Published in final edited form as:
Expert Rev Mol Diagn. 2009 July ; 9(5): 423–440. doi:10.1586/ERM.09.31.

MMTV mouse models and the diagnostic values of MMTV-like sequences in human breast cancer

Pankaj Taneja,
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Donna P Frazier,
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Robert D Kendig,
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Dejan Maglic,
Departments of Pathology, Cancer Biology, and Molecular Medicine Graduate Program, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Takayuki Sugiyama,
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Fumitake Kai,
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Neetu K Taneja, and
Departments of Pathology, and Cancer Biology, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Kazushi Inoue[†]
Departments of Pathology, Cancer Biology, and Molecular Medicine Graduate Program, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA

Abstract

Mouse mammary tumor virus (MMTV) long terminal repeat (LTR)-driven transgenic mice are excellent models for breast cancer as they allow for the targeted expression of various oncogenes and growth factors in neoplastic transformation of mammary glands. Numerous MMTV-LTR-driven transgenic mouse models of breast cancer have been created in the past three decades, including MMTV-neu/ErbB2, cyclin D1, cyclin E, Ras, Myc, int-1 and c-rel. These transgenic mice develop mammary tumors with different latency, histology and invasiveness, reflecting the oncogenic

†Author for correspondence: The Departments of Pathology, Cancer Biology, and Molecular Medicine Graduate Program, Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157, USA, Tel.: +1 336 716 5863, Fax: +1 336 716 6757, kinoue@wfubmc.edu.

Financial & competing interests disclosure: The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

pathways activated by the transgene. Recently, homologous sequences of the *env* gene of MMTV have been identified in approximately 40% of human breast cancers, but not in normal breast or other types of cancers, suggesting possible involvement of mammary tumor virus in human breast carcinogenesis. Accumulating evidence demonstrates the association of MMTV provirus with progesterone receptor, p53 mutations and advanced-stage breast cancer. Thus, the detection of MMTV-like sequences may have diagnostic value to predict the clinical outcome of breast cancer patients.

Keywords

breast cancer; c-rel; cyclin D1; cyclin E; HMTV; int-1; mouse mammary tumor virus; Myc; neu/ErbB2/HER2; p53; prognosis; Ras; transgenic mouse

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Mouse mammary tumor virus (MMTV; also known as Bittner virus) is an oncoRNAvirus of the *Retroviridae* family, which causes breast tumors once activated [1–4]. Efficient replication of MMTV occurs predominantly in the alveolar epithelial cells of the mammary gland. During lactation, MMTV expression markedly increases under the influence of steroid hormones. Moreover, inbred strains of mice (e.g., C3H) that contain dominantly expressed integrated MMTV genomes (provirus) can also transmit replication competent viruses genetically [1–6]. By contrast, among mouse strains derived from feral animals devoid of integrated MMTV (e.g., Czech II mice), viral infections are transmitted solely by the milk of an infected mother and are not usually transmitted horizontally between adult mice [6–8]. In cases of milk-borne transmission, virions pass through the gut wall and, via the gastric vein, to the spleen where the virus infects lymphoid cells [1]. The MMTV induces premalignant lesions and malignant tumors of the mammary gland by acting as an insertional mutagen or activating transcription of nearby oncogenes [1,2,9]. Molecular analysis of proviral integration sites in tumors has led to the discovery of a number of cellular proto-oncogenes. *Int-1* (*Wnt1*) was the first gene discovered to be activated by an integrated provirus; since then, *int-2–6* genes have also been isolated and characterized [2].

Promoters of *MMTV-LTR*, *WAP*, *BLG* and *C(3)1* have been frequently used to create transgenic mouse models of breast cancer. Among these, MMTV-LTR has most frequently been used to express a gene of interest in the mammary epithelium since the promoter is active in both nonlactating and lactating females.

D-type cyclins (D1, D2 and D3) are induced in the context of a delayed early response to growth factor stimulation. In the presence of mitogens, cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6) are synthesized and assemble with their D-type cyclin catalytic partners [10,11]. Cyclin D-Cdk holoenzymes have two distinct functions in promoting progression through the G1 phase of the cell division cycle: catalysis of the phosphorylation of the retinoblastoma protein (pRb) and accumulation of cyclin D-Cdk holoenzymes that recruit Cdk inhibitors (e.g., $p27^{Kip1}$ and $p21^{Cip1}$) into higher order complexes, thereby neutralizing their effects on other Cdks. This process facilitates the activation of cyclin E-Cdk2 later in the G1 phase and the release of E2F proteins from the constraint of Rb and histone deacetylases [10,11]. $p19^{Arf}$ is an alternative reading frame gene product generated from the *Ink4a/Arf* locus that directly binds to Mdm2, thereby stabilizing and activating p53. Since this single genetic locus encodes two independent tumor-suppressor proteins that regulate the p53 and the Rb pathways, it is frequently (~40%) disrupted in human cancers [10,11].

As of the date of this publication, more than 50 transgenic mouse models of breast cancer have been generated. This review gives a brief description of MMTV-*neu/ErbB2*, *cyclin D1*, *cyclin E*, *Ras*, *Myc*, *int-1/Wnt1* and c-*rel* models and their relevance to human breast cancer. *neu/*

ErbB2, *cyclin D1*, *cyclin E*, *Ras*, *Myc*, *int-1/Wnt1* and c-*rel* have been selected based on the fact that these genes are frequently overexpressed or mutated in human breast cancers. Moreover, the products of these genes influence the expression levels of cyclin D1, which plays critical roles in breast cancer development. Tumor-free survival, accelerating factors and histopathology of single and compound transgenic mice are summarized in Table 1. The signaling cascades involving these molecules are depicted in Figure 1.

MMTV-*neu* & MMTV-*ErbB2* models

ErbB2 (neu/HER2), along with ErbB1 (HER1), ErbB3 (HER3) and ErbB4 (HER4), comprise the EGFR family of receptor tyrosine kinases (RTKs), which form homo- and hetero-dimers in response to ligand stimulation [12,13]. HER1–HER2 and HER2– HER3 dimerization leads to activation of MAP kinase and phosphatidyl inositol 3-kinase (PI3K) pathways, respectively, leading to cell cycle proliferation signals. *ErbB2* genomic amplifications have been reported in 20–30% of human breast cancers [14–16]. Furthermore, its overexpression is observed in invasive human ductal carcinomas and is observed in benign breast disorders, such as hyperplasias and dysplasia, although less frequently [17,18]. It is also important to note that *ErbB2* overexpression serves as a clinically useful prognostic marker. Rat *neu* possesses a valine to glutamic acid substitution in its transmembrane domain that results in the constitutive aggregation and activation of the receptor in the absence of ligand [19–23]. Although the transmembrane point mutation has not been detected in primary human breast cancers overexpressing ErbB2, studies have detected an alternative splice form in human breast cancers [24,25]. Additionally, MMTV-*neu* breast tumors exhibit sporadic mutations in *neu* resulting in its constitutive activation. Mutations commonly encountered include multiple frame deletions, insertions or point mutations in the extracellular domain of the neu protein. Formation of intermolecular disulfide bonds further promotes the transforming ability of this transgene [29,30]. Transgenic mice (MMTV-*neu*) expressing activated *neu* develop multifocal mammary tumors at a median age of 5–10 months with frequent potential to metastasize to the lung [26–28]. Typical pictures of mammary tumors from MMTV-*neu* mice (7 months old) and immunohistochemical staining of cyclin D1, Cdk4 and Ki-67 are shown in Figure 2. In males, bilateral enlargement of parotid glands and epididymis has been reported [26]. This phenotype was exhibited in carriers of TG.NF and TG.NA strains and was characterized by both hypertrophy and hyperplasia in the salivary gland epithelium. Although both mucous and serous glands showed some degree of dysplasia, none of these animals developed overt neoplasia [26]. Dysplasia of Harderian glands has also been reported in TG.NF, TG.NA and TG.NB strains of MMTV-*neu* mice (Table 2). It should be noted that these tissues of extramammary tumor development correspond to those where the MMTV-LTR is known to be transcriptionally active [26].

Mice strains bearing the wild-type *ErbB2* allele under the control of the *MMTV* promoter have also been established (MMTV-unactivated *neu*; MMTV-*ErbB2* in Table 1) [31]. In contrast to the rapid tumor progression observed in several transgenic strains carrying the activated *neu* transgene, expression of unactivated *neu* in the mammary epithelium resulted in the development of focal mammary tumors with longer latency than wild-type *neu* (8–12 months dependent on the strain, Table 1). Overexpression of neu in these mammary tumors was associated with elevated intrinsic tyrosine kinase activity of neu and the *de novo* tyrosine phosphorylation of several cellular proteins. Interestingly, many of the tumor-bearing transgenic mice developed secondary metastatic lesions in the lung. These observations suggest that overexpression of the unactivated neu protein can induce metastatic disease after long latency [31]. Li *et al.* examined the role of mutations in *p53* in mammary tumor development in these mice [32]. They found that 37% of tumors arising in these mice had missense mutations in *p53*. They have directly tested for cooperativity between wild-type ErbB2 and mutant p53 in mammary tumorigenesis by creating bitransgenic mice carrying MMTV-*ErbB2* (line

N#202) and WAP-*p53–172H*. In these bitransgenic mice, tumor latency was shortened to 154 days, indicating strong cooperativity (Table 1) [32]. Tumors arising in the WAP-*p53–172H*; MMTV-*ErbB2* bitransgenic mice were anaplastic and aneuploid and exhibited increased apoptosis, in contrast to tumors arising in *p53*-null mice. In these bitransgenic mice, Li *et al*. recapitulated the two common genetic lesions that occur in human breast cancer and showed that *p53* mutation is an important cooperating event in wild-type neu/ErbB2-mediated oncogenesis [32].

$p16^{INK4a}$ is deleted in 40–60% of human breast cancer cell lines, and $p16^{INK4a}$ inactivation by DNA methylation occurs in 20–30% of human breast cancers [33]. To test the effects of *Ink4a/Arf* inactivation in ErbB2-induced breast carcinogenesis, MMTV-*ErbB2* mice were crossed *Ink4a/Arf*-null mice [33]. Compared with MMTV-*ErbB2; Ink4a/Arf*$^{+/-}$ mice, MMTV-*ErbB2; Ink4a/Arf*^{wt} mammary tumors showed increased $p16^{Ink4a}$, reduced Ki-67 expression and reduced cyclin D1 protein, but increased mammary tumor apoptosis with no significant change in breast tumor-free survival. Since Eμ-*Myc*-induced lymphomagenesis was reported to be accelerated in *Ink4a/Arf*$^{+/-}$ mice, their study demonstrated that the contribution of *Ink4a/Arf* heterozygosity to tumor progression was tissue-specific *in vivo* [33]. The contribution of homozygous deletion of the *Ink4a/Arf* locus was not clear in their study due to the fact the *Ink4a/Arf*-null mice spontaneously developed other tumors faster than MMTV-*ErbB2* mice [33].

In human breast cancer with *HER2* amplification, the c-*ErbB2* gene is controlled by its own promoter. To test whether expression of activated *neu* under the control of the endogenous promoter in the mammary gland contribute to breast cancer development, knock-in mice for activated *neu* were created, which showed accelerated lobulo-alveolar development and formation of focal mammary tumors after a long latency period (Table 1) [34]. However, expression of activated *ErbB2* under the endogenous promoter was not sufficient for the initiation of mammary carcinogenesis. Interestingly, relative to the wild-type allele, all tumors isolated from these mice bear amplified copies (from two to 22 copies) of the activated *ErbB2* allele and express highly elevated levels of the *ErbB2* transcript and protein. Thus, similar to human HER2-positive breast carcinomas, mammary tumorigenesis in this mouse model requires the amplification and elevated expression of the *ErbB2* gene and protein [34–36]. Interestingly, in comparison to MMTV-*neu* mice, the *ErbB2*-knock-in mice showed significantly increased tumor latency and an extremely low metastasis rate [34]. Gene-expression profiling of tumor RNA from MMTV-*neu* and *ErbB2*-knock-in mice revealed distinctive and nonoverlapping patterns of gene expression. Consistent with the noninvasive nature of the mammary tumors induced by expression of *neu* under the endogenous promoter, the tumors from *ErbB2*-knock-in mice expressed a number of markers characteristic of a highly differentiated state. In addition, these knock-in tumors expressed elevated levels of two genes (*Grb7* and *Cab1*) closely linked to *ErbB2* that are often co-amplified in human noninvasive ductal carcinoma *in situ* [35,36]. These studies demonstrate that the promoter used to drive *ErbB2* expression, a key regulator of gene/protein expression levels, developmental stages, and the cell types where *ErbB2* is expressed, significantly affects breast tumor phenotypes. In summary, like HER2-positive human breast carcinomas, mammary tumorigenesis in mice requires gene amplification and elevated expression of the *ErbB2* gene.

MMTV-*cyclin D1*

Cyclins are the major regulators of activation of the cyclin-dependent kinases (Cdks), inducing cell cycle progression, S-phase entry and DNA replication [10,11]. Endogenous cyclin D1 expression is essential for normal alveolobular mammary gland development [37]. The human *Cyclin D1* gene is located on human chromosome 11q13 and is amplified in approximately 15% of breast cancers, which is associated with poor prognosis of estrogen receptor (ER)-

positive patients [38,39]. On the other hand, the cyclin D1 protein is overexpressed in more than 50% of human breast cancers and is associated with improved relapse-free and overall survival, possibly because breast tumors without cyclin D1 expression have mutations of other genes, such as RB [40–42]. Thus, the interpretation of prognostic studies for cyclin D1 in breast cancer is complicated, and further investigations with a larger number of patients and a longer follow-up period are required to ascertain the true prognostic value of cyclin D1 overexpression in breast cancer.

Overexpression of cyclin D1 through the *MMTV* promoter results in proliferative abnormalities leading to the onset of focal mammary tumors around 18–22 months (Table 1) [43,44]. As with the *c-Myc* transgenic models, the long latency and focal nature of these tumors suggests that although cyclin D1 can promote mammary tumorigenesis, additional genetic changes are needed for the development of overt mammary carcinomas. Moreover, activation of Src kinases, integrin linked kinase and ErbB2 induces a cyclin D1 neoplastic response [45]. *cyclin D1* is also a critical target in MMTV-*neu*- and MMTV-*ras*-induced breast carcinogenesis, but not in MMTV-*Myc* or -*int-1* mice (Figures 1 & 2) [46]. Mice lacking *Cdk4* have resistance to mammary carcinomas triggered by the *ErbB2* oncogene [47]. In fact, cyclin D1–Cdk4 complexes are needed to induce breast tumor cell proliferation [47]. Collectively, these observations suggest that cyclin D1 plays a critical role in both normal mammary gland development and mammary tumorigenesis.

Cyclin D1 levels are maintained at steady state by phosphorylation-dependent nuclear export and polyubiquitination by SCF (FBX4-αB crystallin) [44]. The contribution of stable cyclin D1 to breast cancer development has not been evaluated. Lin *et al.* generated transgenic mice where expression of a stable cyclin D1 allele (D1T286A) is regulated by MMTV-LTR [44]. MMTV-D1T286A mice developed mammary adenocarcinomas at a significantly increased rate (18 months) relative to MMTV-D1 mice (22 months) (Table 1). Similar to human cancers that overexpress cyclin D1, D1T286A tumors were ER-positive and exhibited estrogen-dependent growth. Their results indicated that temporal control of cyclin D1 subcellular localization and proteolysis is critical for maintenance of homeostasis within the mammary epithelium [44].

It has been reported that the basal-like subtype of human breast carcinomas is associated with invasiveness, high rates of post-surgical recurrence and poor prognosis. Little is known about the molecular mechanisms that cause basal breast carcinomas. Corsino *et al.* created heterogeneous MMTV-*cyclin D1-Cdk2* (MMTV-*D1K2*) transgenic mice and found that the mammary tumors contain regions of spindle-shaped cells expressing both luminal and myoepithelial markers [48]. Cell lines cultured from these tumors exhibited the same luminal/myoepithelial mixed-lineage phenotype that is associated with human basal-like breast carcinomas and expressed a number of myoepithelial markers. The MMTV-*D1K2* breast tumor-derived cell lines formed highly invasive tumors when injected into mouse mammary glands [48]. Invasion was associated with loss of E-cadherin expression or E-cadherin localization to the cytoplasm. They also showed that cytoplasmic E-cadherin correlated with lack of colony formation *in vitro* and β-catenin and p120(ctn) localization to the cytoplasm. Together, their results suggest that abnormal cyclin D1 and Cdk2 activation may contribute to the formation of basal-like breast carcinomas [48].

MMTV-*cyclin E*

Cyclin E is a G_1 cyclin necessary for the transition from G_1 to S phase of the normal cell cycle [35,36]. Overexpression of cyclin E accelerates the entry of the cells into S phase, but causes inefficient G_1–S progression. The untimely expression of cyclin E has been shown to interfere with the replication complex assembly as cells exit mitosis [49]. Oncogenic roles for cyclin E

have been demonstrated by studies with *cyclin E*-deficient cells that are resistant to transformation by Myc alone, Myc and mutant Ras, dominant-negative p53, or E1A, suggesting that cyclin E is a key component in oncogenic signaling [50]. Constitutive overexpression of cyclin E protein at all phases of the cell cycle is one of the features observed in breast cancer and is considered to result in premature DNA replication, genomic instability and, ultimately, breast carcinogenesis [51–53].

In several breast cancer cell lines, amplification of the *cyclin E* gene and constitutive expression of the protein have been identified [54–56]. Keyomarsi *et al.* reported that some of the breast cancer cell lines overexpressed up to five low-molecular-weight (LMW) isoforms in addition to overexpressing the full-length 50-kDa cyclin E protein [54–56]. These LMW isoforms are unique to tumor cells, suggesting that they may play specific roles in tumorigenesis. A study with 395 breast cancer samples showed that high levels of total or LMW cyclin E were found in 32.2% of the samples and was the most powerful parameter of short disease-free and overall survival of breast cancer, outperforming criteria currently used clinically, including positive nodes, late-stage (stage III–IV) disease and negative ER status [57].

To study the oncogenic potential of the LMW forms of cyclin E in breast carcinogenesis, transgenic mice expressing full-length cyclin E alone, full-length and the EL4 isoforms, or the EL2/3 isoforms of cyclin E were generated under the control of MMTV-LTR (Table 1) [58]. Compared with full-length cyclin E, LMW cyclin E overexpression induced delayed mammary growth during the pubertal phase and abnormal cell morphology during lactation. Both primary mammary tumor formation and metastasis were markedly enhanced in LMW cyclin E transgenic mice. LMW cyclin E overexpression in mammary epithelial cells of mice was sufficient by itself to induce mammary adenocarcinomas in 34 out of 124 (27.4%) animals compared with seven out of 67 (10.4%) mice expressing only the full-length cyclin E ($p < 0.05$) (Table 1). In addition, metastasis was seen in 25% of LMW cyclin E tumor-bearing animals compared with only one out of seven (14%) of tumors in the full-length cyclin E background ($p < 0.05$) (Table 1). Moreover, LMW cyclin E overexpression selected for inactivation of *p53* by loss of heterozygosity and spontaneous and frequent inactivation of $p19^{Arf}$, canceling the protective checkpoint function of the Arf-p53 pathway and accelerating progression to breast cancer [58].

MMTV-*Ras*

Activating mutations in the *Ras* oncogenes are found in approximately 30% of human malignancies [59–63]. Mutant H-*Ras*, N-*Ras* and K-*Ras* occur in varying frequencies in different tumor types, for reasons that are not completely understood. Other members of the Ras superfamily may also contribute to human cancer development. Mutations also occur in downstream pathways of Ras signaling, notably B-Raf, PTEN and PI3K. These pathways interact at multiple points, including cyclin D1, and act synergistically. Drugs blocking elements of these pathways are in different stages of clinical development [63]. MMTV-*Ras* mice have been created by placing an activated v-Ha-*Ras* under the control of the MMTV-LTR (Table 1) [64]. Approximately 20% of MMTV-v-Ha-*Ras* transgenic mice develop bilateral exophthalmos that is the result of progressive enlargement of the Harderian glands (Table 2). Malignant tumors arose stochastically among transgenic mice between 6 and 12 months and histological examination consistently revealed the presence of mammary adenocarcinomas. Both male and female MMTV-*Ras* transgenics develop mammary and salivary gland tumors (Tables 1 & 2) [64]. Crossing MMTV-*Ras* mice with *p53*-knock-out mice resulted in dramatic acceleration of mammary tumor progression (Table 1) [65]. Mammary tumors found in these MMTV-*Ras; p53*-null compound mice also displayed higher histological grades, increased growth rates, extensive genomic instability and dramatically shortened survival (~3 months) compared with those found in the MMTV-*Ras* transgenic alone

(9–10 months; Table 1) [65,66]. In another study with MMTV-*Ras* mice, $p21^{cip1}$-deficiency resulted in an acceleration of both mammary and salivary tumor onset [67]. In addition, it was shown that activated *Ras* has greater transforming potential in keratinocytes from $p21^{Cip1}$-deficient mice than from $p21^{Cip1}$ wild-type mice, suggesting that $p21^{Cip1}$ suppresses tumorigenesis by Ras [68,69].

MMTV-c-*Myc*

Myc proteins regulate oncogenic response by their ability to both activate and repress target genes [70,71]. Myc, along with Cdc6 and Orc-1, regulates the initiation of DNA replication [72]. Expression of the c-*Myc* protooncogene is upregulated in as many as 80% of human breast cancers and c-*Myc* amplification is associated with poor prognosis of patients [73]. MMTV-*Myc* mice develop spontaneous mammary adenocarcinomas by 10–15 months of age (Table 1) [74,75]. Furthermore, the *WAP* promoter can elevate expression of Myc and accelerate the formation of tumors in multiple mammary glands as early as 2 months [75].

In another experiment, interbreeding of MMTV-v-Ha-*Ras* strains with the MMTV-c-*Myc* mice produced bitransgenic mice expressing both c-*Myc* and activated *Ras*. This new strain developed focal mammary tumors with a dramatically shortened latency period (Table 1), indicating that c-*Myc* and activated *Ras* cooperate to accelerate mammary tumor development with pronounced malignancy [64]. Simultaneous overexpression of c-*Myc* and mutant *Ras* also changed the tumor spectrum in extra-mammary tissues, with increased incidence of B-cell lymphomas (38% in bitransgenic mice and 3% in Ha-*Ras*-transgenic mice) and occurrence of seminal vesicle neoplasia (5% in bitransgenic mice) (Table 2) [64]. Of note, one out of 21 bitransgenic mice developed three different tumors in the same animal and one out of 21 developed four independent primary tumors [64]. Together, their results indicated that the incidence of B cell lymphomas dramatically increased as a consequence of coexpression of the v-Ha-*Ras* and the c-*Myc* transgenes [64].

Generally, overexpression of c-*Myc* in normal cells induces apoptosis with simultaneous activation of the Arf-p53 pathway and Apaf-1 caspase-9 [76,77]. Conversely, the *Ras* oncogene has been demonstrated to inhibit cellular susceptibility to apoptosis in several experimental systems [59–63]. MMTV-*Myc* mammary tumors had much higher levels of spontaneous apoptosis than MMTV-*Ras* tumors, whereas intermediate levels were observed in MMTV-*Myc/Ras* double transgenic mammary tumors. Significant differences in cell cycle characteristics were also observed in tumors from mice of the three genotypes [69]. Tumors from MMTV-*Myc* mice had lower G_1- and higher S-phase fractions than MMTV-*Ras* tumors, with intermediate values again observed in the MMTV-*Myc/Ras* tumors.

The cell cycle-promoting phosphatase cdc25A is an activator of Cdks and one of the downstream targets for the Chk1-mediated checkpoint pathway [78]. The Myc/Max heterodimer binds to elements in the *cdc25A* gene and activates transcription. Similar to Myc, *cdc25A*, itself a protooncogene, can induce apoptosis in cells depleted of growth factor. Myc-induced apoptosis also requires *cdc25A*. These findings indicate that *cdc25A* is a physiologically relevant transcriptional target of c-Myc [79]. Whereas cdc25A overexpression is observed in various human cancers, it has not been determined if deregulated cdc25A expression triggers or promotes breast tumorigenesis *in vivo*. Ray *et al.* created MMTV-*cdc25A* mice and showed that the transgenics exhibit alveolar hyperplasia in the mammary tissue, but do not develop spontaneous mammary tumors [78]. However, the MMTV-*cdc25A* transgene markedly shortened the latency of mammary tumorigenesis in MMTV-*ras* mice, consistent with the fact that *cdc25* is a critical target of c-Myc in cell cycle progression [78,79].

MMTV-*Myc* mice are also responsive to $p21^{Cip1}$ expression [69]. The paradoxical possibility that $p21^{Cip1}$ can function as either a negative or positive regulator of Cdk activity has been demonstrated by both biochemical and cell culture studies [80,81]. MMTV-*Myc*; $p21^{Cip1-/-}$ tumors displayed a significantly reduced number of cells in S phase, accompanied by a decrease in both cyclin D1- and cyclin E-associated Cdk activity. Also, $p21^{Cip1}$ promotes an increase in G1 cyclin/Cdk activity, leading to the higher S-phase fractions seen in tumors from MMTV-*Myc* mice. McCormack *et al*. reported lack of both *p53* alleles in the presence of c-Myc overexpression resulted in a dramatic hyperplastic alteration in mammary gland development [82]. Specifically, in female bitransgenic MMTV-*Myc*; *p53*-null mice (MMTV-*Myc*; $p53^{-/-}$), lobular hyperplasias were observed at almost every ductal end bud as early as 32 days of age. By contrast, only mild ductal and lobular hyperplasias were seen in MMTV-*Myc* mice that contained both *p53* alleles (MMTV-*Myc*; $p53^{+/+}$); an intermediate phenotype occurred in mice with a single intact *p53* allele (MMTV-*Myc*; $p53^{+/-}$). Spectral karyotyping analysis revealed that there were multiple chromosomal alterations in the c-Myc-overexpressing cells that contained either one or two unmutated *p53* allele(s). Mammary carcinomas arose with a high frequency in MMTV-*Myc*; $p53^{+/-}$ mice; the tumors were comparable in frequency, histology and apoptotic index to the tumors in MMTV-*Myc*; $p53^{+/+}$ mice. Lymphomas also developed with extremely short latency in MMTV-*Myc*; $p53^{-/-}$ mice, precluding study of the fate of their hyperplastic mammary lesions *in situ* [82].

MMTV-*int-1*

The *int* genes were originally identified as oncogenes activated by the insertion of MMTV in virus-induced mammary adenocarcinomas [2]. The *int* genes fall into three groups: the *Wnt* (*int-1/Wnt1*, *int-4/Wnt3*, *Wnt10b*) family, the *Fgf* family (*int-2/Fgf3*, *Hst/Fgf4*, *Fgf8*), and others (*int-3/Notch4*, *int-5/aromatase*, *int- 6*) [2,83–85]. Generally, the Wnt signaling pathway plays critical roles in cell proliferation and differentiation, cell movement and polarity, and the self-renewal capacity of stem cells [86,87]. The canonical Wnt signaling pathway is shown in Figure 1. Once bound by Wnt, the Frizzled (Fz)/low-density lipoprotein-related protein (LRP) coreceptor complex activates the canonical signaling pathway. Fz interacts with Dishevelled (Dsh), a cytoplasmic protein that functions upstream of β-catenin and the kinase GSK3β. Wnt signaling controls phosphorylation of Dsh and induces the phosphorylation of LRP by GSK3β and casein kinase I-γ (CK1γ), thus regulating the docking of Axin. The recruitment of Axin away from the destruction complex leads to the stabilization of β-catenin. In the nucleus, β-catenin displaces Groucho from Tcf/Lef to promote the transcription of Wnt target genes, one of which is *cyclin D1* (Figure 1).

The most convincing evidence for the involvement of Wnt signaling in human breast cancer is the overexpression of nuclear and/or cytoplasmic β-catenin, as assessed by immunohistochemistry, in tumor tissues [88,89]. Two groups independently reported that approximately 60% of human breast cancers showed elevated expression of β-catenin [90, 91], and the former group demonstrated that the β-catenin staining correlated with high expression of Cyclin D1 and poor prognosis of breast cancer patients [90]. Although mutations of β-*catenin*, *APC* or *Axin* genes are rare, several studies reported the overexpression of *WNT* genes (*WNT2*, *3*, *4*, *5A*, *7B*, *10B*, *13* and *14*) in breast cancer relative to normal tissues [88]. In addition to abnormal Wnt overexpression in breast cancer, reduced expression of soluble extracellular Wnt inhibitors has also been reported [88]. Thus, the Wnt pathway is dysregulated in human breast cancer possibly through epigenetic mechanisms.

The *int-1*/*Wnt1* gene is related to the *Drosophila* gene *wingless*, which controls segment polarity during larval development [84]. Transcriptional activation of *int-1/Wnt1* gene by proviral insertion mutations associated with mammary gland hyperplasia has been considered to be a key step in mammary tumor induction by MMTV [9,92,93]. MMTV-*int-1* transgenic

mice exhibit stochastically developed mammary carcinomas, suggesting that additional events are necessary for tumorigenesis [94]. To induce such events and to identify the genes involved, Shackleford *et al.* infected *int-1*-transgenic mice with MMTV, intending to insertionally activate and molecularly tag cooperating protooncogenes [94]. Infection of breeding female *int-1* transgenics decreased the average age of breast tumor onset from approximately 4 to 2.5 months and increased the average number of primary tumors per mouse from one or two to more than five [94]. Analyses of provirus-containing tumors showed induced or altered expression of *int-2/Fgf-3*, *Hst/Fgf-4*, *int-3/Notch* and *int-4*/*Wnt3*, where activation of *int-2* was found in 39% of the breast tumors, *Hst* in 3% and both *int-2* and *Hst* in 3%. Their study provided evidence that *int-2* and *Hst* can cooperate with *int-1*, another secreted factor, in mammary tumorigenesis [94]. Donehower *et al.* crossed MMTV-*int-1* mice with *p53*-deficient mice and observed acceleration of mammary tumorigenesis from 24 weeks to 11.5 weeks (Table 1) [95]. Mammary tumors lacking *p53* displayed less fibrotic histopathology and increased genomic instability with aneuploidy, amplifications and deletions [95]. Interestingly, one tumor showed amplification of chromosome 7 with an ectopic expression of the *int-2/Fgf-3* proto-oncogene, confirming collaboration between *int-1* and *int-2* in this compound mouse model as well [95].

To test the functional significance of cyclin D1 induction by the Wnt pathway (Figure 1), Rowland *et al.* investigated the genetic interaction between transcriptionally active β-catenin (ΔN89β-catenin) and its target gene *cyclin D1* in mouse mammary gland development and tumorigenesis [96]. They showed that cyclin D1 was dispensable for the ΔN89β-catenin-stimulated initiation of alveologenesis in virgin females and for the formation of breast tumors. These data were consistent with previous observations made by Yu *et al.* [46]. Thus, alveologenesis is a two-step process, and cyclin D1 activity during late alveologenesis cannot be replaced by the activity of other β-catenin target genes that successfully drive proliferation at earlier stages [96].

Expression of the *int-3* locus is activated by MMTV as a consequence of insertional mutagenesis. Integration of the MMTV provirus into the *int-3* locus promotes the transcription and translation of flanking cellular *int-3* sequences that showed significant homology with the intracellular domain of the neurogenic *Notch* gene of *Drosophila* [97]. Notch signaling is an evolutionarily conserved pathway that regulates a variety of physiological processes including stem cell maintenance, cellular differentiation, proliferation and apoptosis. There are four mammalian homologues of *Drosophila Notch* (*Notch1–4*). The roles of Notch signaling in breast cancer and tumor angiogenesis have been reviewed [98,99]. Poor differentiation of mammary and salivary adenocarcinomas has been reported in MMTV-*int-3/Notch 4* transgenic mice between 2 and 7 months of age [97]. All female *int-3/Notch4* mice were lactationally deficient and mammary gland development was arrested. The salivary glands, glands of the nasal mucosa and sinus, the extraorbital lacrimal glands, and the Harderian glands of juvenile and adult transgenic mice all contained proliferating, immature ductule cells that were incompletely differentiated. It was also reported that all male *int-3/Notch4* transgenic mice were sterile, apparently the result of severe hyperplasia of the epididymis [97]. In summary, activated *int/Notch* genes cause hyperproliferation of glandular epithelium predisposing to adenocarcinoma [84–89,97–99].

MMTV-c-*rel*

Amplification, overexpression or rearrangement of the c-*rel* gene, encoding the c-rel NF-κB subunit, has been described in breast carcinomas [100,101]. It was reported that c-rel plays an essential role in tumorigenesis of the mammary gland in an MMTV-LTR-driven mouse model [100]. Mammary tumors were detected in 31.6% of MMTV-c-*rel* transgenic mice at an average age of 19.9 months (Table 1) [102]. Histological analysis of these tumors revealed a wide

spectrum of tumor subtypes, including adenocarcinomas, adenosquamous carcinomas, squamous carcinomas, a spindle cell carcinoma and a papillary carcinoma (Table 1) [102]. The MMTV-c-*rel* mammary tumors displayed sustained expression of the c-*rel* transgene mRNA. Increased aberrant nuclear expression of multiple subunits, including c-rel, p50, p52, relA, relB and the Bcl-3 protein was also observed [102]. When compared with mammary glands from wild-type mice or virgin transgenic mice, expression of the NF-κB target genes *cyclin D1*, c-*Myc* and *Bcl-xl* was significantly increased in grossly normal transgenic mammary glands starting with the first cycle of pregnancy; expression increased further in mammary carcinomas [102]. Their results indicated that dysregulated expression of c-rel, as observed in human breast carcinomas, contributes to mammary tumorigenesis.

Interestingly, none of the cell lines established from the MMTV-c-*rel* mice grew in soft agar. To test the hypothesis that a prototypic carcinogen insult can promote a more invasive, mesenchymal phenotype, Shin *et al.* established a cell line from a MMTV-c-*rel* mammary tumor (rel-3983), which was then treated in culture with either 7,12-dimethylbenz(a) anthracene (DMBA) (rel-3983D) or DMSO [103]. Rel-3983D breast tumor cells showed an increased rate of proliferation, displayed growth to a higher cell density, and acquired the ability to grow in soft agar and in Matrigel compared with the parental or vehicle-treated cells [103]. They also showed loss of E-cadherin expression. Compared with control cells, rel-3983D displayed increased NF-κB binding and higher levels of the NF-κB transactivating subunits, c-rel, relA and relB. Ectopic expression of an IκB kinase-resistant super repressor mutant of IκB-α reduced rel-3983D cell growth and invasive morphology in Matrigel, confirming the role of NF-κB in epithelial-to-mesenchymal transition (EMT). Thus, DMBA treatment of c-rel-transformed mammary tumor cells in culture result in EMT via activation of NF-κB [103].

Doxycycline-inducible MMTV models of breast cancer

Constitutive transgenic mouse models that rely on mammary-specific promoters to control transgene expression have limited utility for studying the effect of developmental events on breast cancer risk since the hormonal signals governing these events also markedly influence transgene expression levels. Gunther *et al.* created a novel transgenic mouse system that uses the MMTV-LTR to drive expression of the reverse tetracycline-dependent transactivator (*rt*TA) [104]. They crossed transgenic mice expressing *rt*TA in the mammary epithelium (MMTV-*rt*TA) with reporter lines bearing *tet* operator-controlled transgenes and tested the ability to spatially, temporally and quantitatively control reporter gene expression after administration of doxycycline to bitransgenic mice [104]. They showed that transgene expression using this system could be rapidly induced and de-induced in mammary glands, which could be reproducibly titrated over a wide range of expression levels. Moreover, their system allowed transgene expression to be restricted to any desired stage of postnatal mammary gland development. Moody *et al.* used this doxycycline-inducible system to conditionally express activated *neu* in the mammary epithelium of transgenic mice to determine the impact of tumor progression on the reversibility of *neu*-induced tumorigenesis [105]. When induced with doxycycline, bitransgenic MMTV-*rt*TA, *tet*O- *neu*NT mice developed multiple invasive mammary carcinomas; however, essentially all of the tumors regressed to a clinically undetectable state following transgene de-induction. Their data demonstrated that *neu*-initiated tumorigenesis was reversible. It should be noted that even extensive lung metastases arising from *neu*-induced tumors rapidly and fully regressed following the abrogation of *neu* expression. However, despite the near universal dependence of both primary and metastatic tumors on *neu* transgene expression, most animals bearing fully regressed *neu*-induced tumors ultimately developed recurrent tumors that have progressed to a *neu*-independent state, suggesting that secondary genetic alterations occurred during mammary tumor development [105].

By using this inducible model, the same group showed that targeted downregulation of the Wnt pathway results in the rapid disappearance of essentially all Wnt-initiated invasive primary mammary tumors, as well as pulmonary metastases [106]. Interestingly, tumor regression did not require *p53* and even occurred in highly aneuploid tumors. However, despite the dependence of primary mammary tumors and metastases on continued Wnt signaling and the dispensability of *p53* for tumor regression, they found that a substantial fraction of tumors progressed to a Wnt-independent state and that p53 suppressed this process. Loss of one *p53* allele dramatically facilitated the progression of mammary tumors to a *Wnt1*-independent state both by impairing the regression of primary tumors following doxycycline withdrawal and by promoting the recurrence of fully regressed tumors in the absence of doxycycline. Thus, although p53 itself was dispensable for tumor regression, it played a critical role in the prevention of tumor recurrence [106].

To determine if Ras activation has context-dependent effects in the mammary gland, Sarkisian *et al.* generated doxycycline-inducible transgenic mice that allowed Ras activation to be titrated [107]. Low levels of Ras activation stimulated cellular proliferation and mammary epithelial hyperplasias similar to those found in non-transformed mouse tissues expressing endogenous oncogenic *K-ras2*. By contrast, high levels of Ras activation – similar to those found in tumors bearing endogenous *K-ras2* mutations – induced cellular senescence that was *Ink4a/Arf*-dependent and irreversible following Ras downregulation. Chronic low-level Ras induction resulted in tumor formation, but only after the spontaneous upregulation of activated Ras and escape from senescence checkpoints. They further showed that high-level, but not low-level, Ras activation stimulates tumor-suppressor pathways and triggers irreversible senescent growth arrest *in vivo* [107]. They suggested a three-stage model for Ras-induced tumorigenesis consisting of an initial activating Ras mutation, overexpression of the activated Ras allele and, finally, inactivation of the *Ink4a/Arf*, *p53*-dependent senescence checkpoints [107].

Utility of the FVB strain in MMTV transgenic mouse models

Human breast cancer models using MMTV-LTR mice have often been created in the FVB/NJ (FVB) strain due to its high productiveness of pups, while gene-knock-out/knock-in mice have been developed in the 129/SvJ-C57BL/6 strains due to the capacity of 129/SvJ embryonic stem cells to facilitate germline transmission [108]. Gene-targeted mice are commonly backcrossed into the C57BL/6 background for comparison of tumor spectra and other phenotypes that had been reported. Although FVB/N females do not spontaneously develop breast tumors even at 24 months of age [109], it is generally believed that the FVB/N strain is more susceptible to mammary tumors than mice of the C57BL/6 strain. For instance, FVB MMTV-*ErbB2* (wild-type) females develop mammary tumors between 7 and 12 months of age, whereas FVB × C57BL/6 (F1) MMTV-*ErbB2* mice have tumor latencies greater than 18 months [110]. It should be noted that the expression level of the *ErbB2* transgene was equivalent in breast tumor tissues from both FVB and FVB × C57BL/6 (F1) mice. Furthermore, increased tumor latency did not appear to be associated with a decrease in expression of the *ErbB2* transgene in the normal mammary gland of F1 mice because immunohistochemical staining for ErbB2 protein expression in the mammary glands of 3-month-old virgin female mice revealed similar levels of protein expression in FVB and F1 animals [110]. When F1 animals were backcrossed one more generation into the FVB strain ([FVB × B6] F1 × FVB; F2), a subset of the resulting offspring developed tumors with a latency almost equivalent to that of the pure-strain FVB mice. Statistical analysis of the genetic variability in mammary tumor latency indicated that approximately three independent genes were involved in the latency effect [110]. Mikaelian *et al.* categorized transgenic and spontaneous mouse mammary tumors by using morphologic and architectural criteria [111]. On the basis of the expression of terminal differentiation markers, three types of mouse mammary tumors were identified. The first group is ‘simple carcinomas’, composed exclusively of cells with a luminal phenotype (glandular or solid) that

are characteristic of neoplasms of other WNT mRNAs *neu*, Ha-*Ras*, c-*Myc* (FVB/N), SV40-TAg (C57BL/6) and spontaneous papillary carcinomas in BALB/cJ mice [111]. The second group is 'complex carcinomas', displaying luminal and myoepithelial differentiation that are characteristic of type P tumors arising in mice transgenic for *Wnt1* (FVB/N, B6SJ/L), tumors arising in mice infected with MMTV and spontaneous adenosquamous carcinomas in BLAB/cJ mice [111]. The third category is 'carcinomas with epithelial-to-mesenchymal transition' (EMT), which is a characteristic feature of tumor progression in Ha-*Ras*-, c-*Myc*-(FVB/N) and SV40-TAg (C57BL/6)-induced mammary neoplasms and spontaneous tumors in PL/J and SJL/J mouse strains [111]. They concluded that immunohistochemical profiles of complex mammary tumors are consistent with a stem cell origin, whereas simple carcinomas might originate from a cell committed to the luminal lineage [111]. Their results also suggest that the initiating oncogenic events determine the morphologic features associated with cancer progression because EMT is only observed in certain types of neoplasm.

Relevance of MMTV mouse models to human breast cancer

Mouse mammary tumor virus mouse models (oncogene transgenic mice and insertional mutagenesis) have been informative models for human breast cancer despite morphological, hormonal and lifestyle differences between mice and humans. There are at least three different implications of MMTV mouse models to human breast cancer.

First, MMTV-LTR-driven oncogene overexpression models have been useful in the analysis of the signaling cascades governed by oncogenes that are frequently overexpressed or mutated in human breast cancer. These mice have also been used to test the efficacy of new chemopreventive agents *in vivo*. Crossing these mice with possible tumor-suppressor-knock-out mice has provided valuable information about oncogene–tumor-suppressor gene interactions *in vivo*.

The second implication is the fact that genes that have been isolated by MMTV insertional mutagenesis are often aberrantly expressed in human breast cancers. The human homologue of the *int-2* locus (*WNT2*) has been found to be amplified and highly expressed in 15–45% of breast cancers [2,89]. Durgam *et al.* showed that the cellular gene at the MMTV integration site in the *int-5* locus was identical to the gene encoding aromatase (CYP19), a member of the cytochrome P-450 gene superfamily [112]. MMTV was integrated within exon 10 in the 3′ untranslated region of the *int-5/aromatase* gene, which was expressed in normal mammary gland and overexpressed in mammary tumors. Their results suggested that the overexpression of int-5 may be responsible for breast tumorigenesis, demonstrating that the integration of MMTV in a cellular gene that plays a role in hormone-dependent breast cancers. Indeed, overexpression of the human homologue of *int-5* (*WNT5A*) was reported in 36% of human breast cancer [88]. Bui *et al.* identified the human homologue of the mouse *Wnt10b* gene, *WNT10B*, the expression of which was found to be elevated in three out of 50 primary breast carcinomas [113]. Overexpression of other *WNT* mRNAs (*WNT7B*, *WNT13* and *WNT14*) has also been reported in human breast carcinomas, as described in the MMTV-*int1* section [88]. As a more comprehensive approach, Theodorou *et al.* conducted a microarray with 295 primary human breast carcinomas and determined the expression levels of the human homologues of the MMTV *cis* target genes [114]. Interestingly, the expression of 11 of the 33 *cis* genes were dysregulated in 5–43% of the human breast carcinoma samples they examined [114]. Importantly, most of the MMTV-tagged candidate oncogenes were correlated with one or more clinicopathological tumor characteristics, including angioinvasion, tumor grade and/or lymphatic infiltration [114]. Collectively, genes identified from common MMTV integration sites are often involved in human breast cancer development.

The third implication of MMTV models of breast cancer in human disease is the isolation and characterization of MMTV *env*-like sequences in human breast carcinomas. The hypothesis that a human retrovirus homologous to the MMTV (HMTV) is involved in human breast cancer etiology has fascinated scientists for many years. In 1972, RNA related to MMTV was detected in human breast cancer cells [115]. Detectable MMTV *env* gene-related antigenic reactivity has been found in tissue sections of human breast cancers [116], breast cancer cells in culture [117], human milk [118], in the sera of patients [119], in human breast cyst fluid [120] and in particles produced by a human carcinoma cell line [121]. Wang *et al.* reported that MMTV *env*-like sequences were detectable in 38.5% of human breast cancer samples from the analysis of 314 cases [122], a finding later confirmed by Etkind *et al.* [123]. However, the frequency of detection of a MMTV-like virus in human breast cancer varies from 0 to 100%, depending on the demographic distribution of breast cancer samples, source of materials (paraffin-embedded or frozen), and the sensitivity of the methods used [124]. To circumvent the problem of sensitivity and reproducibility for the detection of MMTV *env*-like sequences, Zammarchi *et al.* established a fluorescence nested-PCR (FN-PCR) method [125]. With this approach, they were able to detect very low copies of the viral genome. They then screened a panel of 45 frozen breast cancer samples obtained by laser microdissection. The MMTV *env* gene-like sequence was found in 15 (33%) of the human breast cancers analyzed, whereas the same sequence was undetectable in both normal tissues and in other types of tumors [125]. Sequence analysis revealed 96% homology with the MMTV genome but no significant similarities with the human genome. They concluded that the combined use of frozen material, microdissected cell populations and FN-PCR provides a novel, very sensitive and nonradioactive methodology for the molecular detection of HMTV [125].

Obviously, MMTV *env*-like sequences alone do not make up an entire provirus. Numerous groups examined human breast cancer samples for the presence of other MMTV-like sequences to the rest of the viral genome. In 2001, Liu *et al.* reported successful amplification of a whole proviral structure from two human breast carcinomas that were *env*-positive [126]. The 9.9-kb provirus was 95% homologous to MMTV, but only 57% homologous to 3.5 kb of the *gag* and *pol* genes of the human endogenous retrovirus K10. The HMTV provirus displayed typical features of a replication competent retrovirus, plus open reading frames for the superantigen and the glucocorticoid-responsive element [126]. FISH with the 2.7-kb *env*-LTR probe revealed that the sequence was present on several chromosomes from breast cancer but, importantly, not on chromosomes from normal breast cells [126]. The origin of the MMTV-like sequences appears to be exogenous because they were undetectable in normal tissues, the similarity between the two isolates was very high (96%) and they maintained open reading frames [126]. The same group later reported that MMTV-LTR-like sequences were detectable in 42% of human breast cancer [127]. Their data indicated that the human superantigen that is encoded by an open reading frame located 3′ of the LTR of the provirus was fully functional to activate human T lymphocytes, suggesting a role in human breast cancer pathogenesis.

To investigate the possibility of an association between breast cancer and MMTV-like viruses, histological characteristics of invasive ductal human breast cancer specimens were compared with archival MMTV-associated mammary tumors from C3H experimental mice [128]. The presence of MMTV-like *env* DNA sequences in the human breast cancer specimens was simultaneously studied by PCR and Southern blotting. MMTV-like *env* gene sequences were identified in 22 out of 59 (37.3%) human breast cancer specimens, consistent with previous reports from other groups. In total, 17 out of 43 (39.5%) invasive ductal carcinoma breast cancer specimens and four out of 16 (25%) ductal carcinoma *in situ* specimens had some histological characteristics that were similar to Dunn type A or type B MMTV-associated mouse mammary tumors. However, these similarities were not associated with the presence or absence of MMTV-like gene sequences in the human breast tumor specimens. On the other hand, they reported a significant ($p = 0.05$) correlation between the lower grade of the human

breast cancer and similarity to the mouse mammary tumors [128]. Based on these observations, they proposed a 'hit and run' mechanism where an MMTV-like virus initially acts in concert with other viruses to cause breast cancer and then leaves no trace after it has initiated oncogenic change.

Recently, Etkind *et al.* conducted an intriguing study involving family members diagnosed with breast cancer to determine if MMTV might be implicated in breast cancer development in a familial context [129]. The diagnosis of late-onset breast cancer in a father, mother and daughter living in the same house for decades suggested the possibility of an environmental agent as a common etiological factor. They detected MMTV-like *env* and LTR sequences containing the MMTV superantigen gene (*sag*) in the malignant tissues of all three family members. The amplified *env* gene sequences showed 98.0–99.6% homology to the MMTV *env* sequences found in the GR, C3H and BR6 mouse strains [129]. Interestingly, the amplified LTR sequences containing *sag* sequences segregated to specific branches of the MMTV phylogenetic tree and did not form a distinct branch of their own. The presence of MMTV-like DNA sequences in breast cancer tissues of all three family members suggested the possibility of MMTV as an etiological agent. Phylogenetic data suggested that the MMTV-like DNA sequences were mouse in nature and not human-derived and that the ultimate reservoir of MMTV was most likely the mouse. Although the route by which these family members came to be infected with MMTV was unknown, the possibility exists that such infection might have resulted from a shared exposure to mice [129].

All the aforementioned studies clearly suggest that MMTV-related genes may play a very important role in the etiology and pathogenesis of human breast cancer.

Diagnostic values of MMTV-like sequences & proteins in human breast cancer

There have been several reports on the prognostic value of MMTV *env*-like sequences in human breast cancer [130–133]. As the presence of MMTV-like sequences in breast cancer has been associated with laminin receptor expression, a marker of poor prognosis, it was predicted that MMTV-*env* positivity would predict poor outcome for breast cancer patients. Wang *et al.* studied the frequency of *env* gene-like sequences in both sporadic and gestational breast cancer samples [130]. Gestational breast cancer is generally associated with rapid growth and increased mortality. Whereas the target sequences could be identified in 30–38% of sporadic breast cancer samples, the prevalence in gestational breast cancer is 62%. Their findings indicate that hormonal response elements present in the MMTV-like LTR may play a role in promoting cell growth as they do in the mouse system [130].

In another study, a cohort of 128 Australian female breast cancer samples was screened for MMTV-like DNA sequences using PCR [131]. The presence of progesterone receptor, ER and nuclear accumulation of p53 protein was studied in the same samples using immunohistochemical staining. Nuclear accumulation of p53 was significantly more prevalent ($p = 0.05$) in archival human breast cancers containing MMTV-like DNA sequences. The presence of progesterone receptor was significantly higher in MMTV-positive than MMTV-negative breast cancers ($p = 0.01$). No correlation between ER and MMTV-like DNA sequences was found. The positive association between MMTV-like DNA sequences and progesterone receptor indicates steroid hormones and MMTV may play a role in human breast cancer. Mutations of the tumor suppressor gene *p53* have been reported in approximately 20% of human breast cancers and are associated with poor prognosis of patients [132]. Since nuclear p53 accumulation is usually suggestive of *p53* mutation, the association of MMTV-like DNA sequences with higher grades of cancer and nuclear accumulation of p53 clearly warrant additional investigation [132].

The same research group screened a larger and more diverse cohort of female breast cancer samples and revealed a correlation of MMTV-like sequences with the severity (grade) of breast cancer [133]. In total, 34% (43 out of 136) of female breast cancer samples were positive for MMTV-like sequences when screened using PCR. A significant gradient of MMTV positivity was observed with increasing grade of breast cancer, from 23% of infiltrating ductal carcinoma (IDC) grade I tumors to 34% of IDC grade II tumors ($p = 0.00034$) and 38% of IDC grade III tumors ($p = 0.00002$). They also reported the detection of MMTV-like sequences in 62% (8 out of 13) of male breast cancer samples and 19% (10 out of 52) of male gynecomastia samples analyzed. Interestingly, MMTV-like sequences were detected in various premalignant breast lesions of females, including fibroadenoma (20%) and fibrocystic disease (28%) at a significantly higher prevalence than that in normal breast tissue (1.8%; $p = 0.00001$). In a study examining a cohort of female breast cancer patients, MMTV-like sequences were discovered to be associated with breast tumors but were not present when tumor cells were not detectable by histopathological examination. Their results support the involvement of MMTV-like sequences in the development of breast tumors in men and women and the association of MMTV with increasing severity of breast cancer [133]. Although no prospective studies have been conducted, it is possible that MMTV *env*-like sequences will represent a novel prognostic marker for breast cancer.

With regard to humoral response to MMTV antigens in breast cancer, it was reported that sera from 56 to 137 (40.9%) patients with breast carcinoma were positive for antibodies to MMTV [134,135]. Positive reactivity was also found in sera from five to 27 (18.5%) patients with benign breast disease, in seven out of 60 (11.7%) patients with malignancies other than breast carcinoma, but only in two out of 56 (3.6%) female controls [134]. Antibodies to gp52 have been detected in the sera of 18.6% (27 out of 145) of breast cancer patients in the USA [119], indicating immunoreactivity. One report from Israel showed that gp52 was detectable in 128 out of 204 breast carcinomas tested (62.7%), but the immunological reaction was not detected in normal breast tissue, benign breast tumors, ductal hyperplasia or in other types of cancers. Importantly, the frequency of detection of gp52 was higher in stage IV breast cancer (80%) compared with stage I (15%), suggesting that the gp52 cross-reacting antigen is a useful biomarker to predict the poor prognosis of breast cancer [136,137].

Expert commentary

Breast cancer is the most common type of cancer in women. Despite major advances in its diagnosis and treatment, several major clinical and scientific problems remain unsolved. It is of critical importance to identify genetic alterations responsible for the initiation of malignant transformation of the breast epithelium in order to identify targets for novel drug discovery. Overexpression of HER2/neu, cyclin D1, cyclin E, c-Myc and WNT1, and mutation of Ras have been reported in human breast cancer and, in the case of HER2/neu, cyclin D1, cyclin E and c-Myc, are associated with poor prognosis of patients. In mouse models of human breast cancer, the MMTV-LTR promoter has most frequently been used, and among MMTV LTR-driven mouse models, MMTV-*neu/ErbB2*, -*cyclin D1*, -*Ras*, -*Myc* and -*int-1* models have been especially well studied and characterized. These transgenic models of breast cancer have provided invaluable insights into the molecular mechanisms of breast carcinogenesis and tumor progression.

It was concluded by a panel of experts in mammary gland pathology that these genetically engineered mouse models often develop breast carcinomas that are indistinguishable from human neoplasia. However, there are critical differences between mice and humans. Approximately 50% of human breast cancers are positive for ERα and responsive to estrogen, while tumors that arise in genetically engineered mice are usually ERα-negative and hormone-independent. The exception is MMTV-*cyclin D1T286A* mice, which are ER-positive. Another

issue is that mouse mammary tumors are much less fibroblastic compared with human breast carcinomas (i.e., human mammary glands consist mainly of connective tissue stroma while mouse mammary glands are mainly composed of adipose tissues). Moreover, most mammary gland-induced tumors metastasize to the lung in mice, while breast cancer metastases tend to appear in the lymph nodes, lungs, bones and liver in approximately the same proportion in humans. Since metastatic disease is usually the cause of breast cancer-related death, generation of mouse models that reflect metastatic site preference of human breast cancer remains to be established.

Published studies report detection of the *env*-like DNA sequence of MMTV in 35–40% of human breast cancer samples but only in 0–2% of normal tissues. Based on these studies, the viral sequence is highly specific to breast cancer and, thus, the MMTV *env*-like gene could be an excellent molecular marker for breast cancer. Indeed, several studies have shown that the presence of MMTV-like sequences was associated with progesterone receptor, nuclear p53 and advanced clinical stages. However, the frequency of detection of MMTV-like sequences greatly varies from 0 to 100% depending on the specimen, the country where the study is conducted, and the methods used. Scientists have tried PCR followed by Southern blotting, nested PCR or FN-PCR with laser microdissection to detect MMTV *env*-like and other sequences, but so far there is no general consensus which method is the best for the detection of HMTV in human breast cancer samples. Thus, the establishment of highly sensitive and reproducible methods for HMTV provirus DNA detection is essential to determine the diagnostic values of HMTV in human breast cancer.

Five-year view

Constitutive transgenic mouse models that depend on mammary-specific promoters to control transgene expression provide invaluable information on the signaling pathways involved in breast cancer development. However, they may have limited utility for studying the effect of developmental events on breast cancer risk since the hormonal signals governing these events also influence transgene expression levels. Novel transgenic mouse systems that use the MMTV-LTR to drive expression of the *rt*TA and the regular tetracycline-dependent transactivator (*t*TA) have recently been reported and reviewed in this paper [104–107,138]. Transgene expression using these *tet*-controlled systems can be rapidly induced and de-induced, are highly specific to mammary glands and are reproducibly determined over a wide range of expression levels. These *tet*-controlled systems will be more widely used in the near future to study the effects of oncoprotein expression in mammary glands. Another future direction is the use of the Cre-LoxP1 recombination system. With this system, it has now become possible to delete tumor-suppressor genes selectively in the mammary gland [139, 140]. With these techniques, more physiological models of breast cancer will be created within the next 5 years.

Notwithstanding these technical advances, the value of MMTV-LTR-driven mouse models of breast cancer has been re-evaluated since the discovery of MMTV *env*-like sequences in the DNA from human breast carcinomas, which are typically absent in normal breast tissues or other types of cancers. Although the entire 9.9-kb proviral structure has been clarified for HMTV, cloning of HMTV insertion sites in human carcinomas has not been reported. This can potentially be attributed to the technical difficulties encountered in the associated studies. Identification of HMTV target genes and creation of mouse models for the candidate gene will be essential for understanding the roles of HMTV in human breast carcinogenesis. The prognostic value of MMTV *env*-like sequences, envelope protein gp52 and LTR superantigen should be determined in a large prospective study since a significant percentage of breast cancers are positive for these markers.

If some human breast cancers have been caused by integration of MMTV-like retrovirus, the tumor cells will exhibit viral antigens both internally and on their surface. As a consequence, viral antigens in tumors will represent a potential antigenic target that is clearly different from normal tissues. As a matter of fact, MMTV-*env*-like sequences have not been detected in normal tissues. If HMTV is found to possess a *bona fide* role in human breast cancer development in the near future, the possibility of developing vaccines to prevent infection against such a virus becomes reasonable. This is potentially an extremely exciting discovery that may lead to a reduction in morbidity and mortality from human breast cancers.

Key issues

- Mouse mammary tumor virus (MMTV)-transgenic mice are invaluable models for human breast cancer.
- There are three different breast cancer models for neu/ErbB2 mice. They show different latency for tumor development and invasiveness dependent on the gene expressed (mutant or wild-type ErbB2) and on the promoter used (*MMTV-LTR* or endogenous *ErbB2* promoter). Similar to HER2-positive human breast carcinomas, mammary tumorigenesis in mice requires gene amplification and elevated expression of the *ErbB2* gene.
- MMTV-*cyclin D1* mice show much longer latency for breast tumor development (~22 months) than MMTV-*neu* or MMTV-*ErbB2* mice, but the tumorigenesis is significantly accelerated in the MMTV-*D1T286A* strain that expresses stable cyclin D1.
- LMW cyclin E overexpression strongly selects for spontaneous inactivation of the Arf-p53 pathway *in vivo*, thereby canceling its protective checkpoint function and accelerating progression to breast cancer.
- Bitransgenic mice expressing both c-*Myc* and activated *Ras* under the control of MMTV-LTR developed focal mammary carcinomas with a dramatically shortened latency period, indicating c-*Myc* and activated *Ras* cooperate to accelerate mammary tumor development with pronounced malignancy.
- The *int* genes were originally identified as oncogenes activated by the insertion of MMTV in virus-induced mammary adenocarcinomas. MMTV-*int-1* transgenic mice exhibit stochastically developed mammary carcinomas, suggesting that additional events are necessary for tumorigenesis.
- MMTV *env*-like sequences have been isolated from approximately 38% of human breast cancer DNA, but not in normal breast tissues or other types of carcinomas.
- A 9.9-kb HMTV provirus with 95% homology to MMTV has been amplified from two human breast cancer samples.
- MMTV-like LTR superantigen was reported to be present in 42% of human breast cancer, suggesting a role in human breast carcinogenesis.
- The association of MMTV-like DNA sequences with progesterone receptor, nuclear p53 accumulation suggestive of *p53* mutation, and advanced stage of breast cancer indicates that tumors with MMTV-like sequences are associated with hormonal response and prognosis of patients.

Acknowledgments

We thank Elizabeth Fry for technical assistance.

Kazushi Inoue has been supported by NIH/NCI 5R01CA106314, American Cancer Society RSG-07-207- 01-MGO and Wake Forest University Golfers against Cancer grant P30CA12197GAC. Pankaj Taneja is currently supported by the Susan G Komen Foundation postdoctoral fellowship KG080179. Donna P Frazier was supported by the Ruth L Kirschstein National Research Service Award Institutional Research Training Grant (5T32CA079448, F. Torti) from the NIH.

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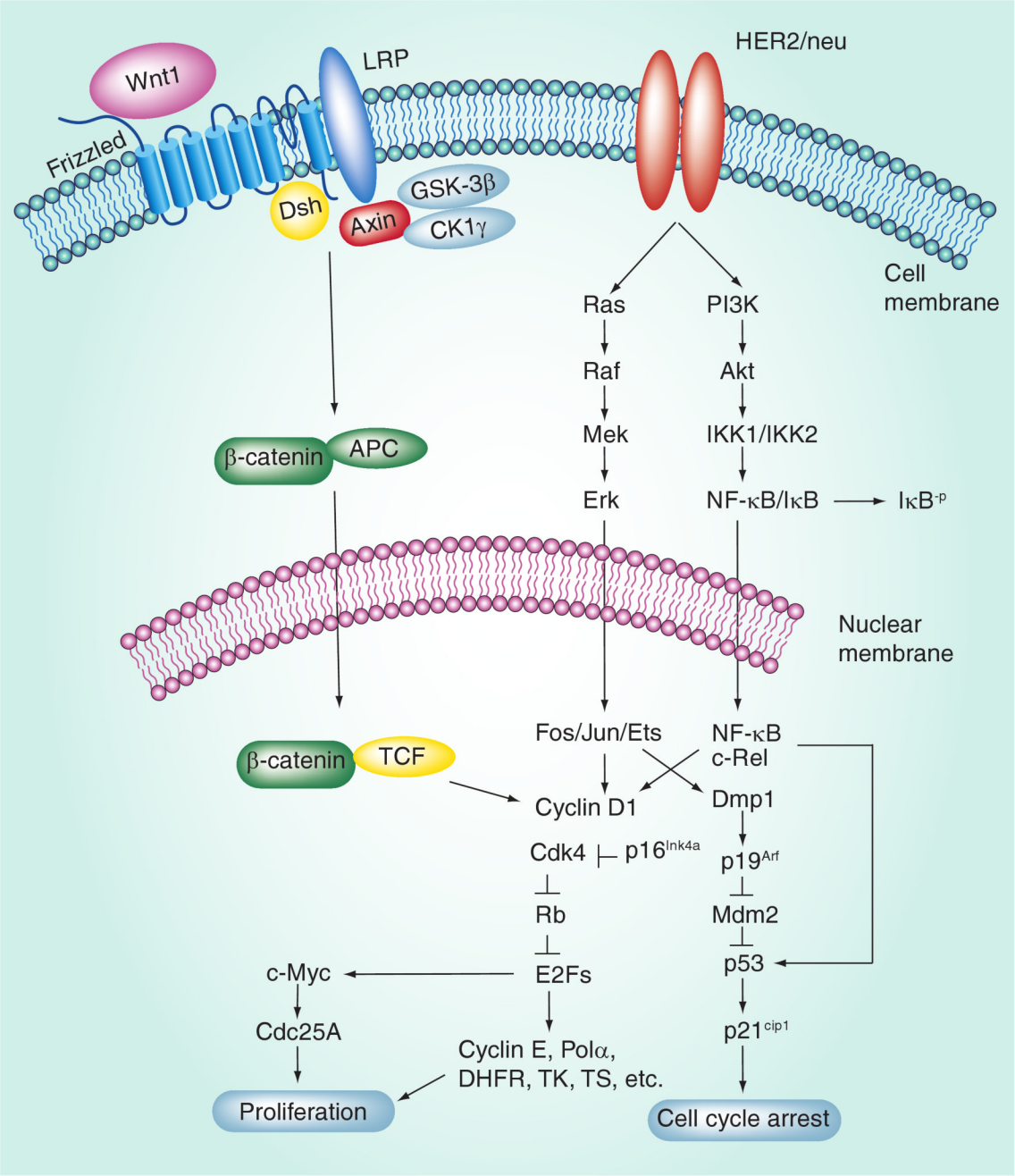

Figure 1. Signaling pathways involving target proteins overexpressed in mouse mammary tumor virus (MMTV) breast cancer models

HER2/neu is an orphan receptor that can be activated by overexpression or mutation of the transactivating domain (rat neu). Overexpression of HER2/neu results in enhanced cell survival and mitogenicity and its deregulation can lead to breast tumorigenesis. Erk activation by the Ras–Raf pathway leads to cell proliferation through the activation of a number of nuclear targets. Activation of the PI3K–Akt pathway results in enhanced survival through inhibition of proapoptosis proteins, such as Bad, GSK3 and the transcription factor FKHR-L1. A major mitogenic player acting downstream of ErbB2 is cyclin D1 [46]. Once bound by Wnt, the Frizzled/LDL-related protein (LRP) coreceptor complex activates the canonical signaling

pathway. Frizzled interacts with Dsh, a cytoplasmic protein that functions upstream of β-catenin and the kinase GSK3β. Wnt signaling controls phosphorylation of Dsh. Wnts are thought to induce the phosphorylation of LRP by GSK3β and CK1γ, thus regulating the docking of Axin. The recruitment of Axin away from the destruction complex leads to the stabilization of β-catenin. In the nucleus, β-catenin displaces Groucho from Tcf/Lef to promote the transcription of Wnt target genes, one of which is cyclin D1. As indicated, a number of pathways lead from the receptors to enhanced activation of cyclin D1, thereby promoting cell cycle progression. c-Myc is one of the direct targets of E2Fs. Although both c-Myc and cyclin D1 rescue the CSF-1 response and regulate each other [141], MMTV-*Myc*-induced breast cancer development is not dependent on cyclin D1 [46]. Dmp1 is a unique transcription factor that regulates the Arf-Mdm2-p53 tumor surveillance pathway [142–151].

APC: Adenomatosis polyposis coli; Cdc25: Cell division cycle 25; Dsh: Dishevelled; DHFR: Dihydrofolate reductase; GSK3β: Glycogen synthase kinase 3β; IKK: IκB kinase; LEF: Lymphoid enhancer-binding factor; Polα: DNA polymerase α; TCF: T-cell factor; TK: Thymidine kinase; TS: Thymidylate synthase.

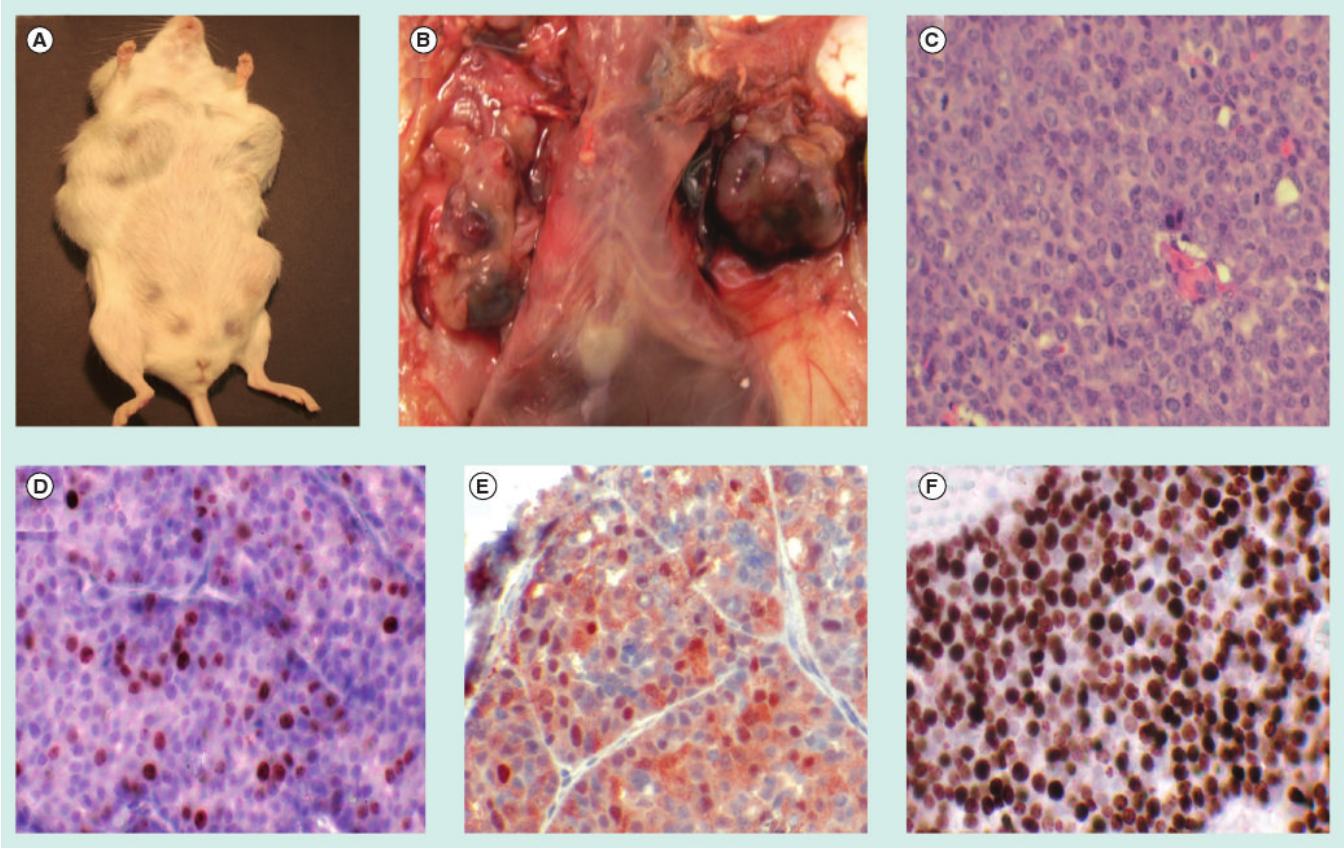

Figure 2. Breast cancers found in mouse mammary tumor virus (MMTV)-*neu* mice

Wild-type MMTV-*neu* (rat neu with mutation of the transmembrane domain) mice develop multiple breast tumors at approximately 7 months in FVB/NJ background. Breast carcinogenesis is accelerated to 4–5 months when both alleles contain the mutant *neu* transgene. Induction of cyclin D1 and Cdk4 plays critical roles in breast carcinogenesis by *neu*. **(A & B)** Macroscopic picture of a tumor-bearing MMTV-*neu* mouse. **(C)** H&E staining of breast tumors. **(D–F)** Detection of **(D)** cyclin D1, **(E)** Cdk4 and **(F)** Ki67. Both cyclin D1 and Cdk4 are induced by c-ErbB2/neu and play essential roles in breast carcinogenesis. Magnification: ×40.

Table 1

Tumor-free survival, accelerating factors and histopathology of mouse mammary tumor virus transgenic breast cancer models

Transgene	Mouse model	Tumor-free survival (months)	Tumor formation in compound mice	Pathology	Ref.
neu/ErbB2	MMTV-*neu* (mutant)	5–10		Adenocarcinoma, multifocal, metastatic	[26–28]
	MMTV-*ErbB2* (wild-type)	7–12 (205–367 days)*	5 months in WAP-*p53–172H*	Adenocarcinoma	[31,32]
			No change in *Ink4a/Arf*$^{+/-}$		[33]
	ErbB2 knock-in (mutant)	14.7		Adenocarcinoma, rare metastasis	[34]
cyclin D1	MMTV-*cyclin D1*	18–22		Papillary adenocarcinoma	[43,44]
	MMTV-*cyclin D1T286A*	18		Secretory glandular adenocarcinoma, multifocal	[44]
cyclin E	MMTV-*cyclin E*	17		10.4% adenocarcinoma, 14.3% metastasis	[58]
	MMTV-*cyclin E EL1/EL4*	18.6	11.6 months in *p53*$^{+/-}$	27.4% adenocarcinoma, 16.7% metastasis	
	MMTV-*cyclin E T1*	17.2	10.7 months in *p53*$^{+/-}$	30.2% adenocarcinoma, 25% metastasis	
Ha-Ras	MMTV-*Ras*	5.6 (168 days)‡	1.5 months with MMTV-c-*Myc*	Adenocarcinoma	[64]
		9.0‡	2.3 months in *p53*$^{-/-}$	Higher histological grade, genomic instability	[65]
		5.1 (154 days)‡	3.1 months in *p21cip1*$^{-/-}$		[67]
		10.0‡	6 months in *p21cip1*$^{-/-}$		[69]
c-Myc	MMTV-*c-Myc*	10.8 (325 days)		Adenocarcinoma	[74]
		7	No change in *p21cip1*$^{-/-}$		[75]
int-1/Wnt1	MMTV-*int-1*	5–6 (virgin females)	2.9 months in *p53*$^{-/-}$	Stochastically develop adenocarcinoma	[93,94]
		4 (breeding females)			[94]
c-rel	MMTV-*c-rel*	20 (32% of transgenics)		Adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma, spindle cell, papillary carcinoma	[102]

The results for cross of MMTV mammary tumor models with other transgenic mouse models (WAP-*p53–172H*; MMTV-c-*Myc*) or tumor-suppressor gene-knockout mice (*Ink4a/Arf*-null, *p53*-null or *p21*^{cip1}-null) are shown in the column of 'Tumor formation in compound mice'.

*Tumor-free survival of MMTV-*ErbB2* mice is dependent on the strain (i.e., 205 days in N#202, 261 days in N#721 and 367 days in N#510).

[‡]There are a significant differences in tumor-free survival of MMTV-*Ras* mice dependent on the genetic background. The mean tumor onset is 5–6 months in FVB/N and 9–10 months in FVB/N × C57BL/6/× BALB/c mixed genetic background.

MMTV: Mouse mammary tumor virus.

Table 2

Tumor spectra and the frequency of extra-mammary gland tumors reported in mouse mammary tumor virus transgenic models

Transgene	Mouse model	Other types of tumors	Ref.
neu/ErbB2	MMTV-*neu* (mutant)	Salivary gland hyperplasia and hypertrophy (TG.NA, TG.NF) Papillary hyperplasia and hypertrophy of the epididymis (TG.NA, TG.NB, TG.NF) Harderian gland dysplasia (TG.NA, TG.NB, TG.NF)	[26]
	MMTV-*ErbB2* (wild-type)	Not reported	[31,32]
	ErbB2 knock-in (mutant)	Not reported	[34]
cyclin D1	MMTV-*cyclin D1*	Not reported	[43,44]
	MMTV-*cyclin D1T286A*	Not reported	[44]
cyclin E	MMTV-*cyclin E* MMTV-*cyclin E EL1/EL4* MMTV-*cyclin E T1*	Not reported	[58]
Ha-Ras	MMTV-*Ras*	Harderian gland hyperplasia, 20% in TG.SH and 14% in TG.SQ Salivary gland adenocarcinoma: 17% Lymphoblastic lymphoma: 3%	[64]
c-Myc	MMTV-*c-Myc*	Not reported	[64]
c-Myc/Ras	MMTV-*c-Myc/Ras*	B-cell lymphoma: 38% Salivary gland adenocarcinoma: 19% Harderian gland hyperplasia: 19% Seminal vesicle neoplasm: 5% 1 out of 21 mice showed triple neoplasia 1 out of 21 mice showed quadruple neoplasia	[64]
int-1/Wnt1	MMTV-*int-1*	Salivary gland adenocarcinoma in 1 out of 24 females (line 303)	[93,94]
c-rel	MMTV-*c-rel*	Not reported	[102]

MMTV-LTR has been shown to be active in the salivary glands, Harderian glands and epididymis, and contributes to tumor formation in these tissues. In addition, increased occurrence of lymphomas has also been reported in c-Myc/Ras double transgenics. These extramammary gland tumors have not been reported in MMTV-*ErbB2* (wild-type, mutant knock-in), *cyclin D1* (wild-type, T286A) *cyclin E*, *c-Myc* or *c-rel* mice.