

Genetically Engineered Mouse Models of Mammary Intraepithelial Neoplasia

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Foci of atypical mammary epithelium have been associated with breast cancer in many species including mouse and man. The advent of targeted genomics has led to the creation of numerous genetically engineered mice (GEM)⁵ which display focal atypical lesions associated with mammary cancer. Some early lesions in GEM have a remarkable morphological similarity to pre-cancers in humans. While the malignant potential of atypical foci have been thoroughly documented in the non-GEM by tissue transplantation, a review of the literature reveals that precursor lesions in GEM remain incompletely described and only partially documented. Their validation as appropriate models of human breast preneoplasia awaits classical transplantation studies. Here, we review the literature characterizing early lesions of GEM models of mammary cancer, discuss the principles of the Focality, Atypia, and Association and present an introduction of mammary transplantation for model Validation.

KEY WORDS: Focality; atypia; association; dysplasia.

INTRODUCTION

Early lesions believed to be precursors to mammary cancer in Genetically Engineered Mice (GEM) have been recognized for more than a decade. The literature now exceeds 70 peer reviewed articles with descriptions and/or photographic images of hyperplasia, hyperplasia with atypia and/or carcinoma *in situ* in mammary glands of GEM. While numerous articles have discussed and reviewed the concept of neoplastic progression in mouse models, none have critically

reviewed the precancerous mammary lesions in GEM (1–15).

In March 1999, a panel of distinguished pathologists was asked by the NIH Breast Cancer Think Tank to develop a classification of GEM breast lesions based on their examination of 39 mouse models of mammary cancer (8). The meeting, in Annapolis Maryland, resulted in a consensus report from the Pathology Panel (8) that points to the mammary lesions of GEM as distinctive and divisible into three distinct categories: 1) lesions that resemble those found in spontaneous mouse mammary tumorigenesis, 2) lesions that have a unique “signature” tumor phenotype that was specific for the transgene, and 3) lesions that resemble those found in human breast diseases (8). The Pathology Panel further developed and recommended the application of a simplified descriptive terminology to the classification of GEM lesions.

The consensus report also recommended a nomenclature for potential precursor lesions. The recommendation to apply the term mammary intraepithelial neoplasia (MIN) to all hyperplasias that were associated with cellular atypia was made, but with the

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⁵ Abbreviations: Genetically engineered mice (GEM); mammary intraepithelial neoplasia (MIN); mouse mammary tumor virus (MMTV); hyperplastic alveolar nodule (HAN).

prerequisite that they be associated with cancer (8). The consensus report suggested that these atypical foci would be precursor lesions with the potential to develop into cancer (1). However, the Pathology Panel felt that the potential precursor lesions of GEM were insufficiently characterized and recommended more thorough molecular and biological analysis before establishing a definitive diagnostic nomenclature (8).

This review will summarize the criteria of focality, atypia and association suggested by the Annapolis Panel. The review will also present the methodology of tissue transplantation (Test-by-Transplantation) as a means for defining biological potential of precursor lesions and validating GEM models of MIN that are currently presumed to have malignant potential. Finally, we will discuss the current literature on GEM models of mammary cancer that have characterized early lesions adhering to the recommendations of the Annapolis Panel.

Annapolis Pathology Panel

The Annapolis Pathology Panel was brought together by the organizers of the Workshop on Mouse Models of Human Breast Cancer on March 3–5, 1999. The organizers all from the NIH Breast Cancer Think Tank included, Lothar Henninghausen, Lalage Wakefield, and Jeffrey E. Green. They assembled a Panel of nine expert pathologists (M. Anver, J. Ward, M. Merino, B. Gusterson, J. Russo, R. Jensen, F. Tavassoli, S. Rehm, and R. Cardiff) to develop a classification of GEM mammary tumors suitable for comparison with human breast cancer. The Pathology Panel was provided with 175 slides representing 39 transgenic model systems involving 26 genes, five promoter systems and two species. In addition, three transplantation models, two carcinogen-induced models, and spontaneous mouse mammary tumor virus (MMTV)-induced lesions were represented. The slide collection represented about 90% of the published GEM models of human breast cancer available at the time. The Panel received and studied the slides in advance of the meeting. The workshop featured presentations of the various models by the investigators. On July 28 and 29, 1999, the Annapolis Pathology Panel met at NIH for final discussions leading to the consensus report.

The basic assumptions supported by the Pathology Panel were that all cancers arise as progeny of a single cell and that the earliest histological lesions can

be detected as focal or multifocal areas in the terminal ductules or the alveoli or both (8). This assumption more or less eliminates the possibility of mammary cancers originating in the collecting ducts. As a result, the use of the term *ductal* in relation to carcinoma *in situ* was discouraged in the characterization of mammary lesions in the mouse.

The Panel's report indicated that the major problem in the histological interpretation was insufficient information concerning the natural history of neoplastic progression. To remedy the situation, the panel recommended the following (8):

1. A recording of the evolution or natural history of neoplastic progression. For example, systematic collection of whole mount and histological samples from GEM with age- and parity-matched wild type controls during development (4, 8, 12, and 14 weeks after birth) and during the cycle of pregnancy, lactation and involution cycles (8). In addition, the panel signified the importance of following mice after surgical resection of the primary lesions to establish the metastatic potential of GEM lesions. Documentation of the early events of tumorigenesis with transplantation and molecular analysis **is needed** to validate the use of the term MIN (intraluminal epithelial proliferations with significant nuclear atypia) which implies malignant potential (8). It was suggested that this nomenclature be considered a transition terminology until more precise biological, or epidemiological data is collected.
2. The Annapolis Consensus Report further states:

“Until these studies are completed and the biology understood, the Panel recommends a simplified classification for MIN. In this simplified classification, the MIN lesions are to be classified as low or high grade. The low grade lesions are characterized by the presence of hyperchromatic duct, luminal and/or myoepithelial cells with little cytoplasm, more than one layer of atypical cells, intact basement membrane and an increased mitotic rate. High-grade lesions show more layers of epithelium, pleomorphism of nuclei and/or epithelial cells and/or an increase in mitotic figures. Filling of the lumen by these proliferative cells can occur but the basement membrane remains intact.

The term MIN infers that such lesions are not tumors, neither adenomas nor carcinomas, but rather morphological intermediates between normal and cancer. They are putative preinvasive lesions with characteristics of both benign and malignant cells. While they are probably immortalized and probably

not reversible, little research has been done to support this concept. They are thought to be essentially biologically identical to similar lesions seen in other conventional mouse mammary pre-cancers such as the HAN. The MIN in GEM frequently resembles many lesions in human epithelial tissues prior to or accompanying invasive carcinoma. MIN should, therefore, be considered neither as benign nor malignant lesions but rather a preneoplastic, protoneoplastic or premalignant state.” (8)

CHARACTERIZATION OF EARLY MOUSE MAMMARY LESIONS

GEM MIN, as with other potential precursor lesions, can be identified initially using the criteria of focality, atypia and association. Grading might be used to predict relative biological risk. While validation of their biology will ultimately require test-by-transplantation for proof of biological potential (8), the individual components of these criteria for morphological classification are reviewed later.

Focality

Since mammary tumors in all systems arise as a subset of cells from the epithelial population and there are no documented examples in which the entire epithelial population becomes malignant, it is reasonable to assume that precursor lesions will also arise as subpopulations (16). Therefore, diffuse hyperplasias and various diffuse or global examples of mammary dysmorphogenesis are unlikely to be preneoplastic. On the other hand, some types of transgenes, such as *wnt1* (40), lead to diffuse hyperplasias, with secondary atypical lesions that stand out from the background because they have dense cellularity or atypical cytology (17).

Such lesions may be precursors. However, not all focal lesions in the mammary gland are necessarily pre-malignant. For example, the wild type parous mouse develops occasional inflammatory nodules or squamous nodules that have rarely been associated with cancer and cannot be considered pre-malignant.

Atypia

The principle of focality implies an area of morphological abnormality. As pointed out before, not all focal abnormalities lead to cancer. The criteria of cy-

tological atypia are primarily nuclear. Large nuclei, variation in size and shape (pleomorphism), hyperchromasia and abnormal chromatin patterns are all considered components of atypia. Increased proliferative indices (mitotic index, Ki67 staining, S-phase, BrdU labeling and similar indices) might also be important. However, mouse mammary glands normally have a high mitotic rate relative to humans and atypical mitosis might be a more reliable morphological criterion.

Since these criteria are relative gauges, the investigator must compare the lesion with the background mammary gland and utilize the wild-type proliferating pre-lactating mammary gland as a control for ‘normal’ physiological hyperplasia. These normal, proliferating cells are naturally larger than in the quiescent gland and have larger nuclei with a more open chromatin. Cells in the lactating and early involuting gland frequently have hyperchromatic nuclei and the collapsed ducts will have a papillary configuration. Injury in the mouse mammary gland may also be associated with hyperchromatic and somewhat pleomorphic nuclei. Yet, it is important to note that these are normal phenomena. These changes in the nuclear structure are considered the “dysplasia” of repair and may be frequently observed in the nonneoplastic inflammatory nodules. Several papers have mislabeled images of such phenomena as neoplastic atypia. Adequately trained investigators should be utilized to make the distinction between these types of atypia.

Association

As implied from the preceding paragraph, association of a given type of lesion with a known or existing neoplasm has long been an accepted criteria for premalignancy. Yet, it represents only guilt-by-association and does not define causality. Using *association* as a criterion may be sounder if the suspected lesion is in direct continuity with an identifiable malignancy or a cytological continuum exists between early lesions and the cancer. Morphological continuums and associations were initially used to identify precursors to human and animal cancers. They were validated as precursor lesions only after subsequent retrospective and prospective studies (18–26). This precedent in many tumor systems emboldened the Annapolis Pathology Panel to assume that the combination of focality, atypia and association to justify the designation of MIN (8).

Grading

The Annapolis Pathology Panel recommended that MIN lesions be graded as “high grade” and “low grade” (8). Low grade MIN would consist of any lesion in alveoli or ducts with one or two layers of atypical luminal epithelium. The classical non-GEM HAN and their GEM counterparts would be examples of low grade GEM. The majority of illustrations of GEM MIN in the literature have multi-layered epithelium and should be considered high grade. However, it might be useful to consider a subclassification within the high grade lesions. Some multi-layered atypical foci are cystic and/or have alveolar differentiation within the layered epithelium (27). These lesions have been described in association with a longer tumor latency period and might be regarded as “intermediate grade.”

Validation

Atypical lesions found in the mouse mammary gland, in contrast to human, can be visualized in situ, isolated, surgically removed and transplanted into a gland cleared mammary fat pad of a syngeneic recipient mouse. The mammary nodule transplant has been used to test the biological potential of a great variety of focal lesions and provides operational proof of neoplastic potential (10,11,16). The test of biological potential by transplantation should be the *sine quo non* of validation. Not all lesions possess the same biological potential and through the selection of morphologically distinct lesions, high and low risk lesions can be isolated. The ability to identify, isolate, transplant and characterize the mouse lesions provides a method of validation not available in the human. The biology of serially transplantable atypical hyperplasias can be correlated with tissue organization and cytological features such as nuclear size, pleomorphism, chromatin, mitotic rate, S-phase, nuclear/cytoplasmic ratio, and cytoplasmic differentiation. As this information is gathered, the abnormalities of structure can be correlated with functional genomics to provide a more integrated, comprehensive view of neoplastic progression. Since such experimental techniques are not available for the study of human breast cancer, the mouse will continue to provide excellent opportunities for reverse modeling. This technology is elaborated in more detail later in this paper.

PROTONEOPLASTIC MAMMARY LESIONS IN MICE

Protoneoplasia in Non-GEM Mice

Two major types of precancerous lesions occur in murine mammary tumor virus (MMTV)-infected mice. Both were initially identified as focal atypical lesions that were associated with a high incidence of breast cancer. The most common lesion, the hyperplastic alveolar nodule (HAN), was first described by Apolant and identified and associated with mammary cancer by Haaland in 1911 [reviewed by Nandi and McGrath (28)]. The other type of lesion, the plaque, is limited to specific strains, such as GR/A, RIII, and DD, that harbor an expressed endogenous virus (16,29).

The HAN is a focal alveolar hyperplasia that stands out from the background of the nonlactating mammary gland as 1 to 5 mm nodules that closely resembles the histology of the normal prelactating mammary gland but generally have more hyperchromatic nuclei. They are clonal lesions, are typically cytological low-grade lesions with slightly abnormal nuclei, and have increased DNA synthesis and mitotic rates. The HAN has been the standard model of preneoplasia and has been extensively characterized by tissue transplantation (10,11,30,31).

Similar to HANs, the plaque is also a clonal proliferation that can progress to malignancy with hormone independence and invasion (29). The plaque is induced by the insertion of the MMTV, leading to activation of the *FGF3* gene product (29). This lesion starts as a circumscribed ductal proliferation found during pregnancy. It is hormone-dependent and regresses after weaning or withdrawal of the hormone. It is a flat but palpable lesion with radiating ducts. The termini and ducts are filled with cells. The connective tissue stroma is increased but generally delicate. Following multiple pregnancies or repeated stimulus with hormones, some of these lesions will continue to grow to eventually form invasive carcinomas that differ in morphology from the hyperplastic lesions. Plaques have also been referred to as type P tumors (32).

Protoneoplasia in GEM

The first transgenic mouse with mammary tumors was reported by Stewart *et al.* (33) in 1984. Although Andres *et al.* described multiple “neoplastic” foci in their WAP promoted bigenic myc and ras mice

(34), the first mention of hyperplasia as an initiating event was in a 1988 paper by Tsukamoto *et al.* describing an *int-1* transgenic GEM (35). However, Varmus, when reviewing the *int-1* transgenic, described a diffuse hyperplasia and focal malignancy (14). In the same year, Muller *et al.* described "... mammary adenocarcinomas that involve the entire epithelium in each gland..." in activated *c-neu* transgenic mice (36). This observation led some investigators to believe that malignant transformation in transgenic mice was a one step process. Using the criteria outlined in this review, the lesions Muller *et al.* described were most likely activated *c-neu* hyperplasias and probably not adenocarcinomas (4).

The earliest histological recognition, description and images of focal mammary hyperplasia of potentially premalignant lesions in GEM should probably be attributed to Bouchard *et al.* who, in 1989, explicitly suggested that lesions found in their MMTV-LTR/*neu* mice resembled human carcinoma *in situ* (37). Since then, numerous types of hyperplasias have been reported. By 1991 numerous review articles were already acknowledging and discussing the use of GEM for the study of neoplastic progression (3,12,15).

Many investigators have now claimed to identify premalignant mammary lesions in GEM. However, the first description of criteria for characterizing early lesions in the mammary gland of GEM mice appeared in a paper by Halter *et al.* documenting the histopathology of their TGF α model (17). In this 1992 paper, the authors described focal and diffuse cystic and solid hyperplasias with and without atypical nuclei. Lesions with atypical nuclei were described as *dysplastic* (17). The authors were of the opinion that the atypical lesions did not have definitive evidence of malignant transformation and the use of *dysplasia* was appropriate until experimental proof established their biological potential (17).

The word "*dysplasia*" has been used as a precedent in many subsequent publications to describe atypical hyperplastic foci without proven biological potential. However, other investigators have developed their own descriptive nomenclature. For example, Shibata *et al.*, in a series of C3(1)/SV₄₀ Tag papers used descriptive terms of atypical hyperplasia and nodular atypical hyperplasia to describe the intraluminal lesions (38,39). They published excellent images of whole mount and histological preparations that clearly illustrated intraluminal proliferation of undifferentiated cells in the terminal branches of the mammary tree (38,39). They concluded that the atypical intraluminal hyperplasias in their system preceded the

development of overt adenocarcinomas. As a result, the atypical lesions were thought to be pre-malignant. These early lesions were never transplanted, and, thus, were never conclusively demonstrated to be immortalized by the indefinitely serial transplantable criteria.

Although a number of groups have now called attention to focal atypical, dysplastic or *in situ* lesions in association with mammary cancer in their GEM (see Table II), their hypotheses have rarely been confirmed biologically by serial transplantation. On the other hand, when the slides from such model systems are examined in detail, a gradation of lesions between normal and malignant is easily identified, suggesting a series of cytological changes throughout neoplastic progression. Since such model systems usually have many more atypical foci than tumors, it is not clear whether all or only some of the atypical lesions progress to full malignancy.

If proof-by-transplantation is the criterion for establishing the biological behavior of mammary tissue, the current literature contains no proof that any of the lesions described thus far are precancerous. Lin *et al.* published the initial characterization of hyperplastic GEM mammary glands in 1992 (40). Although the outgrowths were hyperplastic, no tumors were reported (40). Smith *et al.* have published the most comprehensive transplant analysis of hyperplastic GEM mammary tissues to date (41). Their experiments dealt with TGF α mice that developed focal hyperplasias in the FVB/N background. Their transplantation experiments provided unexpected results.

"Serial transplantation of these hyperplasias indicated an absence of proliferative immortality. Nevertheless, they gave rise to tumors at a low frequency and after a prolonged latency in virgin hosts; in multiparous hosts, tumors developed earlier and at a high incidence." (41)

Several other groups have also transplanted GEM mammary tumors of various types (41-44). The tumor transplants can be serially passaged, can grow in orthotopic sites and metastasize at various rates.

MIN MODELS IN GEM

A review of the literature ending in January 2000 has revealed 74 articles on GEM associated with mammary hyperplasia describing 40 unique transgene/ promoter combination constructs (Table I). Of these, 47 articles documented the

Table I. Models with Mammary Hyperplasia

		Reference(s)	
Growth factors	<i>FGF-3 (int-2)</i>	(52,82–85)	
	<i>FGF-7 (kgf)</i>	(53)	
	<i>FGF-8</i>	(86)	
	MGF	(87)	
	NDF	(88)	
	SGF	(87)	
	<i>TGFα</i>	(17,41–43,66,67,69,89–92)	
	<i>TGFα X c-myc</i>	(42,43)	
	<i>TGFαX neu</i>	(67,69)	
	<i>TGFαX Stat5a^{-/-}</i>	(89)	
	<i>TGFβ2</i>	(93)	
Receptors	ErbB2/neu	(36,37,45,56–58,67,68,90,94–97)	
	ErbB2/neu X <i>TGFα</i>	(67,69)	
	Met	(98)	
	Ret	(99,100)	
Differentiation	Notch (<i>int-3</i>)	(101,102)	
	<i>Wnt 1 (int-1)</i>	(35,40,82,103)	
	<i>Wnt 1</i> \times <i>int-2</i>	(82)	
	<i>Wnt 1</i> \times <i>Era^{-/-}</i>	(104)	
	<i>Wnt 1</i> \times VP	(103)	
	<i>Wnt 10b</i>	(55)	
Signal transduction	PyV-mT	(60,62,63,90)	
	PyV-mT \times <i>Ets2^{+/-}</i>	(27)	
	PyV-mT \times <i>Grb2</i>	(63)	
	PyV-mT \times <i>Shc</i>	(63)	
	PyV-mT \times <i>src^{-/-}</i>	(59)	
	PyV-mT \times <i>yes^{-/-}</i>	(59)	
	Ras	(34,51,67,72,105–107)	
	<i>Ras</i> \times <i>c-myc</i>	(34,106)	
	<i>Src</i>	(108)	
	PTPe	(109)	
Cell-cycle	<i>Myc</i>	(33,34,42,43,64,110–112)	
	<i>Myc</i> \times <i>p53^{-/-}</i>	(110)	
	<i>Myc</i> \times <i>TGFα</i>	(42,43)	
	<i>Myc</i> \times <i>ras</i>	(34,106)	
	Cyclin A	(113)	
	Cyclin A \times <i>cdk2</i>	(113)	
	Cyclin D1	(114)	
	Cyclin E	(115)	
	SV40-Tag	(38,39,70,71,116,117)	
	SV40-Tag \times <i>Bax^{-/-}</i>	(39)	
	SV40-Tag \times <i>p53^{-/-}</i>	(38)	
	Other	<i>Int 5</i> (aromatase)	(118)
		Str 1	(119,120)

hyperplasia with either photoimages or histopathological descriptors (Table I). The papers vary widely in their breadth of characterization. Most articles offer diagnostic terms rather than descriptions of the histological features to justify the diagnosis. When grouped

into potential model systems, 25 models have descriptions or images of mammary hyperplasia with atypia or dysplasia that could be considered potential proto-neoplasms. A list of the models with descriptions and/or acceptable images of hyperplasia is in Table I.

The types of promoters, the combinations with other genes or knockouts, the variations in the source and construction of the transgenes, and the host animal expand the complexity and numbers of the models. Of the 25 models, nine have been studied in sufficient depth to be considered established models of preneoplasia (Table II).

A variety of promoters, including MMTV-LTR, WAP, MT, BLG, C3(1), UASXGAL4 bitransgenic, MMTV/MT bitransgenic, RSV and H4, have been used to activate or overexpress the constructs (Table II). The MMTV LTR is the most commonly used promoter. Recently, Andrechek *et al.* has utilized a cre-loxP system to obviate necessity of MMTV promotion by placing the neu oncogene under its own promoter (45). Deng *et al.* have described the development of a model for the conditional expression of mutant BRCA1 (46,47). These experiments mark the beginning of new approaches to engineering mouse models of breast cancer. Each oncogene and promoter has their advantages and disadvantages and should be carefully considered in evaluation, yet their relative value will not be the focus of this review.

The FVB strain has many advantages and has been the strain most commonly used for developing GEM (48). It is important to note comparison of GEM models is complicated by the variety of mouse host strains used. Many investigators not only compare oncogenes in different strains but also complicate the comparison by crossing different strains. Hunter and colleagues have extensively demonstrated the variable effects of inter-strain breeding on tumor progression and demonstrated unpredictable synergistic or inhibitory effects on tumor and metastasis kinetics (49). Investigators using any host strain should be well versed in the background pathology of the strain or crosses. For example, over half of elderly FVB will develop tumors of various types (50). In some cases, the appearance of tumors in GEM may have been only accelerated but not created by the transgene. More important, the rigorous investigator will need to be familiar with the background of even the specific sub-strain of the strain being used since separation of a strain for more than several generations will introduce new genetic modifications.

Histopathological Characterization

Ironically, more is known about the molecular biology and biochemistry of many GEM models of MIN than their biological potential. A variety of methods

have been used to examine the altered morphology of the mammary gland. Although histological photomicroimaging should be the cornerstone of published pathological evaluation, there has been inconsistent selection of such images in published accounts. We have excluded all models that did not have illustrations or detailed descriptions of the lesions. Forty-seven out of the 74 publications identified with GEM hyperplasias had acceptable histological evidence of MIN (focality, atypia, and association) with pictures of the hyperplasia.

Whole mount of the mammary fat pad is another useful and standard technique for evaluating the morphology of the mammary gland. It is particularly important in the detection and characterization of the smallest, early lesions. Interestingly, only 26 of the 47 papers included whole mount images. A paper concerning the effects of melatonin introduced a semi-quantitative grading system that scored whole mount preparations of *N-ras* induced "HANs" according to the percent of the mammary fat pad containing lesions (51). Interestingly, the lesions began in the subareolar area and spread to the periphery of the gland. When only the inner 25% of the gland was involved it was scored as Grade 1. Grade 2 involved the mammary gland in inner 50% of the fat pad. Grade 3 involved the inner 75% and in Grade 4 the entire gland had HANs (51).

Surprisingly, with the availability of numerous reagents for specific immunohistochemical staining, only 21 of the published studies included immunohistochemistry or images of RNA *in situ* hybridization sections of hyperplasias. Of note are the 6 characterizations that include ultrastructural evidence.

The increasing numbers of GEM models of mammary tumorigenesis provide a unique opportunity to identify, characterize, categorize and study the biology of neoplastic progression. The Annapolis Panel found that many reports utilize human pathology nomenclature when characterizing early lesions. However, only three articles provided the appropriate descriptions of the early lesions (17,38,51). The combination of incomplete descriptions in the text and incomplete documentation in the illustrations make evaluations for MIN in these models very difficult.

Established Models of GEM Preneoplasia

Of the 44 model systems available that have published evidence of focality, atypia and association, nine potential models of GEM precancer have been

Table II. Models with Evidence of Hyperplasia with Atypia

	Oncogene manipulation	Oncogene species	Promoter(s)	Mouse strains used	Reference(s)	
Growth factors						
	wt TGF α	human	MMTV	C57BL; DBA	(17,66,69)	
	TGF α	wt TGF α	human	MT	FVB; C57BL/6J; CD-1	(41,42,90,92)
	TGF α	wt TGF α	rat	MT	C57BL; SJL	(43,91)
	TGF α	wt TGF α	rat	WAP	C57BL/6; SJL/J	(43,89)
	TGF α \times <i>neu</i>	human TGF α ; rat <i>neu</i>	MMTV	C57BL \times DBA \times FVB	(67,69)	
	TGF α \times <i>c-myc</i>	rat TGF α ; mouse <i>c-myc</i>	WAP	C57BL/6; SJL	(43)	
	TGF α \times <i>c-myc</i>	human TGF α ; mouse <i>c-myc</i>	MT/TGF α , MMTV/ <i>myc</i>	FVB; CD-1 \times C57B6/J	(42)	
	TGF α \times Stat5a ^{-/-}	wt TGF α	rat	WAP	SvEv129; C57B6	(89)
	<i>FGF-3 (int-2)</i>	wt <i>FGF-3</i>	mouse	GAL4/UAS	FVB/N	(52,85)
	<i>FGF-3 (int-2)</i>	wt <i>FGF-3</i>	mouse	MMTV	FVB/NHd; C57BL \times CBA	(83,84)
Receptors						
	ErbB2/ <i>neu</i>	activated c-ErbB-2	human	MMTV	C57BL/6 \times CB6	(121)
	ErbB2/ <i>neu</i>	wt <i>neu</i>	rat	MMTV	FVB; Balb/c	(56,57,67,68,90,94)
	ErbB2/ <i>neu</i>	Activated <i>neu</i>	rat	MMTV	FVB; CD1; Balbc	(36,37,56,57,67-69,94,95)
	ErbB2/ <i>neu</i>	Knock-in wt <i>neu</i>	rat	Endogenous <i>neu</i>	Balb/c/129 \times FVB	(45)
	ErbB2/ <i>neu</i> \times TGF α	Activated <i>neu</i> , wt TGF α	rat <i>neu</i> , human TGF α	MMTV	C57BL \times DBA; FVB	(67,69)
Differentiation						
	<i>Wnt-1</i>	wt <i>Wnt-1</i>	Mouse	MMTV	C57BL; SJL; FVB/N	(35,40,82,103)
	<i>Wnt-1</i> \times <i>Int-2</i>	wt <i>Wnt-1</i> , wt <i>Int-2</i>	Mouse	MMTV	C57BL; SJL; FVB/N	(82)
	<i>Wnt-1</i> \times ERa ^{-/-}	wt <i>Wnt-1</i>	Mouse	MMTV	C57BL; SJL; FVB/N	(104)
	<i>Wnt-1</i> \times VP	wt <i>Wnt-1</i> , wt VP	mouse <i>Wnt-1</i> , rat VP	MMTV	C57BL; SJL; FVB/N; Balb/c	(103)
	Notch (<i>int-3</i>)	Truncated <i>int3</i>	Mouse	MMTV	FVB/N	(101)
	Notch (<i>int-3</i>)	Truncated <i>int3</i>	Mouse	WAP	FVB/N	(102)
Signal transduction						
	PyV-mT	wt PyV-mT	Virus	MMTV	FVB/N	(27,60,67,90)
	PyV-mT	shc decoupled	Virus	MMTV	FVB/N	(62,63)
	PyV-mT	PI-3' decoupled	Virus	MMTV	FVB/N	(62)
	PyV-mT \times Ets2 ^{+/-}	wt PyV-mT	Virus	MMTV	FVB/N; Swiss/Black	(27)
	PyV-mT \times <i>src</i> ^{-/-}	wt PyV-mT	Virus	MMTV	FVB/N	(59)
	PyV-mT \times <i>yes</i> ^{-/-}	wt PyV-mT	Virus	MMTV	FVB/N	(59)
	Ha- <i>ras</i>	Activated Ha- <i>ras</i>	Human	WAP	C57BL/6 \times SJL	(34)
	c-Ha- <i>ras</i>	activated c-Ha- <i>ras</i>	Human	WAP	C57BL/6 \times SJL	(72)
	v-Ha- <i>ras</i>	v-Ha- <i>ras</i>	Not specified	MMTV	C3H \times C57BL/6; BALB/ B6D2; CD-1	(105,106)
	N- <i>ras</i>	wt N- <i>ras</i>	Not specified	MMTV		(51,107)
	Ha- <i>ras</i> \times <i>c-myc</i>	Activated c-Ha- <i>ras</i> , wt <i>myc</i>	human <i>ras</i> , mouse <i>myc</i>	WAP/ <i>ras</i> , MMTV/ <i>myc</i>	CD1; C57BL/6; SJL	(34)
	v-Ha- <i>ras</i> \times <i>c-myc</i>	v-Ha- <i>ras</i> , wt <i>myc</i>	<i>ras</i> (Not specified), mouse <i>myc</i>	MMTV	CD-1; C57BL/6J	(106)
Cell cycle						
	<i>c-myc</i>	wt <i>c-myc</i>	Mouse	MMTV	CD-1 \times C57B6/J	(33,42,64,110,111)
	<i>c-myc</i>	wt <i>c-myc</i>	Mouse	WAP	C57BL/6 \times SJL	(34,112)
	<i>c-myc</i>	MuMTV infected, wt <i>myc</i>	Mouse	MMTV	CD-1 \times C57B6/6J	(64)
	<i>c-myc</i> \times p53 ^{-/-}	wt <i>c-myc</i>	Mouse	MMTV	CD-1 \times C57B6/6J \times 129/J	(110)
	<i>c-myc</i> \times TGF α	wt <i>c-myc</i> , wt TGF α	Mouse <i>myc</i> , human TGF α	MMTV/ <i>myc</i> , MT/TGF α	FVB; CD-1 \times C57B6/J	(42)
	<i>c-myc</i> \times TGF α	wt <i>c-myc</i> , wt TGF α	mouse <i>myc</i> , rat TGF α	WAP	C57BL/6; SJL	
	<i>c-myc</i> \times Ha- <i>ras</i>	Wt <i>c-myc</i> , activated Ha- <i>ras</i>	mouse <i>myc</i> , human <i>ras</i>	WAP	C57BL/6 \times SJL	(34)
	<i>c-myc</i> \times v-Ha- <i>ras</i>	wt <i>c-myc</i> , v-Ha- <i>ras</i>	mouse <i>myc</i> , <i>Ras</i> (Not specified)	MMTV	C3H \times C57BL/6; BALB	(106)
	SV40-Tag	wt SV40	Viral	WAP	NMRI	(116)
	SV40-Tag	wt SV40	Viral	C3(1)	FVB/N	(117)
	SV40-Tag \times Bax ^{-/-}	wt SV40	Viral	C3(1)	FVB/NsC57BL/6	(39)
	SV40-Tag \times p53 ^{-/-}	wt SV40	Viral	C3(1)	FVB/N \times 129/Sv	(38,39)

described in sufficient detail and with adequate experimental documentation to warrant its discussion as a prototypical model of GEM MIN (Table II). Space does not permit a detailed summary of each model. However, some points of interest are summarized here.

FGF3 and FGF8

MMTV-LTR/Int2(FGF3) (52) and MMTV-LTR/KGF(FGF8) (53) models are informative in that they produced signature lesions that resembled the pattern seen in hormone dependent lesions (plaques or Type P tumors) of GR3/A mice. The lesions in MMTV-infected mice form circumscribed complex radiating ducts in dense connective tissue and are associated with insertional activation of *FGF3* by MMTV (29). Over-expression of the same and related oncogenes resulted in similar morphological lesion when inserted as transgenes, validating the concept that specific oncogenes can result in specific or unique phenotypes (8,54). The production of these proliferative ductal lesions in GEM models has allowed the study of their evolution into invasive neoplasms. When induced by the virus in the mouse strains RIII, DD and GR/A, the lesions are hormone sensitive, reversible tumors (29). When induced by a transgene, the lesions are irreversible, multifocal and benign. The hormone sensitivity of these lesions has never, to our knowledge, been tested. The invasive neoplasms that emerge from these focal lesions have a phenotype distinctly different from the precursor lesion and do metastasize (53). Thus, the early ductal hyperplasias may be precursors but cannot be considered malignancies. Although they grow as dysplastic lesions in short term transplants, their biology has not been verified using long term transplantation experiments. The *wnt1* and *wnt10b* hyperplasias have not been studied in the same detail but they do resemble the microacinar lesions found in the MMTV induced hyperplasias and tumors (40,55). Unfortunately, most of these lesions do not resemble human atypical hyperplasias.

ErbB2

The best documented MIN lesions appear to originate in the TDLU or lobule (7). DiCarlo *et al.* have presented detailed morphological and immunohistochemical evidence that the lesions in

MMTV-LTR driven erbB2 GEM originate from lobules (56). The lesions created with other constructs of erbB2 and in other host strains result in similar lobular hyperplasias (7,8,57,58). Other MMTV-LTR driven transgenes also produce focal lobuloalveolar hyperplasias (src, PyV-mT, TGF α ect) (17,59,60).

Since erbB2 has been implicated in human breast cancer, it has been a logical candidate for extensive modeling (7). The gene and a number of activated variants have been placed behind a number of different promoters and in several mouse strains (see Table II). All of the variants, promoters and host strains have resulted in the same signature lesions (5,8,54). The various types of erbB2 hyperplasias share common cytological features amongst themselves and with classical human ductal carcinoma *in situ* (7). However, the mammary lesions produced by erbB2 in the rat are quite different, perhaps giving dramatic illustration of the modifying influence of the host species (61). It is important to note that the human and mouse lesions share the overexpression of erbB2 and now also share downstream molecular pathways (4,9,15).

PyV-mT

The Polyoma Virus middle T (PyV-mT) model is of interest because the transgene produces atypical mammary hyperplasias within three weeks of birth and metastatic adenocarcinomas as early as five weeks (60). Since the PyV-mT triggers many of the same pathways as erbB2 with converging signal transduction at the PI(3)' kinase activation site and has much more rapid tumor kinetics, it has been used as a surrogate for erbB and other receptor tyrosine kinases. The PyV-mT develops mammary tumors that resemble the papillary and scirrhous carcinomas of humans (59,60). Although identified and described, the premalignant lesions have not been described in detail (27,59,60,62,63). However, interactions of the PyV-mT transgenics with a large number of knockouts and other transgenes have defined many of the pathways that are required for neoplastic progression (see Table II).

Myc

Myc is another transgene that has been used in a variety of host strains, with different promoters and different companion genes (see Table II).

Myc also produces a characteristic “signature” tumor phenotype (54). In our experience, *myc* is associated with a variety of early lesions (64). Some are associated with proliferative glandular and stromal reactions that bear some resemblance to human breast disease. However, these early lesions have not been thoroughly characterized biologically or pathologically. Less extensively studied models such as activated *src* also produce sclerotic lesions with early foci resembling either HAN or sclerosing adenosis (59).

TGF α

The hyperplasias in TGF α transgenics have been extensively studied with a variety of promoters, hosts and in combination with other GEM (see Table II). It is clearly associated with hyperplasias and malignancies (17,41,65,66). However, as a transgene, TGF α is associated with a bewildering variety of hyperplastic lesions. Some of the lesions are apparently associated with reduced expression of the transgene (17). This is perplexing since other atypical hyperplasias such as *myc* and *erbB* clearly over express the respective transgene (64,67,68). Further, although some of the lesions progress into cancer, by transplant criteria not all of the lesions are protoneoplastic because they rarely result in a stable transplantable line (41). The situation is somewhat compounded by the suggestion that TGF α is not a dominant oncogene. When combined with *erbB2* in bi-transgenic mice, the tumors and atypical hyperplasias all over expressed the *erbB2* transgene and were of the *erbB*-phenotype (67,69). The interpretation of these conflicting results will require the identification, isolation and transplantation of specific focal lesions (8).

SV40-Tag

The SV40-Tag system has very well documented intraluminal lesions (38,39). They have been thoroughly analyzed with histology, immunohistochemistry, whole mounts and electron microscopy (38,39). These early lesions are typically solid masses of poorly differentiated cells with relatively compact hyperchromatic nuclei and scanty cytoplasm. They resemble some forms of human intraductal carcinomas (70,71).

Although *ras*, *notch*, *wnt1* and others produce recognizable focal hyperplasias with atypia, our experience and review of the literature suggests that the

lesions are primarily acinar and tend to not have a counterpart in the human breast. Further, the production of *ras*-associated tumors tends to be sporadic (72) and *wnt1* transplants did not appear to produce tumors (40). Since neither gene is highly associated with human breast cancer, they have not been as actively investigated as model systems for human breast cancer. However, these factors have encouraged their use in combination with other transgenes as potential co-factors (see Table II).

The cellular origin of the hyperplastic lesions might be more related to the promoter used to generate the mice than the transgene. The majority of current model systems use either the MMTV long terminal repeat promoter/enhancer (MMTV-LTR) or the whey acidic protein promoter (WAP) as promoters (see Table II). Although these promoters are expressed in a variety of tissues, they are primarily expressed in the acini and they appear to produce alveolar lesions (56). Some promoters, can produce ductal lesions. For example, the C(3)1-promoted SV₄₀ Tag produces early lesions that appear to originate in the both the collecting ducts and the terminal ductules (70,71). Several other GEM, for example in MMTV/ or WAP/*notch4* and MMTV-LTR/DIINR, also appear to produce lesions of the collecting ducts (8). However, further study of natural history in these models will be required to determine their origin. Again, more thorough study of the earliest lesions using a combination of transplantation of the specific lesion and whole mounts and histology of the whole mammary gland are required to define the origin of these lesions.

VALIDATING GEM MODELS OF PRENEOPLASIA

Validation is a currently popular term used in the setting of mouse models of human cancer. The Annapolis Pathology Panel made a valiant effort to develop a usable nomenclature for classifying lesions. Their survey revealed a lack of information to accurately characterize the biology of potential precancerous lesions in the mouse mammary gland (8). The literature review conducted here confirms the lack of compelling experimental evidence to support the hypothesis that any given lesion is pre-malignant. Much of the terminology is borrowed from older classifications that are based on mouse mammary tumor virus infected strains or human breast pathology that is based on large epidemiological studies. The

majority of investigators resort to inferential, guilt-by-association, arguments. We have not found a single GEM paper that has validated the “precursor” hypothesis by serial transplantation. The scientific community has clearly missed a golden opportunity to validate their claims.

Mammary transplantation is the standard method used to determine the biological potential of precursor lesions and provides the operational definition of protoneoplasia (16). The test-by-transplantation is the “Koch’s Postulates” of tumor biology: Identification, isolation, transplantation, and characterization. Non-GEM hyperplasias are composed of genetically altered clonal populations that are indefinitely serially transplantable and, thus, meet the criteria of immortalization. While immortalization by itself is not sufficient for malignancy, it meets a basic requirement for oncogenesis. Nodules that are immortalized and give rise to tumors upon transplantation are traditionally considered to be “preneoplastic,” yet they are more accurately referred to as “pre-malignant” or protoneoplastic (16).

Although classical non-GEM lesions are reviewed elsewhere in this issue, it is important to point out here that both the HAN and plaque described earlier have a thoroughly documented and proven malignant biological potential. It is of interest to note that its verification has come from transplantation of these protoneoplastic lesions into gland-cleared mammary fat pads and subcutaneous tissue.

In evaluating a putative preneoplastic lesion, the separation of non-precancerous from precancerous mammary tissue requires direct transplantation into the gland-cleared mammary fat pad. The malignancy of a tissue is readily determined by its ability to grow in any ectopic transplantation site. Typically a subcutaneous location has been used. Tissues that cannot grow are defined as nonmalignant.

Transplants of normal mammary ducts will grow in the gland-cleared fat pad but not in the subcutaneous tissue. Transplantation of normal ducts results in the outgrowth of normal ducts in the cleared fat pad but they do not develop into tumors. Further, ductal outgrowths eventually die out on serial transplantation, demonstrating their limited growth potential. In a like manner, transplants of “protoneoplastic” nodules, tissues or plaques do not grow in the subcutaneous tissue. However, their transplantation into gland-cleared fat pads results in abnormal hyperplastic outgrowths, with eventual progression to malignancy and invasion. A distinguishing characteristic of a preneoplastic lesion is its ability to be indefinitely

maintained by serial mammary fat pad transplantation, demonstrating their immortality.

Using the preneoplastic tissue from serially transplanted mouse mammary outgrowth lines, extensive attempts have been made to find morphological or other markers that correlate with malignant potential (8,10,30). The sobering conclusion has been that there are no morphological characteristics and no biological markers that can be used to reliably predict the probability that a given outgrowth is at high risk of undergoing malignant transformation (31). The conclusion that only the test-by-transplantation has provided the proof of biological potential must be kept in mind during the analysis of the hyperplasias in GEM models of human breast cancer.

In spite of the dearth of experimental data, we can confirm that some GEM mammary glands do have focal lesions with cellular atypia and resemble human lesions that carry the diagnosis of atypical hyperplasia and carcinoma *in situ*. Like the human, the murine lesions can be associated with a high risk of malignant transformation. However, the type of morphological continuum so carefully described and documented in the human mammary gland has not been described in the GEM or non-GEM gland. In fact, the evidence in the non-GEM hyperplastic outgrowths suggests that the degree of atypia is not related to biological potential (31).

Mammary Intraepithelial Neoplasia in GEM

We suggest that, for the present, the term Mammary Intraepithelial Neoplasia (MIN) be applied to focal hyperplastic lesions with atypia in the mammary glands of GEM (8). This designation represents a temporary lowest common denominator approach that hopefully will become unnecessary once additional knowledge is gained from serial transplantation studies regarding the biological potential of these lesions. As these data become available, it may then be appropriate to further subdivide this broad designation into specific categories with attendant precise histologic definitions, molecular correlates, and certain knowledge of risk potential. Ideally, this subclassification would more or less correspond to lesions of similar risk potential as described in the human breast, but at this time there is no guarantee of this (18,20,21). For example, it will, hopefully, be possible to identify high risk lesions that, in GEM, can be designated carcinoma *in situ* because, in serial transplantation studies, they, like the human counterpart, lead to a high

risk of developing invasive carcinoma (say, 50% of such lesions) (18,20). Lower risk lesions that, in serial transplantation, develop carcinoma 5–10% of the time could be designated atypical hyperplasia (18,20). Only time will tell if such a division is possible.

In the interim, a simple morphological subdivision into high and low grade MIN has been suggested (8). Since most lesions currently described as atypical mammary hyperplasias thus far in GEM usually have multiple layers of atypical epithelium, they will be classified as “high grade.” At the present time, the criteria for a grading system are not well developed. The one published proposal is based on percent mammary gland involved rather than on cytological criteria (51). Since the morphological mammary abnormalities in the non-GEM mice do not necessarily correlate with biological behavior, it may be difficult to develop cytological criteria for the GEM models of MIN.

On the other hand, this is unexplored territory and might provide great insight into the morphology of neoplastic progression. The ability to identify, isolate, transplant and characterize the mouse lesions provides a method of validation not available in the human. The biology of the atypical hyperplasias can be correlated with tissue organization and cytological features such as nuclear size, pleomorphism, chromatin, mitotic rate, s-phase, n/c ratio, and cytoplasmic differentiation. As we gather this information, the abnormalities of structure can be correlated with functional genomics to provide a more integrated, comprehensive view of neoplastic progression.

COMPARATIVE PATHOLOGY

Potentially preneoplastic mammary hyperplasias have been described in a number of species (reviewed in Cardiff *et al.* (30)). Many of these studies were produced by students of K. B. DeOme who developed the cleared mammary fat pad and provided the test-by-transplantation paradigm (16).

Wellings and his colleagues published the initial studies directly applying the technology and understanding gained from murine breast pathology to the morphological study of early lesions in human breast cancer (reviewed in Cardiff and Wellings (7,73)). A student of DeOme's, Wellings sought the homologue of the mouse HAN in the human by applying the subgross (whole mount) techniques from the mouse to the human mammary gland (7,73). His work clearly established that the earliest atypical lesions found in both species originated in the terminal ends of

the mammary tree. In the human mammary gland, with its well developed lobules, the origin appeared to be in the Terminal Ductal Lobular Unit (TDLU) (24,74,75). The mouse lesions were clearly in the same topographical region referred to as the LA (7). In 1984 Russo, Wellings and colleagues developed what has to be considered the most definitive comparison of the morphology of spontaneous, virus-induced and carcinogen-induced mouse, rat and human mammary lesions (7).

Wellings and colleagues published a series of papers demonstrating that the atypical hyperplasias identified in their preparations of the human breast were correlated with a high probability of breast cancer (18,24–26,75–79). However, they were never able to provide definitive experimental proof that the lesions were pre-cancer (80). This inference has been supported by subsequent epidemiological studies (18,20,21,76). Thus, the human breast lesions comply with the classical criteria of Focality, Atypia and Association with cancer. The details of preneoplasia in the *human* breast are reviewed elsewhere in this issue.

The Annapolis Pathology Panel noted:

“Our understanding of the pathology of human breast cancer is based on the correlation of each morphological entity with molecular and clinical-pathological parameters. Because large numbers of cases of individual types of human breast cancer have been studied within the context of clinical trials, it has been possible to produce predictive information on likely survival and metastatic potential for an individual case.” (8)

The classical mouse HAN lesions are primarily acinar or alveolar (16). While the human lesions originate in the same topographical site (the TDLU), they rarely resembled the murine lesions morphologically (74). In fact, lesions morphologically analogous to the hyperplasias with atypia in humans, present within collecting ducts, terminal ducts or alveoli of mice have been rare until the advent of GEM. Nonetheless, images of comparable lesions have been published and discussed in this Journal and others (5–8).

Bouchard *et al.* were the first to point out the similarities between the early lesions in MMTV-LTR-neu GEM mammary glands and human carcinoma *in situ* (37). It has been pointed out, in several articles, the striking similarities between some erbB2 lesions and human carcinoma *in situ* (6,7). The erbB2 protein is differentially expressed in atypical cells and atypical pre-malignant lesions. Ironically, these mouse lesions have not been biologically characterized and, as in the

case of human pathology, the arguments are still based upon association. Some experiments suggest that they possess at least some of the molecular changes seen in pre-invasive carcinomas of humans (81).

Since precancerous breast disease in human is so heterogeneous, the multitude of GEM models of MIN might be all the more appropriate. Each model system has the potential to teach us more about the human condition. At this time, we need more rigorous and more thorough exploration of the preneoplastic condition in the GEM.

CONCLUSIONS

In conclusion, foci of atypical epithelium have been observed in the mammary glands of many species including mouse and man. These foci are thought to be precursors of invasive mammary carcinoma. The origin of these lesions, in most species, appears to be in the terminal ductal units of the mammary tree. The malignant potential of these atypical foci has been thoroughly documented in the non-GEM mouse by transplantation. The advent of targeted genomics has created a number of GEM with focal atypical lesions associated with mammary cancer. Since many of these lesions are the result of the addition of genes known to be activated in human breast cancer, the molecular biology in mice appears to be similar to human. Some early lesions have a remarkable morphological similarity to pre-cancers in humans. However, few have been described in detail and their biological potential has not been validated by transplantation. The utilization of the test-by-transplantation is a necessary step towards understanding neoplastic progression of the breast, and will provide valuable information in the continued quest to unravel the biology of preneoplasia.

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