the ductal epithelial cells of normal breast (Moller et al., 1989; Wiesen, Young, Werb, & Cunha, 1999). EGFR is overexpressed in about 30–50% of human breast cancers, and elevated EGFR expression correlates with poor prognosis and increased invasiveness (Koenders, Beex, Kienhuis, Kloppenborg, & Benraad, 1993).

To evaluate the role of EGFR in mammary tumor development, transgenic mice expressing human EGFR under the transcriptional regulation of both the MMTV and BLG promoters were developed by the Theuring laboratory (Brandt et al., 2000). As would be expected from our understanding of the respective promoters, the BLG-EGFR mice expressed the EGFR transgene exclusively in female mammary glands, while in the MMTV-EGFR mice, the transgene was expressed in other organs such as salivary glands, ovary, and testis as well. Mammary gland development was affected in both virgin and lactating female transgenic MMTV-EGFR and BLG-EGFR animals. Mammary epithelial hyperplasia was observed in the virgin females, while dysplasia and tubular adenocarcinomas were observed in lactating

female transgenic mice. With increasing number of pregnancies an increase in the number of dysplasias were observed. Small tubular adenocarcinomas were observed in one out of six multiparous BLG-EGFR female transgenic mice and in two out of six multiparous MMTV-EGFR female transgenic mice. Transgenic mice expressing human EGFR under the transcriptional regulation of the MMTV promoter were also generated in the Parsons laboratory to determine the role of EGFR on multiple steroid hormone-responsive tissues (Marozkina, Stiefel, Frierson, & Parsons, 2008). Although the EGFR transgene was expressed in the mammary gland, only a few visible tumors developed over 2-years. Fifty-five percent of the female transgenic mice developed hyperplasia, hypertrophy, or slight dysplasia in the mammary glands, as was observed in the earlier transgenic models. Interestingly, hyperplasia, hypertrophy, or slight dysplasia was also observed in 89% of the female transgenics in the uterus or uterine horn and in 100% female transgenics in the ovaries or oviduct. Thus, although EGFR overexpression caused impaired mammary gland development and induced epithelial cell transformation, it was not able to singly transform the mammary gland.

2.2.3 PyVmT transgenic models—Polyoma virus, like SV40, is a DNA tumor virus that encodes a potent transforming protein, Polyoma virus middle tumor antigen (PyVmT). Infection of newborn or athymic mice with the Polyoma virus results in formation of mammary adenocarcinomas along with multiple other tumors types (Asch, 1996), and it was demonstrated that the middle T antigen is required for tumor induction (Mes & Hassell, 1982). PyVmT binds to and activates a number of signaling proteins, including c-Src, Shc, protein phosphatase 2A (PP2A), and PI3K, leading to activation of multiple downstream signaling pathways, including the Ras/MAPK pathway (Dilworth, 2002).

The Muller laboratory generated transgenic mice with mammary-specific expression of PyVmT under the transcriptional control of the MMTV promoter/enhancer (Guy, Cardiff, & Muller, 1992). Along with mammary expression of PyVmT, PyVmT was also observed at lower levels in salivary glands and ovaries in females, and in salivary glands, epididymis, and seminal vesicles in males but tumors were only observed in the mammary gland. Interestingly, in older animals, PyVmT was also detected in the lungs in female and male transgenic mice and correlated with the appearance of multifocal lung metastases. Synchronous multifocal mammary adenocarcinomas involving all the mammary glands were observed in MMTV-PyVmT mice. The mice develop tumors with a short latency, 100% incidence and majority of tumor-bearing mice develop secondary metastatic tumors in the lungs by 3-months of age. Transplanting the primary mammary tumor into the fat pads of normal syngeneic mice resulted in metastatic foci in the lymphatics or the lungs, further validating the metastatic capabilities of this PyVmT model. Male mice also develop mammary tumors although tumor onset was delayed compared to females. Virgin female transgenic mice developed multifocal mammary tumors indicating that tumor initiation was independent of pregnancy. The simultaneous appearance of multifocal tumors, the short tumor latency, and short latency between transgene expression and malignant transformation suggests PyVmT is sufficient for transforming the mammary epithelium with requirements for few, if any, additional genetic events. Important insights regarding metastatic progression might be obtained using this model since expression of PyVmT was closely

associated with pulmonary metastases. The MMTV-PyVmT model has a far greater metastasis incidence as compared to other MMTV-oncogene bearing transgenic mice, where metastasis is only rarely observed. Since metastatic tumors retain the expression of mammary markers such as β -casein, they likely originated from the primary tumors. One of the advantages of the MMTV-PyVmT transgenic mouse is that the mice develop focal tumors at the various mammary glands. In contrast, in the MMTV-ERBB2 the entire mammary gland coalesces into a large tumor. Although the MMTV-PyVmT model mimics breast cancer observed in patients, certain aspects of the model do not correlate with the human disease. Most of the tumors derived from MMTV-PyVmT mice are estrogen receptor negative and metastasis occurs primarily in the lungs, with some metastasis observed in the lymphatics. In contrast, human tumors show varying estrogen receptor expression and metastasize most commonly to the bone, lungs, and liver. Although another study did show that MMTV-ERBB2 mice develop polyclonal mammary tumors independent of a second event. Of note, both oncogenes ErbB2 and PyVmT are associated with constitutive tyrosine kinase activities that are not subject to normal cellular regulation (Guy, Cardiff, & Muller, 1992). The role of the tyrosine kinase c-Src in PyVmT-mediated tumorigenesis was evaluated by crossing MMTV-PyVmT mice with mice with a disrupted c-Src. In the absence of functional c-Src in MMTV-PyVmT transgenics, mice rarely developed mammary tumors. Following a long latency, abnormal hyperplastic mammary tissue was observed. Thus, c-Src might be required for PyVmT induced mammary tumorigenesis (Guy, Muthuswamy, Cardiff, Soriano, & Muller, 1994). Additionally the role of various genes in the inhibition of metastasis can be evaluated using MMTV-PyVmT. For example, silencing the expression of metastasis-stimulating protein, S100A4 (S100A4^{-/-}/PyVmT mice), resulted in a reduction of the metastatic burden in lungs (Grum-Schwensen et al., 2010).

2.2.4 Wnt transgenic models—*Wnt-1* is a secreted protein that is cysteine-rich, glycosylated, and located on chromosome 12q13 in humans. Wnt-1 functions primarily by stabilizing and increasing the levels of cytosolic β -catenin. Wnt-1 was first identified as a protooncogene activated by viral insertion in mouse mammary tumors. Wnt-1 is normally absent in the mammary gland and it has not been directly implicated in human breast cancer. However, other members of the Wnt family are expressed in the breast and are overexpressed in breast cancer (Li, Hively, & Varmus, 2000).

MMTV-Wnt-1 transgenic mice were developed in the Varmus laboratory (Tsukamoto et al., 1988). MMTV-Wnt-1 mice show extensive ductal hyperplasia early in life and mammary adenocarcinomas were observed in about 50% of the female mice by 6-months of age. Potent mitogenic effects were evident in the mammary epithelium upon ectopic expression of Wnt-1 and extensive ductal hyperplasia resulted in an inability of female transgenic mice to feed their pups. Female transgenic mice develop mammary adenocarcinomas with pregnancy causing a slight increase in tumor onset. Although metastasis was not observed at the time tumors were detected, lymph node and/or lung metastasis were observed after removal of primary tumors in these mice. About 15% of male mice also develop mammary gland tumors with extensive hyperplasia by 12-months of age. The genetic background of the transgenic mice does not seem to influence the latency of Wnt-1-mediated tumor development. The long latency between ductal hyperplasia in the mammary gland and the

appearance of mammary tumors suggests that MMTV-Wnt-1 mice might require additional cooperative genetic events for tumor formation. Studies conducted on ovariectomized MMTV-Wnt-1 mice demonstrated that Wnt-1 functions independently of estrogen signaling during mammary tumorigenesis (Bocchinfuso, Hively, Couse, Varmus, & Korach, 1999). Tumors were still observed although with delayed onset. Additionally, tumors formed after an increased latency in estrogen receptor α -null mice.

In cooperation with another oncogene, fibroblast growth factor 3 (FGF3), Wnt-1 showed increased tumorigenic abilities. MMTV-FGF3/ MMTV-Wnt-1 double transgenic female mice developed tumors faster than either of the individual transgene bearing mice. The effect was more pronounced in double transgenic males (Kwan et al., 1992). To determine whether Wnt-1 cooperates with TGF- β during the tumorigenic process, MMTV-Wnt-1/ MMTV-TGF- β double transgenic mice were generated. MMTV-Wnt-1/MMTV-TGF- β double transgenic mice did not show an increase in tumor incidence or latency, indicating TGF- β cannot inhibit Wnt-1-mediated mammary tumorigenesis (Li, Hively, & Varmus, 2000). MMTV-Wnt-1 mice were also crossed with p53 null mice to determine the influence of p53-deficiency on mammary tumorigenesis (Donehower et al., 1995). The MMTV-Wnt-1/p53^{-/-} mice developed tumors earlier than MMTV-Wnt-1/p53^{+/-} and MMTV-Wnt-1/p53^{+/+} mice suggesting that inactivation of p53 is important in Wnt-1-mediated tumorigenesis.

Like *Wnt-1*, *Wnt-10b* is also located on chromosome 12q13 in humans. Wnt-10b, like Wnt-1, was also identified as a protooncogene activated by viral insertion in mouse mammary tumors. In mice, Wnt-10b is expressed during the formation of the mammary rudiment in embryos and its expression continues through puberty. The Leder laboratory generated Wnt-10b transgenic mice with Wnt-10b under the transcriptional control of the MMTV promoter/enhancer (MMTV-Wnt-10b) (Lane & Leder, 1997). Overexpression of Wnt-10b resulted in profound developmental alterations in the mammary gland of both virgin females and males, with increased susceptibility to mammary adenocarcinomas. The mammary adenocarcinomas that formed in the MMTV-Wnt-10b mice were solitary indicating Wnt10b was necessary but not sufficient to bring about tumorigenesis. The FGF family of transcription factors tends to collaborate with Wnts, however FGF expression was not elevated in MMTV-Wnt-10b tumors. Crossing MMTV-Wnt-10b mice with MMTV-FGF3/int-2 mice produced sterile offspring with highly disorganized mammary epithelium, suggesting potent interaction between Wnt-10b and FGF3 signaling pathways.

2.3. Modeling angiogenesis through the vascular endothelial growth factor family

Angiogenesis is the development of tumor-associated vasculature that aids tumor growth and maintenance by supplying nutrients and oxygen and eliminating metabolic wastes and carbon dioxide. The growing tumor causes normal vasculature to sprout new vessels to sustain tumor growth and the process also aids in metastasis (Folkman, 1992; Hanahan & Weinberg, 2011). Transgenic mouse models based upon expression of vascular endothelial growth factor (VEGF) will be discussed in this section.

2.3.1 VEGF transgenic models—VEGF is a glycosylated protein that is located on chromosome 6p12. VEGF primarily affects endothelial cells and induces changes such as increased vascular permeability, promotes cell migration, inhibits apoptosis and induces angiogenesis, vasculogenesis and endothelial cell growth. Breast cancer patients show overexpression of VEGF (Yoshiji, Gomez, Shibuya, & Thorgeirsson, 1996) and VEGF is a prognostic indicator of relapse free and overall survival in node-negative breast cancer (Obermair et al., 1997).

Transgenic mice expressing human VEGF under the transcriptional control of the MMTV promoter (MMTV-VEGF) were generated by the Thorgeirsson laboratory (Schoeffner et al., 2005). VEGF expression was observed at high levels in the mammary gland and at lower levels in the salivary glands, lungs, and spleen in female transgenic animals. Only female transgenic mice were used in this study. Another laboratory reported that, in addition to mammary gland expression, MMTV-VEGF transgenic males expressed VEGF in the testis and epididymis that resulted in infertility (Korpelainen et al., 1998). Preliminary studies using heterozygous VEGF expressing females observed over a 2-year period showed no significant mammary gland abnormalities despite high levels of VEGF expression (Schoeffner et al., 2005). However, when MMTV-VEGF mice were crossed with MMTV-PyVmT mice in an attempt to induce metastatic mammary carcinomas in the double transgenic animals (MMTV-VEGF/ MMTV-PyVmT), mammary tumor latency was decreased as compared to the MMTV-PyVmT mice, and an increase in vasodilation in the tumors was observed at 4-weeks of age. By 6- to 8-weeks of age, mammary tumor incidence, multiplicity (number of tumors/mouse), and tumor weight was increased in the double transgenic mice compared to that of MMTV-PyVmT mice. As would be expected, double transgenic mice showed a dramatic increase in metastatic abilities as compared to MMTV-PyVmT mice. Thirty-three percent of tumor-bearing double transgenic mice had macroscopic and microscopic lung metastases by 6-weeks of age and by 8-weeks of age this increased to 100%. An increase in microvascular density and an increase in tumor cell proliferation and survival were responsible for enhanced tumor growth. The study helped elucidate that VEGF contributed to mammary tumor growth by a combination of increasing neovascularization, autocrine stimulation of growth, and inhibition of apoptosis.

2.4. Modeling tumor suppression mediated by tumor suppressor genes

Tumor suppressor genes play a crucial role in regulating and limiting cell growth and proliferation. Various tumor suppressor genes have been identified which function by several mechanisms including regulation of cell cycle progression, inhibition of uncontrolled growth factor receptor signaling, promoting apoptosis, and regulating DNA repair mechanisms. In general, the loss or inactivation of both alleles of a tumor suppressor gene promotes tumor development. The following transgenic models will be discussed in this section: tumor suppressors BRCA1 and p53.

2.4.1 BRCA1 knockout models—BRCA1 (breast cancer 1) is a nuclear phosphoprotein involved in maintaining genomic stability. Located on human chromosome 17q21, *BRCA1* acts as a tumor suppressor. Individuals carrying germline mutations in the *BRCA1* gene have a dramatically increased susceptibility to both breast and ovarian cancers. Germline

mutations of *BRCA1* are detected in about 50% of hereditary breast cancer patients and about 90% of hereditary breast and ovarian cancer patients (Casey, 1997; Hill, Doyle, McDermott, & O'Higgins, 1997). *Brcal* expression is elevated in mammary epithelial cells during pregnancy and downregulated during lactation in mice (Marquis et al., 1995). Homozygous *Brcal* knockout results in early embryonic lethality in mice, indicating the importance of *Brcal* in normal development (Gowen, Johnson, Latour, Sulik, & Koller, 1996; Hakem et al., 1996; Liu, Flesken-Nikitin, Li, Zeng, & Lee, 1996).

In order to investigate the role of *Brcal* loss in mammary tumorigenesis in mice, the Deng laboratory developed mammary gland specific conditional *Brcal* knockout mice using the Cre-loxP system (Xu et al., 1999). These mice carried one *Brcal*-null allele and one *Brcal* conditional knockout (floxed) allele, as well as either an MMTV-Cre or WAP-Cre transgene. Increased apoptosis and abnormal ductal development was observed in mammary epithelial cells in these animals. Mammary tumors were observed at a low frequency and following a long latency. The tumors exhibited genomic instability, including both aneuploidy and chromosomal rearrangements, resulting in genomic imbalances similar to those observed in human breast cancer (Weaver et al., 2002). It has been reported that *BRCA1* familial tumors frequently contain *p53* mutations (Deng & Scott, 2000). Thus, to investigate the potential role of *p53* in *Brcal*-associated tumorigenesis, the *Brcal* conditional knockout mice described above were bred to *p53* heterozygous knockout mice to generate mice lacking one *p53* allele, which resulted in a significant increase in both tumor frequency and kinetics of onset (Xu et al., 1999).

2.4.2 *p53* Transgenic and knockout models—The *p53* tumor suppressor gene, located on human chromosome 17p13.1, is the gene found to be most frequently mutated in human tumors overall, and is mutated in approximately 25% of breast tumors (Olivier, Hollstein, & Hainaut, 2010; Petitjean, Achatz, Borresen-Dale, Hainaut, & Olivier, 2007). In response to a wide range of cellular stressors, *p53* normally becomes activated and subsequently promotes several possible cellular responses, including cell cycle arrest, apoptosis, senescence, and DNA damage repair. Tumor-associated *p53* mutations lead to loss of these protective mechanisms, resulting in genomic instability and a dramatically increased susceptibility to cellular transformation. Patients with Li-Fraumeni syndrome, who carry a germline mutation in one *p53* allele, have a strong predisposition to development of a wide range of tumor types, the most frequent of which is breast cancer (Olivier et al., 2010; Petitjean et al., 2007). Additionally, patients with mutations in *BRCA1* and *BRCA2* also tend to show somatic *p53* abnormalities, illustrating that *p53* plays an important role in suppression of breast tumors (Blackburn & Jerry, 2002).

When *p53* knockout mice were originally generated, it was expected that complete *p53* deficiency would result in embryonic lethality, as is the case for many other tumor suppressor genes. It was therefore surprising to find that mice homozygous for the *p53* null allele did not display developmental abnormalities. However, both *p53*^{-/-} and *p53*^{+/-} mice developed a variety of tumor types, most notably lymphomas, and to a lesser extent, sarcomas, with a mean age of 6 months in *p53*^{-/-} mice (Donehower et al., 1992). However no mammary tumors were observed. Thus, although these mice model the overall tumor susceptibility seen in Li-Fraumeni patients, the tumor spectrum is quite different. Several

approaches have therefore been taken to create mouse models for examining the role of p53 mutation in mammary tumorigenesis. One approach was to backcross the p53 null allele onto a BALB/c mouse background, a strain that exhibits a higher rate of radiation-induced mammary tumorigenesis than many other strains (Ullrich, Bowles, Satterfield, & Davis, 1996). As with the original p53 null mice, both BALB/c-p53^{-/-} and BALB/c-p53^{+/-} mice developed multiple tumor types, with a shorter latency in the homozygous knockout mice (Kuperwasser et al., 2000). BALB/c-p53^{+/-} mice had a longer life span (54-weeks) as compared to BALB/c-p53^{-/-} mice (15.4-weeks). While the tumor spectrum in the BALB/c-p53^{-/-} mice was similar to that seen in the original p53^{-/-} mice, including primarily lymphomas and sarcomas, 55% of female BALB/c-p53^{+/-} mice developed mammary carcinomas, which was the most common tumor type seen in these mice. Further, the mammary tumors from the BALB/c-p53^{+/-} mice showed frequent loss of the remaining wild-type p53 allele, as do tumors arising in Li-Fraumeni patients. Thus, the BALB/c-p53^{+/-} mice represent a relatively faithful model of human Li-Fraumeni syndrome.

Another widely used approach for investigating the role of p53 mutation in mammary tumorigenesis has been to cross the p53^{-/-} mice with various transgenic mouse mammary tumor models to examine the effects of p53 deficiency on tumor properties in these lines of mice. For example, Hundley et al. crossed MMTV-v-Ha-ras transgenic mice with p53 null mice to generate MMTV-v-Ha-ras/p53^{+/+}, MMTV-v-Ha-ras/p53^{+/-} and MMTV-v-Ha-ras/p53^{-/-} mice (Hundley et al., 1997). As expected, the MMTV-v-Ha-ras/p53^{-/-} mice developed tumors more rapidly than mice of the other two genotypes. However, there was a surprising shift in the tumor distribution in the p53-deficient mice. While MMTV-v-Ha-ras/p53^{+/+} and MMTV-v-Ha-ras/p53^{+/-} mice developed primarily mammary tumors and few salivary tumors, the MMTV-v-Ha-ras/p53^{-/-} mice developed primarily salivary tumors, indicating that the acceleration of tumor onset resulting from p53 deficiency was more dramatic in the salivary gland than the mammary gland. Tumors arising in MMTV-v-Ha-ras/p53^{-/-} were also of a higher histologic grade and displayed increased tumor growth rates and increased genomic instability and heterogeneity, as compared to MMTV-v-Ha-ras/p53^{+/+} mice. The increased tumor growth rates in the MMTV-v-Ha-ras/p53^{-/-} mice were found to result from increased tumor cell proliferation rather than decreased apoptosis, indicating that p53-mediated tumor suppression can occur independent of its role in promoting apoptosis (Hundley, Koester, Troyer, Hilsenbeck, Subler, et al., 1997). Numerous other transgenic mouse tumor models have similarly been crossed with p53 null mice, as with the MMTV-Wnt-1 mice described above, generally resulting in accelerated tumor kinetics, and altered tumor properties consistent with the poorer prognosis seen in patients whose tumors carry p53 mutations.

The p53 models described above all employed p53 null alleles, from which no p53 protein is produced. While the majority of tumor-associated mutations seen in most tumor suppressor genes are null alleles, p53 is unique in that the majority of human tumor-associated mutations, including those in breast tumors, are missense mutations that result in the production of a full-length protein carrying a single amino acid substitution (Petitjean et al., 2007). Almost all of the missense mutations occur in the central half of the protein encoding the DNA binding domain, particularly, at certain “hot-spot” residues. These mutations all result in impaired DNA binding and transcriptional activation of target genes, and can thus

be considered loss-of-function mutations. However, many of these missense mutants also appear to possess gain-of-function properties, that is, they have acquired oncogenic properties that promote tumorigenesis and tumor progression (Muller & Vousden, 2013). To investigate the functions of p53 gain-of-function mutants, several lines of transgenic mice expressing mutant p53 genes under the control of cell type-specific promoters have been generated. Of relevance to this review is a line of transgenic mice developed by the Rosen laboratory in which mouse p53-R172H (carrying an arginine to histidine substitution at amino acid residue 172) was expressed under the transcriptional control of the WAP promoter (Li et al., 1998). This mutation in the mouse *p53* gene is equivalent to p53-R175H in the human *p53* gene, corresponding to one of the most common “hot-spot” mutations. The transgene did not affect normal mammary gland development and spontaneous tumors were not observed in these mice. However, the mice were more susceptible to mammary tumor development upon administration of the chemical carcinogen dimethylbenz(a)anthracene than wild-type littermates, and the tumors showed increased genomic instability.

3. DEVELOPING NOVEL THERAPEUTICS AND IMAGING TECHNIQUES USING TRANSGENIC ANIMALS

3.1. Evaluating the use of novel therapeutics using transgenic animals

The *in vivo* evaluation of potential anticancer therapies has traditionally been carried out almost exclusively using human tumor xenografts, mouse allografts, or tumor tissue explants grown in immunocompromised mice. There are numerous advantages to the use of such models, including the ability to study effects of investigational agents on actual human tumor cells carrying all of the genetic alterations present in the primary tumors from which they are derived (Neve et al., 2006), the availability of a large number of tumor cell lines representing a broad array of tumor types, and the ability to reproducibly generate a large cohort of tumor-bearing animals at one time. However, there are also several drawbacks to xenograft models, most notably that the host animals must be immunocompromised to permit engraftment of human cells, thus creating an artificial setting that does not model the effects of the immune system on tumor cell growth or response to treatment. In addition, for most xenograft approaches, the microenvironment surrounding the inoculated tumor cells very poorly models the normal microenvironment of a developing tumor. The use of GEM models for drug development can potentially provide a complementary approach that eliminates some of the drawbacks of xenograft models, since tumors arise in immunocompetent animals, and arise stochastically in the presence of a normal microenvironment. In addition, GEM models afford the unique opportunity to tailor the genetics of the tumor to specific targeted therapies being investigated. Because they have an intact immune system, GEM models are also well suited for evaluating vaccine approaches for the prevention of cancer.

Transgenic animals have been instrumental in testing the efficacy and mode of action of chemotherapeutics that are predicted to target the driving oncogene, either directly or indirectly. For example, cyclooxygenase 2 (COX-2) is overexpressed in HER-2/neu-positive human breast cancers and in mammary tumors from MMTV-neu mice. Hence celecoxib, a

selective COX-2 inhibitor, was evaluated for efficacy of tumor reduction in MMTV-neu transgenic mice (Howe et al., 2002). Treatment of MMTV-neu mice with celecoxib resulted in a decrease in the incidence of mammary tumors along with a 50% reduction in mammary prostaglandin E₂ levels. Similar results were obtained by another group that tested both COX-1 and COX-2 inhibitors (SC560 and celecoxib, respectively) in MMTV-neu mice (Lanza-Jacoby et al., 2003). Only celecoxib was able to significantly lower mammary tumor incidence, number of tumors, and increase tumor latency. As in the earlier study, the protective effect of celecoxib was thought to be mediated by reduction in prostacyclin and prostaglandin E₂.

Another group of chemotherapeutic agents, farnesyltransferase inhibitors (FTIs), have been tested in the MMTV-v-Ha-ras transgenic mouse model. Farnesyltransferase is an enzyme that mediates a posttranslational modification of ras required for its activity. Because of the prevalence of ras mutations in human cancer, considerable effort has been directed toward developing FTIs that could potentially block the function of mutant ras in tumor cells. The efficacy of the FTI L-744,832, developed by investigators at Merck Research Laboratories, was investigated in tumor-bearing MMTV-v-Ha-ras mice as a proof-of-principle that tumors carrying Ha-ras mutations would be sensitive to farnesyltransferase inhibition (Barrington et al., 1998). L-744,832 indeed promoted dramatic tumor regression in MMTV-v-Ha-ras mice, primarily *via* induction of apoptosis. This response was largely p53 independent, as tumors from MMTV-v-Ha-ras/p53^{-/-} mice also rapidly regressed in response to L-744,832. Tumors from MMTV-v-Ha-ras/ MMTV-c-myc mice also initially regressed in response to L-744,832, although the response was less durable than in the MMTV-v-Ha-ras mice. In contrast to each of the models carrying an activated Ha-ras transgene, no tumor regression was observed upon treatment of MMTV-c-neu mice with L-744,832. These studies demonstrated the utility of GEM models for evaluating novel agents that target a genetic component of the model.

Additionally, both novel and clinically utilized chemotherapeutics can be evaluated alone and in combination with other therapeutics in GEM models to determine treatments that would synergize and allow for enhanced tumor reduction. The tyrosine kinase inhibitor SU11248 was assessed both as a single agent and in combination with “standard of care” therapeutics in MMTV-v-Ha-ras transgenic mice and xenograft models (Abrams et al., 2003). SU11248 is an oral multitargeted tyrosine kinase inhibitor that has antitumor and antiangiogenic properties. Treatment of tumor-bearing MMTV-v-Ha-ras mice with SU11248 caused a dramatic regression of mammary tumors. In the xenograft models, treatment with SU11248 in combination with docetaxel, 5-fluorouracil or doxorubicin caused a 50% reduction in tumor growth.

To evaluate therapeutics for potential use in estrogen receptor-negative breast cancer patients, treatment with an EGFR inhibitor was evaluated in MMTV-neu (unactivated neu) mice, since these mice form estrogen receptor-negative mammary tumors. Gefitinib (Iressa or ZD1839) is an oral EGFR tyrosine kinase inhibitor. Treatment of MMTV-neu mice with Gefitinib, starting at 6 months of age, resulted in a suppression of mammary tumors with increased tumor latency. Gefitinib reduced proliferation of tumor cells and caused an increase in expression of the cell cycle regulator p27 (Lu et al., 2003).

Activities of drugs for chemoprevention, treatment, and inhibition of metastasis have also been evaluated in transgenic animals. For example, dipyridamole was tested as a chemoprevention strategy in MMTV-PyVmT transgenic mice. Dipyridamole treatment beginning at 4 weeks of age delayed onset of palpable tumors, delayed tumor progression, and suppressed lung metastasis (Wang, Schwab, Fan, Seagroves, & Buolamwini, 2013). The ability of an antimatrix metalloproteinase-9 DNzyme to decrease tumor growth of mammary tumors in the MMTV-PyVmT mouse model has also been evaluated. This DNzyme was able to reduce tumor growth and final tumor load when administered weekly through intratumoral injection (Hallett et al., 2013).

Transgenic animals have also been utilized to assess the effectiveness of cancer vaccines. The HER2/NEU protein was assessed for its potential as a tumor antigen for vaccination and a DNA vaccination strategy against Neu was developed and tested in transgenic animals. The neu gene was inserted into a novel plasmid ELVIS containing a Sindbis virus replicon that reproduces multiple copies of mRNA (Lachman et al., 2001). Immunization of MMTV-neu transgenic mice intramuscularly using this ELVIS-neu vaccine was protective against development of spontaneous mammary tumors. The vaccine also showed effectiveness in reducing incidence and number of lung metastasis in BALB/c mice following tumor cell injection in the mammary fat pad or tail vein, respectively. This vaccine was suggested to have potential use as a therapeutic vaccination for breast cancer patients with HER2/neu-expressing tumors to help reduce metastasis.

The efficacy of a novel tripartite cancer vaccine was also tested in transgenic animals. The tripartite vaccine consisted of an immunoadjuvant Pam₃CysSK₄, a peptide T_{helper} epitope, and an aberrantly glycosylated MUC1 peptide, designed to elicit both recognition by cytotoxic T lymphocytes (CTL) and antibody-dependent cell-mediated cytotoxicity (ADCC) responses against aberrantly glycosylated mucin MUC1 commonly found in breast cancer (Lakshminarayanan et al., 2012). MUC1 transgenic mice that express human MUC1 at physiological levels were first immunized using the tripartite vaccine. The mice were then injected with mammary tumor cells (obtained from tumors derived from MUC1 transgenic mice crossed with MMTV-PyVmT mice). A week after injection with tumor cells, a final immunization was administered to the mice. Interestingly, the vaccine was able to elicit both CTL and ADCC response and resulted in a significant reduction in tumor burden.

3.2. Imaging using transgenic animals

Various imaging techniques have been developed that aid in detecting tumors and metastasis, and noninvasively monitoring efficacy of therapeutic interventions in human breast cancer patients. In fact the field of imaging is rapidly growing. Using computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US), anatomical and physiological information can be obtained and these techniques are widely used both in the clinic and in preclinical applications. Imaging at the molecular level can be obtained using positron emission tomography (PET), single-photon emission computed tomography (SPECT), which are used in the clinic as well as fluorescence reflectance imaging (FRI), fluorescence-mediated tomography (FMT), bioluminescence imaging (BLI), laser-scanning confocal microscopy (LCSM), and multiphoton microscopy (MPM) which are being

optimized for future use in the clinic. Details on these imaging modalities have been reviewed by Condeelis and Weissleder (2010).

MMTV-Neu (activated neu) transgenic mice were utilized to follow the progression of mammary tumors using MRI and ultrasonography (Galie et al., 2004). US imaging was found useful for obtaining basic information such as the size of developing tumors as well as identifying necrotic areas. On the other hand, advanced analysis of morphological aspects was possible with MRI allowing high resolution of differentiation of details of necrotic area such as coagulation, liquefaction, biphasic splitting of cysts, and fibrotic and lipidic infiltration (Galie et al., 2004).

The Segall laboratory developed transgenic animals that specifically express green fluorescent protein (GFP) in mammary tumor cells to evaluate the behavior of tumor cells in mammary tumors (Ahmed et al., 2002). Mice with GFP expression under the transcriptional control of the MMTV promoter were generated. These mice expressed low levels of fluorescence in the mammary and salivary glands of transgenic animals. Next the MMTV-GFP mice were crossed with MMTV-PyVmT mice generating double transgenic MMTV-PyVmT/MMTV-GFP mice. These double transgenic mice developed GFP expressing tumors that showed high metastatic rates. Given the short tumor latency, high penetrance and reproducibility of tumor progression, this model is ideal for monitoring tumor progression and efficacy of therapeutic interventions using imaging. Another transgenic model was also developed by the same laboratory that used a Cre-activatable promoter, CAG-CAT-EGFP in combination with WAP-Cre or MMTV-Cre mice (Ahmed et al., 2002). Even more intense fluorescence was obtained in the mammary glands of virgin female mice. Of note, virgin MMTV-Cre/CAG-CAT-EGFP transgenic females showed more uniform GFP expression than virgin WAP-Cre/CAG-CAT-EGFP transgenic mice. To evaluate the potential of these transgenic mice in following mammary tumors the mice were crossed with MMTV-PyVmT mice and MMTV-neu mice. The mammary tumors that formed showed bright fluorescence. Interestingly, motile cells could be visualized within the tumor using multiphoton microscope and time lapse images showed a cell moving toward a blood vessel followed by intravasation (Ahmed et al., 2002). This study showed that *in vivo* imaging could be used to visualize metastatic properties in living tumors.

The ability of mammary tumor cells to intravasate into blood vessels and the role of macrophages in aiding this process was also evaluated using trans-genic animals (Wyckoff et al., 2007). Interaction between tumor cell and macrophages was observed using two-color fluorescent mice with mammary tumors, MMTV-PyVmT/MMTV-iCre-CAG-CAC-ECFP/c-fms-GFP (Mammary tumors were labeled with CFP (cyan fluorescent protein) and macrophages were labeled with GFP) (Wyckoff et al., 2004, 2007).

Although GFP-based imaging modalities are valuable, autofluorescence, and low tissue penetration of light emitted by GFP can lead to significant drawbacks in using this imaging modality. Bioluminescence based imaging modalities are more sensitive for broader *in vivo* applications. A transgenic mouse model was developed that utilized luciferase and bioluminescent imaging to monitor tumor progression *in vivo* (Zagozdzon et al., 2012). The transgenic mice expressed luciferase (Luc2, the next generation firefly luciferase, optimized

for high expression, and reduced anomalous transcription) under the transcriptional control of the MMTV promoter (MMTV-Luc2). Homozygous MMTV-Luc2 mice were crossed with MMTV- PyVmt mice to generate double transgenic mice with mammary tumors expressing luciferase, allowing visualization of tumor progression using bioluminescent imaging. Of note, metastasis, for example to the lung, was not easily visualizable in these transgenic mice. This double transgenic model is ideal for early detection and monitoring of mammary tumor progression.

Near-infrared non invasive optical imaging was also used for detection of microscopic mammary cancer in ErbB2 transgenic mice (Conti et al., 2013). DA364, a near-infrared fluorescence arginine-glycine-aspartic acid cyclin probe that binds the integrin $\alpha_v\beta_3$ and is then taken up specifically by cancer cells was utilized for imaging. This probe was, particularly, useful as it was not taken up by healthy mammary glands of both virgin and lactating wild-type mice indicating tumor specific uptake. DA364 was useful for non-invasive visualization of atypical hyperplasia and microscopic foci before mammary lesions were palpable. Since DA364 allowed visualization of early microscopic preneoplastic changes in the mammary gland, it was also useful to assess the efficacy of DNA vaccination against ErbB2.

Thus, transgenic animals are very useful models to optimize and develop the various imaging modalities. Future efforts are and will be directed towards developing higher resolution, cancer-specific imaging techniques that will be invaluable tools in the clinic.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

Transgenic mouse models have in the past and continue to provide invaluable insights into our understanding of the biology and molecular mechanisms underlying the progression of breast cancer (Figure 8.3). The ability to specifically overexpress/silence a single transgene has allowed us to determine the role of that transgene in the initiation, progression, and metastasis of breast cancer. Overexpressing multiple transgenes in the mammary gland have helped understand whether the genes act in concert to enhance mammary tumor progression or whether the two genes play differing roles in breast cancer. The influence of hormones and changes in genomic instability upon expression of transgene(s) can also be evaluated. Furthermore, one can evaluate the molecular mechanism of action of the transgene(s), potentially aiding in the design of better, more targeted therapeutics downstream of the transgene for treatment of human breast cancer. Generation of transgenic animals that express alternatively spliced isoforms (e.g., ErbB2) might help identify the biological importance of specific isoforms in breast cancer. Importantly, identification of a specific isoform that might be sufficient to transform the mammary gland will aid in therapeutic targeting by generating antibodies or drugs that specifically target the particular isoform, allowing for rationally targeted therapy of breast cancer. Transgenic animals also aid in our understanding of mechanisms underlying tumor resistance to chemotherapy and tumor dormancy and recurrence. Novel therapeutic interventions can be evaluated for efficacy in transgenic animals. In particular, therapeutic interventions that prove useful in transgenic mice that overexpress ErbB2, for example, will more likely be useful in patients that show ERBB2 overexpression. Consequently, we can target novel therapeutics to patients that are

most likely to respond to the treatment by first testing the drug in a genetically similar transgenic animal. Similarly, cell lines can be generated from the established transgenic mice that can be useful for preliminary screening of drugs and other novel therapeutics *in vitro* for future *in vivo* efficacy studies. Transgenic mice can also be used to evaluate novel vaccines that can prevent breast cancer. Further studies might also help elucidate the mechanisms involved in familial or hereditary breast cancer and will pave the way for therapeutics and early prevention strategies. Finally, development of novel imaging modalities evaluated in transgenic animals that aid in early detection of cancer will be extremely useful in detecting early tumors in patients so that therapeutic intervention is possible before metastatic spread.

In these contexts, while no transgenic animal is fully able to recapitulate the different breast tumors that arise in humans, they have enabled an enhanced understanding of at least some of the complex molecular events involved in the pathogenesis of breast cancer. Current and newer models will help in developing improved methods for identifying, treating, and potentially preventing breast cancer formation and progression to metastasis.

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Figure 8.1. Schematic of embryonic mammary gland development in mice

(A) Cross-sectional view through the trunk of the mouse during mammary gland development. The milk line (orange) is formed by a slight thickening and stratification of the ectoderm (gray) at around embryonic day 10 (E10). The milk line breaks up into individual placodes (orange) and condensation of the underlying mammary mesenchyme (blue) begins on E11.5. Placodes sink deeper into the dermis and the mammary mesenchyme is organized in concentric layers around the mammary bud (orange) over the next few days. Proliferation of the mammary epithelium (orange) begins at the tip and the primary sprout pushes through the mammary mesenchyme toward the fat pad (green) starting on day E15.5. The elongating duct grows into the fat pad and branches into a small ductal system on E18.5. Cells of the mammary mesenchyme form the nipple consisting of specialized epidermal cells (purple). (B) Schematic of the position of the milk line, placodes, and mammary buds along the lateral body wall of early mouse embryos. *Reprinted with permission from MacMillan Publishers Ltd: Nature Reviews Genetics (Robinson, 2007), copyright 2007.*

Figure 8.2. The biology of breast cancer

(a) Breast cancer is a multifaceted disease that is genetically and genomically heterogeneous. The normal breast terminal ductal lobular unit (TDLU) is comprised of lobules and ducts that consist of bilayered epithelium of luminal and myoepithelial cells. Characterized by abnormal cells within the duct or lobule, atypical ductal hyperplasia (ADH) is a premalignant lesion. ADH is thought to be followed by ductal carcinoma *in situ* (DCIS), a noninvasive lesion with abnormal cells. Some of the noninvasive DCIS lesions might eventually give rise to invasive breast cancer (IBC). Finally cells that have gained invasive capabilities might metastasize to a distant area in the body. In breast cancer, the primary site of metastasis (MET) is the lymph nodes. (B) Schematic of the progression of breast cancer. Various factors play a role in the transformation of breast epithelial cells to eventually give rise to metastatic breast cancer. This multistep process involves loss of control of proliferation, gain in survival abilities, increased migration, and aberrant tumor–stromal cell interactions that facilitate transformation. Cells must invade through the basement membrane, intravasate into the blood circulation, survive, extravasate from the blood circulation, and establish a new tumor at a distant site in the body, for metastasis to develop. *Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Vargogogola & Rosen, 2007), copyright 2007.*

Figure 8.3. Applications of transgenic animals in the study of breast cancer

Transgenic animals have greatly advanced our understanding of the biology of breast cancer. They have been invaluable tools in evaluating the role of single or multiple transgenes in the progression of breast cancer and evaluating the impact of factors that might influence the initiation and/or progression of breast cancer. Of particular significance, transgenic animals have been used to evaluate the role of novel therapeutics to target breast cancer, to evaluate novel imaging techniques, and to evaluate the efficacy of newly developed vaccines against breast cancer.

Table 8.1

Mammary gland-specific promoters

Promoter	Origin	Expression	Activation
MMTV-LTR	Mouse mammary tumor virus	Breast epithelial cells, several other tissues	Steroid hormones
WAP	Whey acidic protein	Secretory mammary epithelium	Lactogenic hormones
C3(1)	Rat prostate steroid-binding protein (PSBP)	Epithelial cells of prostate and mammary gland	Estrogen (ductal and alveolar mammary epithelium)
BLG	Bovine β -lactoglobulin	Mammary gland	Pregnancy and lactation
MT	Metallothionein	Most mammary cells	Zn ²⁺

The various mammary gland specific promoters used to drive mammary gland-specific expression of transgenes are shown in this table.

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