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# MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways

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**The study of the mouse mammary tumor virus (MMTV) has provided important insights into the mechanisms of gene transcription regulation by steroid hormones, the mode of action of heritable super antigens and the progressive nature of neoplastic transformation in the mammary gland. Here we describe the current situation with respect to the latter aspect of MMTV biology and the prospects for further advance in our understanding of breast cancer in humans that may be expected from a continued study of MMTV-induced mammary neoplasia. MMTV is a heritable somatic mutagen whose target range is limited. Commonly, the tumorigenic capacity of MMTV is restricted to mammary gland, whereas infection is found in a variety of cell types. In order to replicate, proviral DNA must be inserted into the cell DNA and cell division is required to fix the mutation. Yet only in the mammary epithelium does this lead to neoplastic transformation. This suggests a unique relationship between MMTV and mammary epithelium. In evaluating this relationship, we and others have discovered genes and potential gene pathways that are pertinent in mammary differentiation and neoplasia. In addition, the clonal nature of these progressive events from normal to malignant phenotype has become increasingly clear. The weight of these observations compel us to conclude that mammary neoplasms arise from multipotent mammary epithelial cells through a process of acquired mutations that are reflected in the increasingly malignant nature of the population of progeny produced by these damaged stem cells.** *Oncogene* (2000) **19,** 992–1001.

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## Introduction

A major thrust of breast cancer research has been to understand the genetic basis for the initiation of malignant transformation in breast epithelium. The complexity of genetic alterations which have been associated with primary human breast tumors has confounded the identification of genetic mutations that are paramount to the initiation of the primary cancer from those that are responsible for progression to malignancy and metastasis (reviewed in Bieche *et al*., 1995; Callahan, 1996). Therefore several different experimental models for mammary cancer are being applied in an effort to distinguish gene mutations responsible for early events in malignant transformation from those leading to malignant progression. A venerable and sometimes maligned experimental model for mammary cancer in the mouse has recently received renewed attention from breast cancer investigators (Wang *et al*., 1998). In this model, mouse mammary tumor virus (MMTV) represents an inherited biological carcinogen that induces, by insertional mutagenesis, premalignant lesions and malignant tumors of the mammary gland (Varmus 1982). Genetic analysis of the tumors from virus-infected, high incidence mouse strains have identified genes commonly mutated during MMTV-induced mammary tumorigenesis (reviewed in Callahan, 1996). A wealth of evidence supports the concept that viral induced mammary tumors and hyperplasias in mice are clonal dominant populations and probably represent the progeny of a single cell (Cardiff *et al*., 1983; Cohen *et al*., 1979a,b; Kordon *et al*., 1995; Young *et al*., 1971). Similar conclusions about monoclonality have been reached for human breast cancer, carcinoma of the colon, uterine, cervix and bladder, ovarian teratomas and many hematological neoplasms (Fearon *et al*., 1987, Sidransky *et al*., 1992, Wainscoat *et al*., 1990). This implies that mammary tumors and hyperplasias are developed from tissue-specific epithelial stem cells and therefore represent populations of mutated stem cells and their differentiating progeny. According to the concept of maturation arrest of stem cell differentiation, malignant stem cells arise from the normal tissue-determined stem cells required for tissue renewal and produce tumors that are caricatures of the normal renewal process because of the imperfect differentiation of their proliferation-competent progeny (Sell and Pierce, 1994). In this article we provide an up-date of the MMTV induced mouse mammary tumor model system and a new perspective regarding its relevance to understanding the biology of human breast cancer.

### *Inbred mouse strains having a high incidence of mammary tumors*

The origin of mouse models for breast cancer began 60–70 years ago with the development of the A, DBA, C3H, GR, BR6, and RIII inbred mouse strains which were selected for having a high incidence of mammary tumors that developed with a short latency (reviewed in Hilgers and Sluyser, 1981; Morse, 1978). In genetic crosses between these strains and low mammary cancer strains (with the exception of GR mice), the incidence of mammary tumorigenesis of the mother was consistently favored. This suggested an 'extra-chromosomal' epigenetic influence on the inheritance of susceptibility to breast cancer (Staff of Jackson Laboratory, 1933). Bittner's (1936) demonstration in

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1936 of the 'milk factor' in the mouse strains having a high incidence of mammary tumors, and subsequent studies (Hilgers and Sluyser, 1981; Smith, 1967), suggested that the maternal 'influence' was a highly infectious virus. Subsequent genetic studies revealed host mutations which enhanced the infectivity of the virus or complemented the genes frequently activated by MMTV (reviewed in Weiss *et al*., 1982). Each high incidence mouse strain congenitally transmits highly infectious MMTV through the milk to their offspring. In addition, the C3H (Michalides *et al*., 1981, Smith and Vlahakis, 1982, van Nie and Verstraeten, 1975, Vlahakis *et al*., 1970) and GR (Michalides *et al*., 1985, van Nie *et al*., 1977) mouse strains each contain a dominantly-expressed, genetically transmitted or endogenous MMTV proviral genome (Mtv-1 and Mtv-2, respectively) that encodes an infectious virus that is also present in the milk. Parous C3H females develop pregnancy independent mammary tumors that frequently arise as clonal outgrowths from preneoplastic hyperplastic alveolar nodules (HAN) at 7–10 months of age (Squartini, 1961). Similarly, C3H mice in which the horizontally transmitted MMTV has been removed also develop pregnancy independent mammary tumors as a consequence of infection by the Mtv-1 encoded virus. These Mtv-1-induced tumors mainly arise in the second year of life (Smith and Vlahakis, 1982, van Nie and Verstraeten, 1975, Vlahakis *et al*., 1970). The GR, BR6 and RIII females have a high incidence of pregnancy dependent mammary tumors or plaques that, after one or more parities, progress to a pregnancy independent tumor (Lee, 1968, Squartini, 1961, Van Nie *et al*., 1977). Foulds (1956) and Squartini (1979) described plaques as 'a system of branching tubules often with bulbous ends'. Squartini *et al*. (1963) demonstrated that differences in the manner in which the disease progressed in C3H and RIII females was a function of the particular strain of exogenous virus. In their study BALB/c mice which have a low or no incidence of spontaneous mammary tumors (Squartini, 1961) developed a high incidence of pregnancy-independent tumors when infected with horizontally transmitted MMTV (C3H) and pregnancy-dependent tumors which progressed to pregnancy-independence when infected with MMTV(RIII). Even more surprising was the demonstration by Squartini that the virus could increase mammary secretory differentiation in virgin females and that the intensity of this activity was unique to the strain of MMTV present in the gland (Squartini *et al*., 1983).

### *Feral mouse strains having a high incidence of mammary tumors*

Surveys of feral *Mus musculus* have demonstrated the presence of poorly infectious MMTV in the milk of breeding females (Andervont, 1952; Imai *et al*., 1994). In general, the incidence of mammary tumors in feral breeding females is low, and tumor development occurs late in life (Andervont and Dunn 1962; Rongey *et al*., 1973). In recent years evidence for the expression of MMTV in lactating mammary epithelium has been reported in several subspecies of *Mus* from Europe (Callahan *et al*., 1982) and reproductively separated species of *Mus* from Asia (Escot, *et al*., 1986; Schlom, 1978; Teramoto, 1980). In each case there was a significant incidence of focal mammary tumors. Each of these appeared to be the result of the clonal expansion of a cell containing acquired MMTV proviral genomes.

Our studies have focused on a *Mus musculus musculus* strain (designate CzechII) derived from a single breeding pair of mice trapped in Czechoslovakia. The female antecedent was a carrier of an infectious MMTV transmitted through the milk. This colony has a 20% incidence of pregnancy-independent mammary adenocarcinomas that are histopathologically similar to those induced by MMTV (C3H). Feral mice are generally hemyzigous for genetically transmitted (endogenous) MMTV genomes (Callahan *et al*., 1986). Because of the random assortment of sister chromatids in gametic cells at each generation, some fraction of the offspring will not inherit the endogenous MMTV genomes. In our single breeding pair neither CzechII founder had inherited a genetically transmitted MMTV genome. Thus, no offspring from this mating will bear endogenous MMTV DNA sequences. This characteristic provided our rationale for studying mammary tumorigenesis in this mouse family. From a practical point of view, mice devoid of endogenous MMTV provide a genetic background for the unambiguous identification of somatically acquired proviral genomes (Figure 1). Thus, genes commonly mutated by MMTV integration during mammary tumorigenesis can be readily detected.

### *The MMTV infectious pathway*

The MMTV life cycle begins with the ingestion of infected milk by the pups of a viremic mother (Reviewed in Held *et al*., 1994; Ross, 1998). A shortened gastric vein that empties into the superior hilus of the spleen has been implicated in the individual susceptibility of the pup because it insures the rapid transit of the infectious virus to susceptible lymphoid cells in the spleen (Roubinian and Blair 1980). After a few days the virus infects B cells in lymphoid tissue of the gut, e.g. Peyer's patches. In addition to the gag, pol, and env proteins commonly encoded by retrovirial genomes, the U3 region of the long terminal repeat (LTR) of MMTV encodes a protein designated superantigen (SAg). Sags are presented on the cell surface by major histocompatiblity (MHC) class II proteins in antigen-presenting cells (APCs) such as B cells. The Sag causes the proliferation of cognate T cells, which in turn stimulate bystander B cell proliferation. This develops a reservoir of infection-competent cells from which MMTV spreads to other lymphocyte subsets ($CD4^+$ and $CD8^+$ cells) during the following weeks. Deletion of Sag reactive T cells is detected during the first 2 months of life and seems to have no impact on the health of the animal.

Infected lymphoid cells play a critical role in the infection of mammary epithelial cells. Whether the virus is transferred by cell–cell contact or whether infected lymphoid cell represents a vehicle to deliver virus to the mammary gland is unknown. It is known that MMTV infection also occurs in a variety of other epithelial tissues, including salivary gland, kidney, lung, seminal vesicle, epididymis, and testis (Imai *et al*., 1983; Muhlbock, 1950; Smith, 1965; Tsubura *et al*., 1981). Presumably, all of these tissues are infected in the same

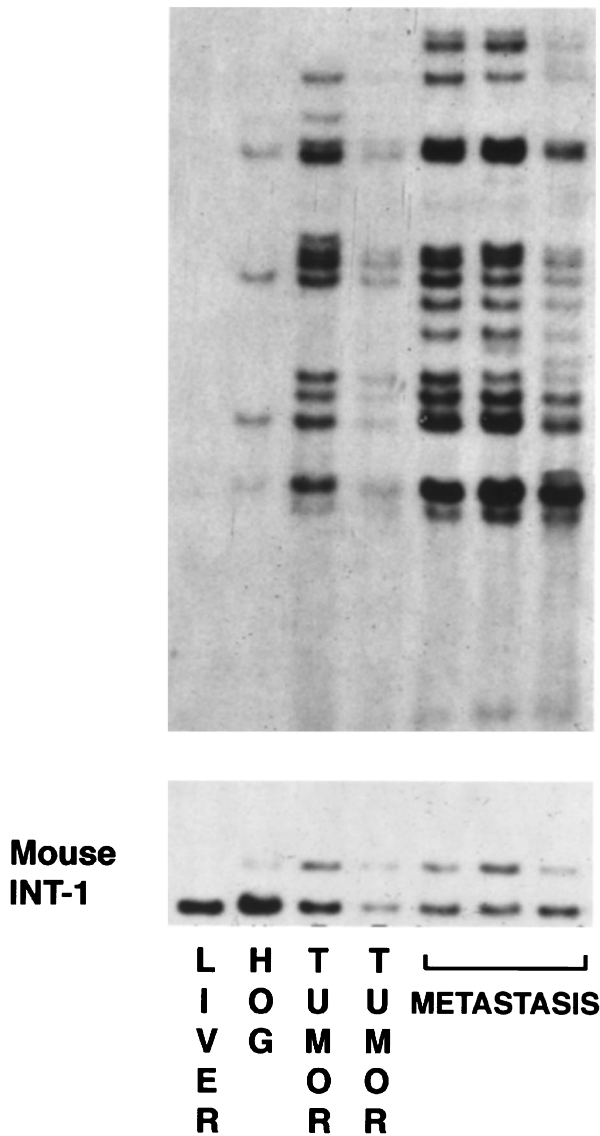


**Figure 1** MMTV proviral genomes in the CZZ2 preneoplastic hyperplastic outgrowth line (HOG) and CZZ2 derived malignant tumors and lung metastasis. CZZ2 HOG and tumor DNAs (10 $\mu$g each) were digested by *Eco*RI, run on a 0.8% agarose gel, and transferred to a nylon membrane. Upper panel, the cellular DNAs were from the CzechII liver (lane 1), CZZ2 HOG (lane 2); CZZ2 derived mammary tumors 1262 (lane 3) and 1263 (lane 4), tumor 1263 derived metastasis #1(lane 5), #2 (lane6), #3 (lane 7). Panel B, the same cellular DNAs were digested with BglII and analysed as above. The membranes coresponding to Panels A and B were hybridized with MMTV LTR and Wnt1 genomic DNA (Marchetti *et al*., 1991) probes, respectively at 65°C using previously described conditions (Gallahan and Callahan, 1987a)

way as the mammary gland. Nevertheless, with few exceptions (Felluga *et al*., 1969) only the mammary epithelium is malignantly transformed subsequent to infection and replication of MMTV. This suggests that mammary epithelium and MMTV share a unique relationship, since proviral insertion (hence, mutation) and viral replication are inseparable. The key to this puzzle seems to be the regenerative activity of the mammary epithelium as compared to the other comparably infected tissues. For example, the epithelium in the mammary gland increases roughly 30-fold each pregnancy only to shrink a like amount following the cessation of lactation during involution (Kordon and Smith, 1998; Nicoll and Tucker, 1965). In all MMTV-infected strains the rate and frequency of mammary tumorigenesis is increased by multiple pregnancy cycles (DeOme *et al*., 1978a,b; Squartini *et al*., 1983). This by itself does not completely explain the occurrence of neoplastic transformation in the mammary epithelium because nearly all of the MMTV-infected cells die by apoptosis at involution. The explanation may be the presence and persistence of multipotent mammary stem cells which are self-renewing and subject to infection by MMTV (Kordon and Smith, 1998). It is these MMTV-infected cells which persist and acquire further mutations that give rise to MMTV-induced hyperplasias and tumors.

*Mammary epithelial stem cells: the targets for MMTV replication in the mammary gland*

DeOme *et al*., (1959) demonstrated that virtually any portion of the mammary gland could recapitulate the entire glandular structure upon transplantation into a cleared mammary fat pad. Similar results are obtained with isolated mammary epithelial cells, although larger numbers of cells are generally needed due to technical considerations (Smith, 1996). Age and hormonal status of the donor does not seem to alter the innate regenerative capacity present in the mammary epithelial population (Young *et al*., 1971). However, regenerative senescence is induced by repeated serial passage of portions of the outgrowth (Daniel *et al*., 1968; 1971a,b; Young *et al*., 1971). The rate of senescence for regenerative growth is strongly linked to the frequency of mitotic activity that has occurred in the transplant fragment that is selected. For example, outgrowths generated from fragments of the peripheral portions of a given outgrowth, which presumably represent cells which have undergone a greater number of mitoses, show a senescent growth phenotype at an accelerated frequency compared to outgrowths of fragments taken from the central portions of the same serially transplanted outgrowths (Daniel and Young, 1971a). Utilizing this technique, DeOme and others demonstrated that MMTV-induced premalignant mammary epithelial lesions were present in the mouse mammary gland (Daniel *et al*., 1968; DeOme *et al*., 1959; Medina, 1973; Smith *et al*., 1984). Premalignant outgrowths do not show regenerative senescence or post-lactational involution and therefore represent epithelial populations which have attained or retained a proliferative 'immortality'. In all likelihood, this characteristic is shared among all premalignant epithelial populations, irrespective of species or tissue type (Sell and Pierce, 1994).

MMTV infects mammary epithelial cells and randomly inserts its proviral DNA into the host somatic cell DNA during its replicative cycle (Ringold *et al*., 1979; Withers-Ward *et al*., 1994). As discussed below some of these random insertions have been shown to cause deregulation of cellular (INT) genes leading to premalignant transformation and subsequently to tumor progression. If Sell and Pierce (1994) were correct in their conclusion that hyperplasias were the result of mutated stem cells, normal mammary epithelial stem cells in CzechII mice might become infected by MMTV and acquire mutations. If stem cells produce all the cells in a mammary gland then normal mammary outgrowths from implanted

fragments might be clonal populations from a single stem cell. To test this hypothesis, Kordon and Smith (1998) transplanted mammary epithelial fragments, chosen randomly, from mature, parous MMTV (CzechII)-infected female mice into gland-free mammary fat-pads of syngeneic mice. Unique virus-host restriction fragments will be detectable and constitute a specific pattern of bands only if the outgrowths are clonal (or nearly-clonal) because they will be present in all (or nearly all) the epithelial cells. They found that 60% of the normal lactating outgrowths from multiparous MMTV-infected Czech implants produced specific patterns of virus-host restriction patterns upon Southern blot analysis, proving that they were clonally-derived from infected mammary stem cells. Serial transplantation of these outgrowths showed identical patterns of virus-host restriction fragments demonstrating that the original MMTV-infected stem cell was capable of self-renewal. If mammary outgrowths are derived from the expansion of many different mammary epithelial cells, then specific MMTV-host restriction fragments will not be detectable, since retroviral DNA insertions occur randomly at multiple sites in the cellular DNA (Withers-Ward *et al*., 1994). Intact, lactating MMTV-infected mammary glands represent polyclonal populations because secretory lobules develop from multiple progenitors at multiple sites along an existing mammary ductal tree. Therefore, only limited locally-developed progeny will reflect a given MMTV insertional event(s). Since none of these patterns is predominant in the whole population, DNA from the host mammary epithelium is not expected to produce a pattern of specific MMTV-host restriction fragments upon Southern analysis. We found this to be the case.

Analysis of mammary outgrowths ($n=16$) derived from fragments of prepubertal ($<3$ week-old) MMTV(CzechII)-infected female glands failed to demonstrate the presence of MMTV insertions in stem cells capable of clonally generating a complete functional gland. We did however occasionally detect the presence of significant amounts of unintegrated MMTV DNA in the DNA preparations, indicating the presence of infectious replicating virus within some of the individual outgrowths and that MMTV replication is not unique to stem cells. Subsequent transplantations of epithelial fragments ($n=18$) from three separate mature virgin females also proved negative by Southern blotting for MMTV-host restriction fragments. However, in these preparations, we were unable to demonstrate the presence of MMTV DNA by PCR analysis suggesting that viral replication was not active in these transplants (Smith, GH manuscript in preparation). In a second experiment, three of 12 outgrowths, produced from random fragments taken from an individual gland of an MMTV-infected virgin Czech mouse, were found positive for MMTV DNA (Smith, unpublished results). These observations suggest that MMTV infection of individual stem cells is more likely to occur during pregnancy and lactation than in the quiescent epithelium of non-breeding females. This may be the reason that the premalignant lesions most often associated with MMTV infection are the well-described and studied hyperplastic alveolar lesions (HAN) found in the involuted glands of multiparous MMTV-infected mice. Our findings are consistent with the observations of (DeOme *et al*., 1978a,b) that show MMTV-infected, nodule-transformed cells in BALB/c mouse mammary glands occur much more frequently in late pregnant, first-pregnancy females than in their virgin littermates as determined by dissociation of the tissue and transplantation of aliquots of viable cells into cleared fat pads (100 000 cells/pad).

Our demonstration, that both normal and nodule-transformed mammary outgrowths are clonally-derived, strongly implicates MMTV-infected, mammary specific epithelial stem cells in the generation of MMTV-induced mammary hyperplasias and malignancies. A similar scenario probably applies to the MMTV induced pregnancy dependent 'plaque' lesions which evolve after a few parities to pregnancy independent tumors. However, the basis for the difference in the etiology of tumors arising from infection with the hyperplastic alveolar nodule (HAN) and plaque inducing strains of MMTV has not been studied at the molecular level. Nevertheless, it is known that the nucleotide sequence of their respective LTRs and ENV genes contain potentially significant differences. For instance it seems possible that the subtle differences present in the nucleotide sequence of the LTRs of different MMTV substrains might modify their interaction with nuclear transcription factors in a way that restricts or enhances MMTV gene expression in certain subpopulations of mammary epithelial cells (Qin *et al*., 1999). Alternatively, differences in the envelope proteins might modify viral interaction with receptors unique to different classes of mammary epithelial stem cells. Recently, a cell surface receptor for MMTV(C3H) has been molecularly cloned and the gene locus assigned to the proximal end of mouse chromosome 19 (Golovkina *et al*., 1998). Although the gene is expressed in several adult tissues, the distribution of expression in the developing mammary gland is not known. In addition, earlier work using the vesicular stomatitis virus pseudotypes containing MMTV envelop proteins to infect mouse-hamster somatic cell hybrids identified mouse chromosome 16 as containing a gene for the MMTV(C3H) receptor (Hilkens *et al*., 1983). An important gap in our understanding of the MMTV infectious pathway is the precise identity of the target cell and the factors which distinguish the etiologies of the HAN and plaque inducing subclasses of MMTV.

We have also observed that mammary tumors and metastases may occur within senescing, serially transplanted mammary outgrowths clonally derived from an MMTV-infected mammary stem cell (Smith GH, manuscript in preparation). The outgrowths from this stem cell appear morphologically and functionally normal and exhibit a senescent phenotype upon serial transplantation. Nevertheless, mutations occurring within either the multipotent stem cell itself or one of its lineage-committed progeny gave rise to a highly aggressive and malignant neoplasm. This transformation did not involve the generation of a stable intermediate, premalignant hyperplastic stage. Rather the tumor arose from a senescencing mammary stem cell. Therefore, malignant transformation of mammary epithelial stem cells need not progress through a morphologically and physiologically-distinct premalignant phase such as the HANs or hormone-dependent plaques.

### *Target host cellular genes and the mechanisms by which MMTV activates/inactivates them*

Early in the 1980s, Nusse and Varmus (1982) and independently, Dickson *et al*. (1984) developed a strategy based on the clonal nature of the MMTV-induced mammary tumors for identifying genes activated by the integration of a MMTV provirus using the viral genome as a 'molecular tag' (Varmus, 1982). Their studies focused on inbred mouse strains having a high incidence of MMTV-induced mammary tumors.

Subsequently, we expanded this approach to the CzechII mouse strain where we could easily follow the progressive acquisition of integrated proviral genomes as a function of the stage of malignant advancement (Gallahan and Callahan, 1987a). As shown in Figure 1 the CZZ-2 CzechII HOG contains two detectable integrated viral genomes, represented by four virus-host restriction fragments in lane 2, upper panel. One of the insertions results in the generation of a larger Wnt1 (INT1) restriction fragment from one of the alleles (lower Panel) because the insertion replaced the host genomic *Eco*RI site adjacent to the gene (Nusse and Varmus, 1982). Two independent tumors which arose from within the CZZ-2 HOG implants located in the contralateral #4 fat pads of the same mouse and contained additional integrated MMTV genomes (upper panel, lane 3 and 4). The tumor represented in Figure 1, lane 4, produced a large lung metastasis. The metastasis was divided into six fragments and transplanted to contralateral inguinal fat pads of three Czech females, DNA from the tumors produced from three of these transplants is shown in lanes 5–7. Each of these metastatic tumors showed unique collateral integrations in addition to those found in the tumor shown in lane 4. All of the lesions possessed the original insertion that caused a rearrangement of Wnt1 present in the premalignant HOG (lower panel, lanes 3–7). Our working hypothesis is that some of the additional acquired proviral genomes represent mutational events which contribute to tumor progression. Using MMTV as a 'molecular tag' (Varmus, 1982), members of four cellular gene families have been shown to be rearranged by MMTV integration. These include members of the Wnt (Lee *et al*., 1995; Nusse and Varmus, 1982; Roelink *et al*., 1990), Fgf (Dickson *et al*., 1984; MacArthur *et al*., 1995; Peters *et al*., 1989), Notch gene families (Dievart *et al*., 1999; Gallahan and Callahan, 1987a, Robbins *et al*., 1992; Sarkar *et al*., 1994) and the gene encoding the p48 component of eucaryotic translation initiation factor-3 (elF-3p48) (Marchetti *et al*., 1995); Asano, 1997 [#35].

### *The* Wnt *and* Fgf *genes*

The *Wnt* genes are members of a family of 12 or more genes related to the *Drosophila* segmented polarity gene, *wingless (wg)* (reviewed in Nusse, 1997). The *Wnt-1* and *Wnt3* genes are normally not expressed in the mammary gland, but are expressed at specific sites and times during embryonic development or in other adult tissues (Gavin *et al*., 1990; Huguet *et al*., 1994). However, several of the other *Wnt* gene family members are expressed at defined times during mammary gland differentiation and development. *Wnt1* encodes cysteine-rich secreted glycoproteins of 41–44 kD which are associated with extracellular matrix and cell surfaces (Smolich *et al*., 1993). Based on studies of the *Drosophila* mutant *wg* (the homolog of *Wnt1*) there is evidence that one function of *Wnt1* is to increase $Ca^2$-dependent cell adhesion through a cellular signal transduction pathway that regulates the $\beta$-catenin intracytoplasmic (or intracellular) pool size and stabilizes its binding to cadherin (reviewed in Klingensmith and Nusse 1994; Nusse and Varmus, 1992).

The *Fgf3*, *Fgf4*, and *Fgf8* genes are members of the fibroblast growth factor (Fgf) gene family (Delli Bovi *et al*., 1987; Dickson and Peters, 1987; Wilkinson *et al*., 1988; Yoshida *et al*., 1987). Like Wnt1 and Wnt3 genes, they too are only expressed during early embryonic development or adult tissues other the mammary gland (Jakobovits *et al*., 1986). Members of the *Fgf* family vary in length, but are homologous to one another within a core of 120 amino acid residues. *Fgf*s exhibit an overlapping, but not identical, range of biological activities that can act as mitogens, chemoattractants, and mediators of cellular differentiation. *Fgf*s are also potent angiogenic factors *in vivo* (reviewed in Basillico and Moscatelli, 1992, Goldfarb, 1990).

The mechanism by which MMTV activates the expression of the *Wnt and Fgf* genes, is primarily a consequence of the effect of enhancer sequences within the long terminal repeat (LTR) of the integrated MMTV proviral genome on the transcriptional promoter of the adjacent affected gene. The activation of a target gene transcription by this mechanism can occur at distance of up to 25 kb 5′ or 3′ of the target gene.

### *The* Notch *gene family*

The *NOTCH* gene family (designated 1–4) is related to the *Drosophila Notch* gene (reviewed in Artavanis-Tsakonas *et al*., 1995; Weinmaster, 1997). Members of this gene family encode transmembrane receptor proteins that are involved in cell fate determinations during development (Muskavitch, 1994). Expression of each of the murine *NOTCH* genes can be detected in mammary glands of virgin, pregnant and lactating mouse mammary glands (our unpublished data). Dievart *et al*., (1999) has found NOTCH1 to be rearranged by MMTV in two out of 24 mammary tumors of (MMTV)/neu transgenic mice. So far *Notch2* and *Notch3* have not been found to be rearranged by MMTV in mouse mammary tumors (unpublished data). Activation of the *NOTCH-4/Int-3* locus (Gallahan and Callahan 1997; Uyttendaele *et al*., 1996) was first detected in the CzechII mouse mammary tumors (Gallahan and Callahan, 1987a; Robbins *et al*., 1992). The locus was defined by the integration of an MMTV proviral genome within a 500 bp region of the cellular genome of five independent mammary tumors corresponding to an exon of the target gene. The *NOTCH4/Int3* locus is located in the class II region of the major histocompatibility (*MHC*) locus on chromosome 17 (Gallahan *et al*., 1987b; Siracusa *et al*., 1991). In each case the transcriptional orientation of the integrated viral genome was in the same direction which was the same as that of the target gene. A 2.3 kb species of RNA was detected in tumors containing a viral

induced rearrangement of *NOTCH4/Int3*. This RNA species was not detected in tumors where the locus was intact nor in the normal mammary gland. All of the viral integration events within *NOTCH4/Int3* occurred within one of three exons encoding amino acid residues just N-terminal to the transmembrane domain of the encoded protein. These viral integration events result in the constitutive overexpression of the portion of the gene encoding the intracellular domain of the protein.

Experiments in which the same region of the *Drosophila Notch* gene are overexpressed demonstrated that this represents a gain-of-function mutation, mimicking the consequences of the interaction between the Notch protein and its ligand (Struhl, *et al*., 1993).

### *The* eIF3p48 *gene*

The *Int6* gene has been highly conserved through evolution (Asano *et al*., 1997; Diella *et al*., 1997; Marchetti *et al*., 1995; Miyazaki *et al*., 1999) and unpublished. The amino acid sequence of the mouse and human *Int6 6* gene products are identical and related sequences are present in *Drosophilia C. elegans* and *Saccharomyces pombe*. The gene encodes the p48 component of the eucaryotic translation initiation factor-3 (eIF-3p48) and, although its function in the translation initiation process is not known, it is expressed in all adult tissues which have been tested including the mammary gland and as early as day 8 of embryonic development (Asano *et al*., 1997; Diella *et al*., 1997). Integration of MMTV into the *eIF-3p48/Int6* gene has been detected in a CzechII preneoplastic hyperplastic outgrowth line (HOG) as well as two independent mammary tumors from unrelated CzechII mice (Marchetti *et al*., 1995). In each case an MMTV genome integrated into an intron *eIF-3p48/Int6* in the opposite transcriptional orientation and resulted in the expression of a truncated RNA species which terminates at a cryptic termination signal in the reverse sequence of the MMTV LTR. The non-rearranged allele of *eIF-3p48/Int6* in these tumors was checked for the presence of a somatic mutation, but none was found. We concluded that MMTV integration into *elF-3p48/Int6* either results in the expression of a biologically activated form of *elF-3p48/Int6* or that it corresponds to a dominant-negative mutation.

### *Biological consequences of target gene expression on mammary gland development and tumorigenesis*

The biological consequences of *Wnt1* and *Fgf3* expression on mammary gland development and tumorigenesis has been evaluated in transgenic mouse strains containing either MMTV LTR activated *Wnt1* or *Fgf3* transgenes (Muller *et al*., 1990; Stamp *et al*., 1992; Tsukamoto *et al*., 1988). Mammary glands of *Wnt1* transgenic virgin females resemble the hormonally stimulated glands normally observed in pregnant animals except that there are increased numbers of terminal branches and alveoli producing a diffuse lobular-alveolar hyperplasia. Focal mammary tumors arise from within these hyperplasias that are indistinguishable from the MMTV-induced disease. This is consistent with the pregnancy-independent nature of mammary tumorigenesis in C3H mice.

In contrast, mammary glands of virgin *Fgf3* transgenic females appeared normal with only microscopic areas of ductal hyperplasia composed of focal aggregates of cells. Mammary hyperplasia was most evident during pregnancy. Three patterns of proliferation were observed: ductal hyperplasias, papillocystic forms, and nodular solid aggregates of cells. After parturition the hyperplastic areas either regressed or remained static, becoming more pronounced in subsequent pregnancies. In this regard the lesions resemble the pregnancy-dependent lesions of the BR6 mice. Focal mammary tumors arose in a fraction of the mice at a late age. The most common tumors were mixtures of ductal hyperplasias composed of irregular, anastomosing, bilayered tubules that exhibited no signs of lactational activity. The hormone-dependence of these tumors has not been established.

Transgenic mice which express MMTV activated *Int3* as a transgene, develop a profoundly altered mammary gland and within 4–6 months 100% have focal mammary tumors (Jhappan *et al*., 1992; Smith *et al*., 1995). In virgin females the mammary ductal epithelium minimally penetrates the mammary fat pad. During the first pregnancy the mammary fat pad fills with ductal epithelium, but there is little lobular-alveolar development. The tumors appear as focal outgrowths derived from intraductal hyperplasias which are common within virgin and parous females. Since *Int3* is expressed during normal mammary gland development, activation of this gene by MMTV appears to either deregulate normal development controls leading to hyperplasia from which tumors develop or provides a force towards malignancy. Interestingly we have not found *Int3* to be rearranged in our panel of HOGs (E Kordon and GH Smith, personal communication), it has only been found to be rearranged by MMTV in feral mouse (Gallahan and Callahan, 1987a; Sarkar *et al*., 1994) and two BR6 inbred mouse mammary tumors (Peters, 1990). We conclude that the effect of expression of the truncated *Int3* protein on mammary gland development and tumorigenesis is exquisitely dependent on the timing of its expression relative to mammary gland development.

### *The effect of the host genetic background on the frequency with which specific genes are activated by MMTV in mammary tumors*

The frequency with which the common integration sites for MMTV are rearranged by the virus in mammary tumors seems to be dependent on the host mouse strain and the strain of virus (Table 1) (Marchetti *et al*., 1991; Peters, 1990). For instance, Wnt1 is activated by MMTV in 75% of the C3H mammary tumors whereas in mammary tumors of other mouse strains infected with MMTV(C3H) or other strains of MMTV the frequency of Wnt1 activation is 26–40%. Similarily, Fgf3 and Notch4/Int3 appear to be preferentially activated in specific MMTV infected mouse strains. Thus Fgf3 was activated in 60–66% of RIII (Table 1) and BR6 (Peters, 1990) mammary tumors, respectively. In other mouse strains infected by MMTV(RIII) or other strains of MMTV, Fgf3 was activated in 4–30% of the mammary tumors. Of the MMTV-infected mouse strains tested Notch4/Int3 is

activated by MMTV only in mammary tumors from the feral CzechII (18%, Table 1), and *M.m. jyg* (43%, (Sarkar *et al*., 1994)), and inbred BR6 (7%, (Peters, 1990)) mouse strains. This suggests that during inbreeding of the high incidence inbred mouse strains or the founders of feral mouse strains, mutations were fixed in the germline that either provide a selective growth advantage to mammary epithelial cells having particular activated genes or broaden the host range within the mammary epithelium for MMTV infection and replication.

Relevant to this discussion are the results of a survey of C3H HANs showing that *Wnt1* is only infrequently rearranged by MMTV (Schwartz *et al*., 1992). This suggests that in the context of the C3H genetic background activation of *Wnt1* plus possibly an *Fgf* gene is sufficient to induce a malignancy in the mammary gland. In this scenario the additional mutation(s) required for malignancy are probably already present in the germline. Consistent with this hypothesis, CzechII mice also develop mammary preneoplastic HANs which we have developed into mammary hyperplastic outgrowth lines (HOGs). A survey of DNA from 31 CzechII HOGs revealed that 22.6% (seven out of 31) had MMTV induced rearrangements of *Wnt1*, none had rearrangements of either *Fgf3* or *Fgf4* (our unpublished data). Since the frequency of MMTV-induced rearrangements of *Wnt1* is similar in both CzechII HOGs and mammary tumors, it seems likely that in the setting of this mouse strain activation of *Wnt1* is primarily an early event in tumorigenesis which disrupts regulatory controls of normal mammary gland development leading to lobular hyperplasia. Mammary tumors arising from within these HOGs frequently contain additional MMTV proviral genomes. This raises the possibility that some of these additional acquired proviral genomes activate/inactivate genes that collaborate with activated Wnt1.

### *Inter-relationships between the signaling pathways activated by common insertions of MMTV in mouse mammary tumors*

Coactivation of Wnt1 and Fgf3 expression by MMTV in C3H and RIII tumors (18% and 28%, respectively) suggests that co-expression of these genes may collaborate in mammary tumor progression. This thesis has been tested in two ways. First, analysis of mammary tumor development in MMTV-Wnt1/MMTV-Fgf3 bi-transgenic mice and second, analysis of mammary tumors arising in MMTV(C3H) infected MMTV-Wnt1 and MMTV-Fgf3 transgenic mice for new common integration sites.

*Wnt1* transgenic mouse strain develops hyperplasias of mammary epithelium prior to tumorigenesis. The *Wnt1/Fgf3* bitransgenic mouse strain develops pregnancy-independent mammary tumors earlier and at a higher frequency than in either parental line (Kwan *et al*., 1992). In 45% of the mammary tumors (36 out of 80) arising in MMTV(C3H)-infected *Wnt1* transgenic mice contained a viral insertion at either *Fgf3* or *Fgf4* (Shackleford *et al*., 1993). Moreover, another 10% of the tumors (eight out of 80) contained a virus integration at *Fgf8* (MacArthur *et al*., 1995). Similarly, eight of 35 (23%) mammary tumors from MMTV(C3H)-infected *Fgf3* transgenic mice contained a viral insertion at *Wnt1* (Lee *et al*., 1995). Another two tumors from this cohort contained a viral insertion at a new related gene, *Wnt10b*. Although in these latter two cases, activation of *Wnt10b* expression, as a consequence of viral integration, was not demonstrated. Taken together, however, these results strongly imply that certain members of the *Wnt* and *Fgf* gene families collaborate in the deregulation of normal control of growth and differentiation toward malignant mammary tumorigenesis when activated by MMTV. The molecular basis for this collaboration remains to be defined.

Another potential collaborative relationship between genes activated in mammary tumors is suggested by studies of MMTV(C3H) infected (MMTV LTR)/*neu* transgenic mice (Dievart *et al*., 1999). In this mouse strain MMTV infection decreases the latency of mammary tumor development. In two out of 24 mammary tumors tested *Notch1* was shown to be rearranged by an acquired MMTV proviral genome in a manner similar to that observed in mammary tumors in which *Notch4/Int3* has been activated by MMTV (Gallahan and Callahan, 1997). Thus the rearrangements of *Notch1* in these tumors represents a gain-of-function mutation. The basis for the collaboration between activated *Notch1* and (MMTV)/*neu* is unclear. However, one possibility may be that the transcription promoter for the wild type c-*erb*-B-2 gene contains binding sites for the transcription factor CBF-1 (Chen *et al*., 1997). The gene product of MMTV activated Notch1 would be expected to bind CBF-1 and upregulate the transcription of the endogenous c-*erb*-B-2 gene. One consequence of elevating the level of c-*erb*-B-2 may be for the cellular concentration of the receptor to reach a critical level which pushes mammary epithelium toward malignant transformation.

Genetic analysis of the role of the *Drosophila* Wingless (*Wg*) and Notch signaling pathways has provided evidence that they both function in many of the same patterning events during development and that their expression has a 'yin – yang' type of influence

**Table 1** Frequency of MMTV insertion into Int Loci[a]

| *Mouse strain* | *Number of tumors* | *Frequency of insertions* (%) *Int1/Wnt1* | *Int2/Fgf3* | *Wnt1 + Fgf3* | *hst/FGF4* | *Int3* |
|---|---|---|---|---|---|---|
| C3H/OuJ | 28 | 57 | 3.5 | 18 | 0 | 0 |
| BALB/cfC3H | 30 | 23 | 23 | 7 | 3 | 0 |
| RIII | 29 | 14 | 31 | 27.5 | 0 | 0 |
| BALB/cfRIII | 40 | 15 | 7.5 | 17.5 | 0 | 0 |
| CZECHII | 45 | 24 | 2 | 2 | 0 | 18 |
| CZECHIIIfC3h | 30 | 30 | 13 | 0 | 0 | 3 |

[a]This data is from Marchetti *et al*. (1991), Peters (1990) and our unpublished data

on these events (reviewed in Axelrod *et al.*, 1996). Genetic analysis of *Drosophila* has identified several genes comprising the Wg signaling pathway (reviewed in Dierick, *et al.*, 1999). A member of the frizzled gene family, *Dfz2*, encodes the Wg receptor. In an early step after ligand-receptor interaction is the activation of the cytoplasmic protein disheveled (*dsh*). Activated Dsh inactivates the serine-thronine kinase Shaggy-Zeste-white 3 (*Sgg-Zw3*). This protein is homologous to mammalian glycogen synthase kinase-3 (*GSK-3*). In the absence of *Wg* signaling *Sgg-Zw3* phosporylates and inactivates Armadillo (*Arm*), the *Drosophila* homolog of $\beta$-catenin. Non-phosphorylated Arm is released from the cellular membrane and activates Wg-dependent gene expression. Expression of the achaete scute gene complex (*AS-C*) is up-regulated by *Wg* signaling. *AS-C* is a complex of genes encoding transcription factors. Activation of *Drosophila* Notch (*N*) by its ligands or by the action of gain-of-function mutations leads to the up-regulation of expression of genes within the Enhancer of Split complex (*E(spl)C*) (reviewed in Artavanis-Tsakonas *et al.*, 1995; Weinmaster, 1997). This action is mediated through a nuclear complex composed of the *N* intracellular domain (ICD) and the transcription factor suppressor of hairless (*Su(H)*). *E(spl)C* encode proteins which repress the expression of *AS-C*. *Wg* activation of *Dsh* also has a direct affect on *N* signaling (Axelrod *et al.*, 1996). Activated Dsh physically binds to the *N* ICD blocking any interaction with Su(H) and thus inhibits E(spl)C expression. Therefore it could be expected that in a situation where activated *N* and *Wg* are co-expressed, the *Wg* phenotype would be dominant.

Uyttendaele *et al.* (1998) have addressed this issue in a study on the effect of activated *Notch4/Int3* and *Wnt1* expression on branching morphogensis of the mouse mammary epithelial TAC-2 cell line. They have shown that, in this setting, Wnt1 expression induces elongation and branching of epithelial tubules whereas activated Notch4/Int3 expression inhibits branching morphogenesis. In addition, they have shown that the minimal part of the Notch4/Int3 ICD required for this phenotype is the region required for binding the Su(H) homolog CBF1. As predicted from the *Drosophila* studies, TAC-2 cells co-expressing activated *Notch4/Int3* and *Wnt1* exhibited the Wnt1 branching ductal morphogenesis.

Genetic and molecular analysis of the function of the 36 EGF-like repeats (EGFLR) of the *Drosophila N* extracellular domain (ECD) have shown that EGFLR 11 and 12 are both necessary and sufficient for binding ligands Delta and Serrate (Rebay *et al.*, 1991). However, these two EGFLR are not sufficient for wild type *N* phenotype (Lieber *et al.*, 1993). For instance, point mutations in EGFLR 2 (*nd*), 14 (*spl*), 24 and 25 ($Ax^9$, $Ax^{59d}$, $Ax^1$), 27 ($Ax^{71d}$), 29 ($Ax^{16}$, $Ax^{E2}$) or 32 ($N^{ts1}$) produce lethality or aberrant Notch function. Interestingly, Wesley (1999) has demonstrated that Wg binds to the *N* ECD and that EGFLR 19–36 are both necessary and sufficient for binding. In fact within this region there are two highly conserved regions comprised of EGFLR 23–27 and EGFLR 31–34 whose functional significance is suggested by the presence of at least one lethal mutation in each region. Wesley compared *Drosophila* S2 cells expressing either *N* or $N^{\delta EGF1-18}$ for the effect of Wg on the expression of other genes downstream of *N* or *Wg*. The intracellular signaling pathways associated with the transduction of signals by *N* and $N^{\delta EGF1-18}$ do not involve upregulation of *E(spl)C* which occurs in response to DI-N interaction nor the stabilization of arm in the cytoplasm in response to Wg-Dfz2 interaction. Thus in *Drosophila* novel signaling pathways seem to be used to transduce signals to the nucleus of S2-*N* and S2-$N^{\delta EGF1-18}$ cells in the presence and absence of Wg. At the present time it is not known whether mammalian *Wnt1* and potentially other members of the *Wnt* family similarly interact with members of the *Notch* family. It is interesting to note however that in the case of Notch4/Int3 EGFLR 14–15, EGFLR 16–17, EGFLR 20–23, EGFLR 26–27, and EGFLR 31–32 each have been fused to to form novel EGFLRs (Gallahan and Callahan, 1997; Uyttendaele *et al.*, 1996). Since *Drosophila N* EGFLR 23–27 and EGFLR 31–34 are required for binding Wg, it may be that Notch4/Int3 has lost the capability to bind Wnt proteins or interacts with a novel ligand(s).

### *The mouse as a model for human breast cancer*

No individual human breast cancer and no individual mouse transgenic model or mouse inbred strain will ever provide an exact replica of the large and complex class of neoplasms that represent breast cancer as a human disease. What we can hope for is to develop insights into the various cellular, molecular and genetic pathways that are most often involved in neoplastic transformation in the breast. One of the major problems in identifying and addressing the impact of somatic mutations on the evolution of breast carcinogenesis is a fundamental lack of information on the identity of the signaling pathways which regulate the growth and development of the mammary gland. It seems likely that the target cells which are susceptible to carcinogenic mutations are those which have been incompletely committed to a particular fate of differentiation, ie. stem cells (Kordon *et al.*, 1995; Medina and Smith, 1990; Smith, *et al.*, 1991, Smith and Medina 1998). However, again the number of molecular tags which identify these cells are limited. The MMTV/mouse model system has provided, relative to other strategies, a productive and experimentally amenable approach to identify genes and signaling pathways which when altered by mutation contribute to the deregulation of normal mammary gland development leading subsequently to mammary tumorigenesis. It seems certain that further analysis of MMTV induced HOGs, HOG derived tumors, plaques, plaque derived tumors, and subsequent distant metastases will lead to the identification of additional MMTV induced genetic alterations. These efforts taken together promise to define pathways of mutations that drive malignant progression to its endpoint, metastasis.

We recognize that the mouse model system may have some potentially important limitations, relative to human breast cancer. For instance there are endocrinological, hormonal and life style differences represented in the two biological systems. In addition, the scientific community has perceived another limitation, i.e. the histopathological descriptions of mouse mammary tumors do not mirror the analogous parameters reported for the most frequent forms of human breast tumors, e.g. invasive ductal carcinomas. However, it is relevant that Wellings (1980) found that

many, if not all, of the human mammary lesions observed are originated in the terminal ductal lobular unit (TDLU). One of these, atypical lobular type A lesions (ALA) are morphologically similar to the mouse mammary HAN lesions. In addition, recent evidence from the late Helene Smith and others have demonstrated that contiguous portions of the ductal systems in the human breast are clonally derived (Tsai *et al*., 1996). Further studies by this group and others have demonstrated that human mammary tumors in situ share with the surrounding normal parenchyma, common genetic mutations, suggesting that progressive malignant changes toward malignancy in the human breast, as in the mouse, occur as the result of acquired mutation in a multipotent cellular antecedent (Deng *et al*., 1996; Rosenberg *et al*., 1997). At the present time the question of whether the genetics of mouse mammary tumorigenesis is directly relevant to human breast cancer remains largely unanswered. *Wnt1* and *Wnt3* appear not be frequently rearranged or amplified in invasive ductal carcinomas (IDC) of the human breast, but other forms of breast cancer have not been extensively studied (Roelink *et al*., 1993; Vande Vijver *et al*., 1989). Similarly, *Fgf3*/*Fgf4* are frequently co-amplified in IDC of the breast, however whether this activity precedes malignancy or is a consequence of it remains controversial (reviewed in Bieche and Lidereau, 1995). The human homologs of the other *Int* genes have not yet been tested for genetic alterations in human breast tumors.

In the short term the MMTV/mouse model system provides an opportunity to identify the genes (or gene families) encoding signaling pathways which are involved in normal mammary gland development. It seems likely that some of these mutations induced by MMTV or the genes involved in the particular signaling pathways will be relevant to human breast cancer. Since the *Int6* type of viral insertion may be functionally similar to LOH, it seems reasonable that the identification of genes affected by this type of viral induced mutation could be prime candidates for mutation in human breast tumors.

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