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Receptor tyrosine kinases are important in cell signal transduction and proliferation. Abnormal expression of tyrosine kinases often leads to malignant transformation. C-met is a tyrosine kinase receptor and its ligand is hepatocyte growth factor (HGF). HGF/c-met plays diverse role in regulation of cell growth, shape and movement. Constitutively activated met, such as tpr-met, is a potent oncogene in vitro, but its carcinogenic role in vivo remains unclear. Our study demonstrates that expression of tpr-met leads to development of mammary tumors and other malignancies in transgenic mice, and suggests that deregulated met expression may be involved in mammary carcinogenesis.

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Timothy A Wagner 10/1/98

PI - Signature Date

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I. Introduction

There has been considerable progress in recent years in our understanding regarding the molecular pathogenesis of breast cancer. Important roles have been established for a number of oncogenes and tumor suppressor genes, including p53, BRCA1, BRCA2, cyclin D-1, HER2/neu, and c-myc (1-4). Like other solid tumors, breast cancers appear to proceed through a multistep process involving a number of genetic alterations that eventually lead to the malignant phenotype.

A number of recent studies suggest that the **c-met** gene may be involved in the pathogenesis of breast cancer. The c-met gene was originally identified as the transforming factor in a MNNG-treated osteosarcoma cell line (HOS), and was activated through a chromosomal rearrangement (5-7). This rearrangement involved the fusion of an upstream promoter sequence (tpr) from chromosome 1 in front of the C-terminus of the met protooncogene on chromosome 7. The **tpr-met** oncogene is activated through dimerization by a leucine zipper domain present in tpr, leading to constitutive activation of the tyrosine kinase region of met (8-10). Chromosomal rearrangement leading to the tpr-met fusion product has been observed in some human gastric tumors (11). However, in other tumors (including gastric cancers), the c-met gene is not rearranged but is amplified and overexpressed, which leads to activation of tyrosine kinase activity and a contribution to cellular transformation (12, 13).

The c-met gene codes for a 190 kDa **receptor**, which consists of two subunits: an intracellular 50 kDa α-subunit and a transmembrane 145 kDa β-subunit (14). The ligand for the c-met receptor has now been established to be hepatocyte growth factor (HGF), also known as scatter factor (15). HGF appears to have diverse biologic effects, including roles in motility, chemotaxis, regulation of cell shape, growth, and invasion (16-18). HGF was originally identified as an important growth factor upregulated during liver regeneration, but recent studies also indicate that HGF is capable of inhibiting tumor growth (19, 20). Nevertheless, overexpression of HGF in transgenic

mice leads to tumor development in a variety of tissues, including mammary gland, skeletal muscle, and melanocytes, suggesting a functional link between mechanisms regulating morphogenesis and those promoting tumorigenesis (21). Most neoplasms, especially melanomas, demonstrated overexpression of both the HGF transgene and endogenous c-met, and had enhanced Met kinase activity, strongly suggesting that autocrine signaling broadly promotes tumorigenesis.

While the c-met gene is widely expressed in many normal epithelial tissues (including breast, intestine, stomach, colon, liver, pancreas, kidney, etc.), levels of c-met gene expression are altered in a number of malignancies. Interestingly, the c-met gene is amplified and overexpressed in some tumors (such as thyroid, gastric, and intestinal tumors), but not in others. In fact, in one study, expression of met was found to be absent in 15 of 15 primary breast cancers, whereas significant met expression was detected in 4 of 4 normal mammmary tissue specimens (22). In another very provocative study, the c-met locus on chromosome 7 (7q21-22) was found to be deleted in 41% of 245 patients with primary breast cancer (23). In addition, patients with LOH on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival. Studies by Vande Woude's group at the NIH have suggested that the HGF-met autocrine loop plays a crucial role in the morphogenesis of breast tissues (24). Taken together, these observations raise the possibility that a region on chromosome 7, possibly the c-met gene, may be the site of an important tumor suppressor gene.

In order to study the role of c-met in breast cancer, we have undertaken wide ranging studies, including genetic and expression analyses of primary breast cancers and breast cancer cell lines; biochemical studies aimed at elucidating protein-protein interactions between the c-met intracellular domain and downstream cellular targets; and mouse models, including transgenic overexpression of the tpr-met chimeric gene and attempts at the generation of c-met null mice. Below, we discuss the results of these studies, and their possible contribution to future research in this area.

II. Body

Our studies have focused on the role of c-met in breast cancer pathogenesis, and over the last 4 years we have pursued four lines of investigation, as outlined in our initial grant proposal. While some of these studies have been successful, a number of these studies have met with limited success, or have been significantly revised or altered as a result of findings from other laboratories which directly impacted our work.

In addition, progress during the last two years has been slowed because of changes in personnel. The original principal investigator, Dr. T. Jake Liang, left the MGH in 1996 and the original co-P.I., Dr. Timothy Wang, assumed the role of principal investigator at that time. However, during this transition, many of the research personnel involved in the study left the project, and momentum was lost. Nevertheless, Dr. Wang and associates have labored intensively on the study to complete the original goals of the c-met project. Listed below is the progress made over the last several years, and the overall conclusions of the study.

Task 1: Genetic alterations involving the c-met gene in breast cancer

Several previous studies suggested that breast cancers show little or no expression of c-met compared with normal breast tissue (22). In addition, loss of heterozygosity (LOH) of the c-met gene appeared to be common in breast cancer (23). Therefore, we undertook a study of DNA, RNA, and protein expression in a number of breast cancer cell lines.

These studies involved a total of 25 separate cell lines. The results are summarized in Table I. Out of 25 cell lines, 11 showed decreased or absent mRNA or protein expression. Out of the 16 "transformed" breast cancer cell lines, 11 showed decreased or absent RNA or protein expression. However, there did not appear to be any significant changes in the c-met gene in any of these cases, as assessed by Southern blot analysis of restriction enzyme-digested genomic DNA.

To address this question of c-met expression in primary tumors (as opposed to cell lines), we obtained several primary breast cancer specimens obtained at the time of surgery. Five (5) primary breast cancers were obtained and processed for DNA, RNA and protein. Three of five primary tumors showed decreased or absent c-met expression by RNA or protein analysis, but again no changes in DNA (i.e. no gross deletions, rearrangements or amplifications) as detected on Southern blot analysis. We have made several attempts at single-stranded conformation polymorphism, but these assays have been mostly negative and it is unclear if our technique is sensitive enough to rule out point mutations. As of yet, we have not carried out direct sequencing of the c-met gene in the tumor DNA. However, at the present time, it is our overall impression that while c-met expression is often decreased in breast cancer, it may be a result of transcriptional silencing rather than through genetic mutation or rearrangement. Our leading hypothesis currently is promoter methylation, as possibility that we would like to address in future studies.

Task 2: Studies on the cellular targets of the c-met protein.

In order to understand the molecular mechanisms of oncogenesis involving c-met, we undertook studies to identify possible cellular targets of the c-met protein. The c-met protein is a receptor tyrosine kinase (RTK) which is involved in the activation of intracellular signaling pathways, presumably through its tyrosine kinase domain and interactions with other cellular proteins. When we undertook these studies, several phosphoprotein had been shown to be involved in the signaling pathway, but no direct physical associations had been shown

In order to identify possible cellular targets of c-met, we utilized the yeast two hybrid system developed at MGH by Dr. Roger Brent. We fused the intracellular domain of c-met to the LexA DNA binding domain, and fused a c-DNA library to the activation domain of LexA. In addition, we utilized a construct in which the LexA binding site was cloned upstream of both the β-

Gal and Leu2 genes. This approach was well outlined in our initial grant application and has been described previously (25, 26).

Using this yeast two hybrid approach, we were able to pull out a number of cDNA clones which appeared to give a strong interaction. Four clones were identified (MAP51, MAP 142, MAP191, MAP282) which resulted in strong β-Gal activity in the presence of galactose. No significant induction of β-Gal activity was observed when the yeast clones were grown in the presence of glucose, or when only the cDNA was transfected (and not the c-met/LexA construct required for DNA binding). Thus, these data indicated that the interaction between c-met and the four clones appeared to be specific.

Over the last year, we have sequenced the four clones. Two of the clones were found to encode for plasma proteins: MAP51 was found to be identical to α-1-antitrypsin, while MAP 191 was found to be identical to apolipoprotein A. It was our judgement that these two proteins were unlikely to be important cellular targets of c-met, given the fact that they are secreted proteins and not intracellular signaling molecules. The other two clones (MAP142 and MAP282) showed no homology to any known sequence in either GenBank or a variety of EST data bases. Both cDNA clones appeared to encode for open reading frames, however, and neither clone showed any homology to the protein Gab1, which is a signal transduction adaptor recently shown to interacts with the cytoplasmic domain of c-met (27).

Given the lack of homology of MAP142 and MAP282 to known signaling molecules, we have begun to try to further define the significance of this interaction through expression of these clones in vitro. The cDNA's were subcloned into pGEX downstream of a GST tag and the constructs transformed into bacteria and lysates analyzed for expression of the fusion protein. Unfortunately, we were not able to detect expression of GST-tagged proteins, suggesting either poor protein stability or insolubility of the fusion protein. We are in the process of trying to express tagged cDNA's using eukaryotic expression vectors (e.g. pcDNA3.1/His) in order to complete these studies. In any case, future studies (not part of this grant project) will hopefully

by carried out to determine the significance of the MAP142 and MAP282 interactions with c-met.

Task 3: Studies on transgenic mice expressing the tpr-met oncogene

We generated four MT1-trp-Met (MTM) transgenic lines, and determined that 3 of the 4 lines showed significant expression of the met oncogene. These three lines were extensively characterized for both expression and for phenotypic effects of tpr-met expression, and the results have been published (Ref. 28, J. Clin. Invest. 97:2872-2877, 1996). To summarize briefly, a high frequency of mammary tumor development was noted in all three lines of mice, with the onset of tumors in multiparous females generally occuring at 10-14 months of age. The histology of these breast cancers resembled closely human mammary tumors, with scirrhous, papillary or nodular patterns of adenocarcinoma. In addition, the mice infrequently developed other types of tumors, including diffuse lymphoblastic lymphoma, thymid lymphoma, spindle cell sarcoma, and giant cell osteosarcoma (28). Thus, these studies confirmed the oncogenic potential of the tpr-met oncogenes, and the MTM transgenic mice represent one of the best histologic models described so far for human breast cancer.

The MTM 1 and MTM 2 lines of transgenic mice were bred to homozygosity, and the lines were sent to the Jackson Laboratory (per request of the Jackson Labs) to be made available to other investigators working on animal models of breast cancer.

In addition, we have carried out additional studies examining possible synergistic interactions between tpr-met oncogene and other breast cancer susceptiblity genes. As part of this approach, we have mated the MTM transgenic mice to mice with genetic alterations in other tumor suppressor genes or oncogenes. We have crossed the MTM-1 line either with p53 knockout mice or with transgenic mice overexpressing cyclin D1 in their mammary epithelium. These MMTV-cyclin D1 have been previously reported by our group (29). While these studies are still ongoing, preliminary results suggest that overexpression of cyclin D1 or loss of one p53 allele may

possibly accelerate the development of breast cancer in these mice. Overexpression of tpr-met alone leads to the development of breast cancer at ~ 12 months of age in multiparous females. In mice carrying both tpr-met and cyclin D1, it appears that the onset of breast cancer occurs somewhat earlier, with several mice developing tumors at ~9 months of age. Mice expressing tpr-met and lacking one copy of p53 appear to develop tumors at ~ 10 months of age. In both case, addtional studies with larger numbers of mice - currently underway - will be required to determine if this acceleration of tumor formation is statistically significant. Nevertheless, it is clear that the tpr-met transgenic model will prove useful for further in vivo studies on the genetic mechanisms involved in the development of breeast cancer.

Task 4: Targeted inactivation of c-met gene.

Previous studies suggested a potential important regulatory role for c-met not only in breast cancers, but also in the morphogenesis of normal mammary epithelium (24). In addition, while overexpression of tpr-met leads to a high rate of induction of breast cancer in our MTM transgenic mouse model, previous observations in humans have suggested that the c-met locus on chromosome 7 may represent a tumor suppressor locus, rather than an activating oncogene. This would imply that the tpr-met protein product was possibly behaving more like a "dominant negative" for the normal tumor suppressing function of the wild type c-met gene. However, a role for c-met as a possible tumor suppressor gene has never been directly investigated.

In order to address this question, we undertook studies to generate c-met deficient mice through targeted deletion of the murine c-met gene. We cloned the murine c-met gene from a mouse SV129 genomic library, and created a targeting vector through replacement of coding exons with the neomycin resistance gene. Transfection of J1 ES cells was accomplished, stable clones selected with G418 treatment, and several clones with homologous recombination were selected by Southern blot analysis of restricted DNA. We then planned to proceed with injection of these

genetically modified ES cell clones into blastocysts, followed by breeding of chimeric mice to generate homozygous knockout mice.

However, at this point, we learned that an identical study involving knockout of the c-met gene had been successfully completed by a group in Germany, and published in Nature (376:768-771). This study suggested an essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud, but the knockout phenotype did result in embryonic lethality (30), such that a possible role in breast development or breast cancer susceptibility could not be assessed. However, given this previous publication and the embryonic lethal phenotype, we decided not to pursue or replicate this "complete knockout" of the c-met gene in the mouse germline.

Instead, in order to address the possible role of c-met in breast development, we have initiated an alternative approach, which is the tissue specific, conditional "targeted" knockout of the c-met gene using the Cre-LoxP approach. My laboratory, in collaboration with Dr. T. Jake Liang and Dr. Brian Sauer of the NIH, are pursuing this avenue of research. My laboratory has generated the MMTV-Cre construct, which is designed to overexpress the recombinase Cre in mammary epithelium. This construct has a high likelihood of success, given the experience our laboratory has had with MMTV targeting vectors. Once these mice have been generated, we would then cross these mice with mutant mice with homozygous knock-in of the c-met gene flanked by LoxP sites. These latter mice will be generated at the NIH by T. Jake Liang and colleagues. These latter studies were, unfortunately, not completed within the time frame of the Army Grant, due to the disruptions resulting from the change in P.I. as well as our being "scooped" by the publication by Birchmeier's group (30). It is clear from the above Nature study that knockout of the c-met gene is not able to answer the question of whether c-met is a tumor suppressor gene for breast epithelium. Nevertheless, we have been able to generate the necessary reagents which will allow future studies which are designed to more directly address this question.

III. Conclusions:

As a result of studies involving breast cancer cell lines and primary breast tumors, we have observed altered expression of the met gene in the majority of cell lines and tumors, consistent with a role for this gene in the pathogenesis of breast cancer. In the majority of cell lines and tumors, met gene expression appears to be decreased or absent, confirming previous studies which have looked at this question. However, Southern blot analysis did not show any clear rearrangements or gross deletions of the c-met gene, suggesting the possibility of epigenetic regulation. However, neither our group nor other groups have ruled out the possibility of point mutations in the coding region (or promoter region) of the met gene, studies that will require considerable effort to complete. Furthermore, based on recent experience with several other tumor suppressor genes (such as MLH1), we would favor the possibility that the c-met promoter may be silenced through a mechanism such as methylation, and this possibility will hopefully be addressed in future studies.

It is likely that met function is mediated through protein-protein interactions, involving primarily the intracellular (kinase) domain of c-met. Several of the downstream cellular targets of c-met have been identified. One such downstream target, and one that interacts directly with c-met, is the protein Gab1 (27), which is related to DOS (for 'daughter of sevenless'). Using the technique of the yeast two-hybrid system, we have isolated and subcloned four candidate clones for c-met interactors. While two of these clones appear to be plasma proteins which are likely not cellular targets of c-met, the other two clones (MAP142 and MAP282) appear to contain novel DNA sequences which have not yet been reported. Future studies with these clones, which will involve expression of the full length cDNA, definition of the met binding domain, and tissue characterization using specific antibodies, will hopefully determine whether these clones represent important c-met targets. In addition, it will be interesting to test whether overexpression of MAP142

and/or MAP282 are sufficient to induce c-Met-specific activities, such as motility or branching morphogenesis.

Extensive studies have now been carried out in the MT1-TPR-MET (MTM) transgenic lines, and these studies have been published (see attached reprint). We have been able to demonstrate induction of tumor formation at 10-14 months in three separate transgenic lines, and histological analysis of these tumors (by our collaborator, Dr. Cardiff, the world's expert on this subject) suggest that the tumor histology resembles rather closely the pathogology of human breast cancer. In addition, the tumors have been shown to upregulate expression of the tpr-met transgene, indicating that mammary adenocarcinoma arises as a direct consequence of tpr-met expression. Similar upregulation of met expression has been demonstrated in other transgenic cancer models, including those involving transgenic overexpression of HGF. The MTM transgenic mice have been made available to other investigators, courtesy of the Jackson laboratory, and further studies are ongoing to determine possible synergy between tpr-met and other genetic factors (cyclin D1 and p53 LOH) in breast cancer.

Finally, the possible role of c-met as a tumor suppressor gene remains unanswered. Knockout of the c-met gene points to an important role in development, but the embryonic lethality of the c-met homozygous knockout limits any possible analysis of c-mets role as a tumor suppresor gene in breast cancer models. We have created MMTV-Cre constructs which will be used to generate transgenic mice, in preparation for studies on conditional knockout of the c-met gene in mammary epithelium.

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V. Appendix

Table 1: Altered c-met Expression in Breast Cell Lines.

Cell Line*	Tumorigenic**	DNA***	RNA#	Protein
75N	-	+	+	ND
70N	-	+	+	+
81NN	-	+	+	ND
21NT	-	+	+	+
21PT	-	+	+	ND
18-2-1	-	+	+	ND
1436N1	-	+	+	ND
HBL-100	-	+	+	+
Hs578Bst	-	+	+	+
MDA-MB-157	+	+	-	ND
MDA-MB-231	+	+	-	ND
MDA-MB-361	+	+	-	ND
MDA-MB-435	+	+	+	+
MDA-MB-436	+	+	+	ND
MDA-MB-468	+	+	-	ND
BT-474	+	+	-	ND
BT-549	+	+	-	-
MCF7	+	+	-	-
MCF10A	+	+	+	ND
T-47D	+	+	-	ND
ZR-75-1	+	+	-	-
ZR-75-3	+	+	-	-
DU4475	+	+	-	-
Hs578T	+	+	+	-
SK-BR3	+	+	+	-

* The primary mammary cells 76N and 70N, and immortalized breast cell lines 21-NT, 21-PT, 18-2-1, and 1436N1 are from Ruth Sager; the remaining cell lines are obtained thru ATCC.

* * "Nontumorigenic" is defined as inability to form tumor in nude mouse.

* * * Absence of gross gene rearrangement by restriction and Southern blot analysis is defined as "+".

\# presence or absence of met mRNA was indicated by + or -.

ND: Not done

Table 2. Interaction of Intracellular Domain of c-met with Candidate cDNA Clones

Yeast Plasmid Construct	β-gal Activity (U) Glucose	Galactose
LexA-MetCD + JG4-5	10	12
LexA-MetCD + JG4-5-MAP51	25	1939
LexA-MetCD + JG4-5-MAP142	26	2789
LexA-MetCD + JG4-5-MAP191	31	1897
LexA-MetCD + JG4-5-MAP282	27	2192
LexA + JG4-5-MAP51	17	19
LexA + JG4-5-MAP142	15	21
LexA + JG4-5-MAP191	11	14
LexA + JG4-5-PMAP282	13	16

Transgenic Expression of tpr-met Oncogene Leads to Development of Mammary Hyperplasia and Tumors

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Abstract

Receptor tyrosine kinases are important in cell signal transduction and proliferation. Abnormal expression of tyrosine kinases often leads to malignant transformation. C-met is a tyrosine kinase receptor and its ligand is hepatocyte growth factor (HGF). HGF/c-met plays diverse roles in regulation of cell growth, shape and movement. Constitutively activated met, such as tpr-met, is a potent oncogene in vitro, but its carcinogenic role in vivo remains unclear. Our study demonstrates that expression of tpr-met leads to development of mammary tumors and other malignancies in transgenic mice, and suggests that deregulated met expression may be involved in mammary carcinogenesis. (*J. Clin. Invest.* 1996. 97:2872–2877.) Key words: c-met • hepatocyte growth factor • receptor tyrosine kinase • carcinogenesis • sarcoma

Introduction

The c-met protooncogene encodes a tyrosine kinase receptor of 190-kD protein. Hepatocyte growth factor (HGF)[1] is the ligand of the c-met receptor (1). HGF is also known as hepatopoietin and is identical to scatter factor, which affects the motility, chemotaxis, and invasiveness of epithelial and endothelial cells in culture (2–4). HGF has been shown to be the most potent growth factor for rat and human hepatocytes in primary cultures (5, 6). In addition to its mitogenic effect, HGF regulates cellular shape as a morphogen and cellular motility as a motogen (3). Recently, met-HGF/SF activation was also shown to mediate mesenchymal to epithelial transition (7).

The oncogenic form of c-met (tpr-met) was identified in a N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)–treated human osteosarcoma cell line HOS (8–10). The activation of the met oncogene was shown to occur via a chromosomal rearrangement, generating a chimeric gene, fusing an upstream promoter-containing sequence (tpr) from chromosome 1 to the carboxyl terminus of the met protooncogene on chromosome 7. The fusion molecule (65 kD) contains the tyrosine kinase domain of the c-met protooncogene. The tpr sequence consists of a constitutive promoter and an open reading frame coding for a protein with strong sequence homology to nuclear oncoproteins fos and jun, transcription factor CREB, and members of intermediate filament multigene family (11). The common feature among these molecules is that they contain a leucine zipper which is required for dimerization and activation of these proteins (12). Recent data demonstrated that tpr-met oncogene was indeed activated through this leucine zipper interaction, resulting in a constitutively phosphorylated and presumably active state of this tyrosine kinase molecule (13).

Overexpression of normal c-met appears to be sufficient to activate tyrosine kinase activity, which may explain the transforming potential of amplified c-met gene in some human tumors (14). Met is frequently amplified and overexpressed in various transformed cell lines and human tumors (15, 16). The identification and characterization of other forms of c-met demonstrated that abnormal processing of the extracellular domain of the protein can also result in constitutive activation of c-met (14). Similar to what has been described for other receptor tyrosine kinases, such as trk and ret protooncogenes (17–19), activating mutations affecting the extracellular or transmembrane domain of c-met may contribute to an oncogenic potential of met in some human cancers. To date, no direct evidence exists demonstrating the oncogenic potential of met in vivo. Therefore, in order to assess met as a candidate oncogene in vivo, we examined the consequences of met oncogene expression in transgenic mice.

Methods

Transgenic construction. The 2.2-kbp cDNA clone of the tpr-met oncogene (9) was inserted into an expression construct containing the 1-kB mouse metallothionein 1 (MT1) promoter (20) and the SV40 small T intron and polyadenylation signal. The MT-tpr-met construct was transfected into COS cells and expression of the tpr-met protein was demonstrated by Western blot analysis (not shown). A linearized fragment from the MT-tpr-met construct was injected into fertilized mouse (FVB/n X FVB/n) eggs following established protocols. 33 potential founder pups were screened for transgene incorporation by Southern blot analysis. Four founder lines positive for the transgene were identified (MTM1-4). Three founders transmitted the transgene to their progeny in a Mendelian fashion, while the fourth line appeared to be a mosaic.

Analysis of tpr-met transgene expression. RNAs were extracted from various tissues using RNAzol (Biotecx Laboratories, Inc., Hous-

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1. *Abbreviation used in this paper:* HGF, hepatocyte growth factor.

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Table I. Tumor Incidence in MTM Transgenic Strains

Transgenic line	Mice in continuous breeding	Mice with mammary tumors*	Independent mammary tumors	Mean age of onset‡	Mice with other tumors§
MTM1*	10	6	7	317	3
MTM2	8	3	7	394	1
MTM3	8	2	3	433	2

Cohorts of mice in each of the MTM transgenic strains were observed for tumor development. Mammary and other tumors were observed in all strains. *Parallel cohort of nontransgenic litter mates (15) were set up for continuous mating as controls and none of the mice developed tumors at one and half years of age. ‡Data reported as mean age in days of detection of breast tumors. §These animals were not included in the cohort of female mice in continuous breeding. One female MTM1 animal developed diffuse lymphoma at 9 mo of age and a male had metastatic spindle cell sarcoma at two years of age. An orbital osteosarcoma was noted in the third male MTM1 animal at 18 mo of age. One MTM2 male mouse was noted to have two lesions: one squamous papilloma and the other rectal spindle cell sarcoma. Two female MTM3 animals developed lymphoma, one thymic and the other diffuse at an early age of 3 mo.

ton, TX), subjected to Northern blot analysis, and hybridized with ^{32}P labeled human c-met cDNA under stringent condition. RT-PCR was performed using an antisense primer from the SV40 polyadenylation sequence (5′ CCTGAAATGAGCCTTGGGA 3′) as the RT primer and a sense primer from the COOH terminus of the tpr-met sequence (5′ TCTTTGCTCCTTGCCATAGG 3′) as the second primer. Control reactions without RT were performed in parallel for each samples. For analysis of protein, tissues were homogenized using a Dounce homogenizer in the presence of RIPA buffer containing protease inhibitors. For analysis of protein phosphorylation, phosphatase inhibitors (100 mM NaF, 20 mM disodium p-nitrophenyl phosphate, 10 mM disodium-bis-glycerophosphate, 10 mM tetrasodium pyrophosphate, 1 mM sodium othovanadate) were also included in the lysis buffer. Cell lysates were cleared of nuclei and other cell debris by centrifugation and subjected to SDS-PAGE (reducing condition) and Western blot analysis with anti-met 19 S monoclonal antibody (from George Vande Woude) in 1:1000 dilution using a chemiluminescence kit (Amersham).

Results

Three lines positive for tpr-met transgene were successfully established. An F1 female animal from the MTM1 line, after breeding for 6 mo, developed a mammary cancer. Nine addi-

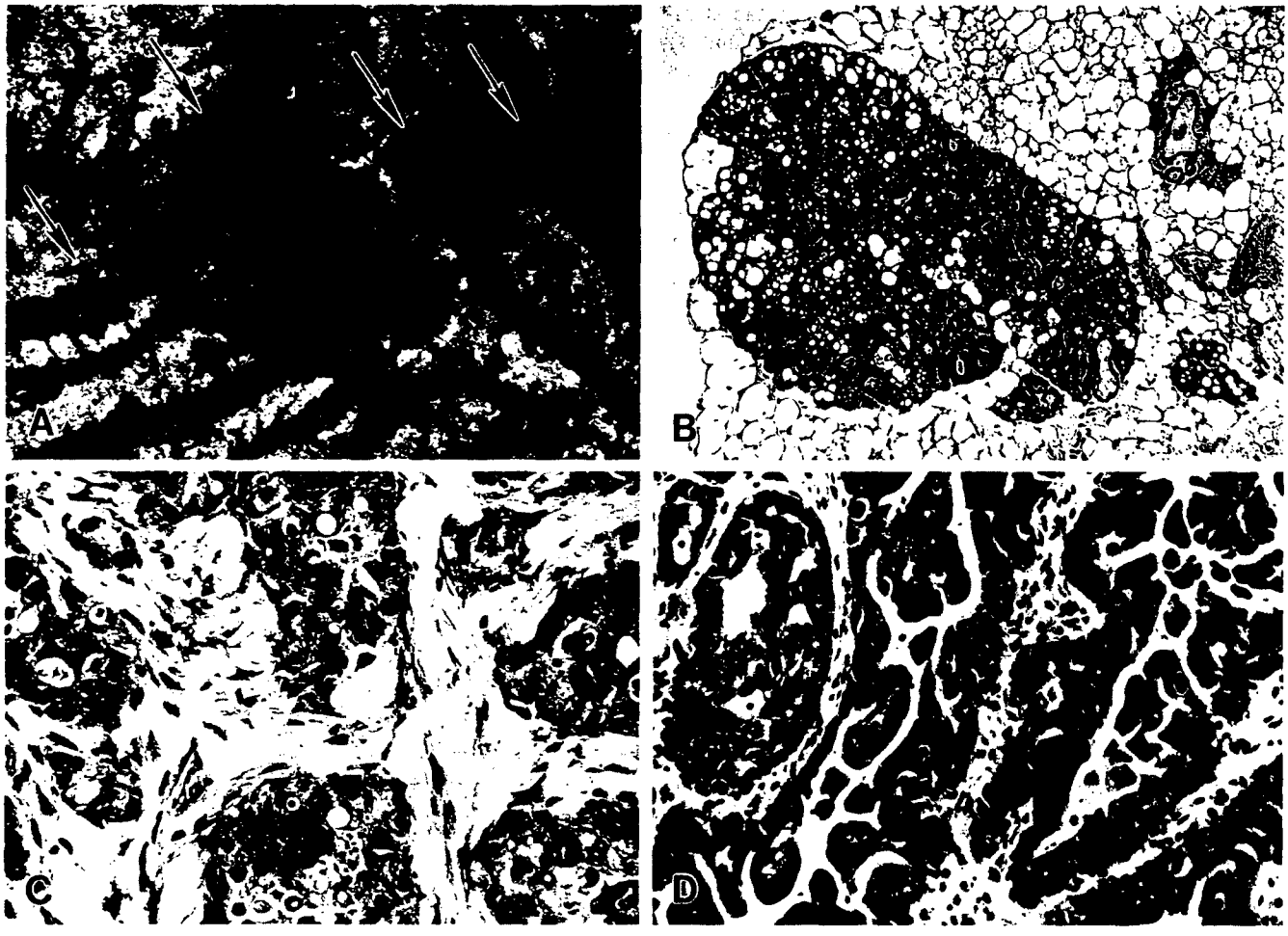

Figure 1. Photomicrographs illustrating the progression from mammary hyperplasia to malignancy in the tpr-met transgenic mouse. (*A*) A fat-cleared, hematoxylin-stained mammary gland whole mount preparation showing large foci of mammary hyperplasia which stand out from the background as dark lobular structures (*arrows*) (×17). (*B*) A photomicrograph showing a focal hyperplastic alveolar nodule which stands out from the general background of mammary fat and ducts (×88). (*C*) A high magnification of a scirrhous carcinoma in a tpr-met transgenic mouse (×220). (*D*) A high magnification illustrating the papillary type of tumor from a tpr-met transgenic mouse (×220).

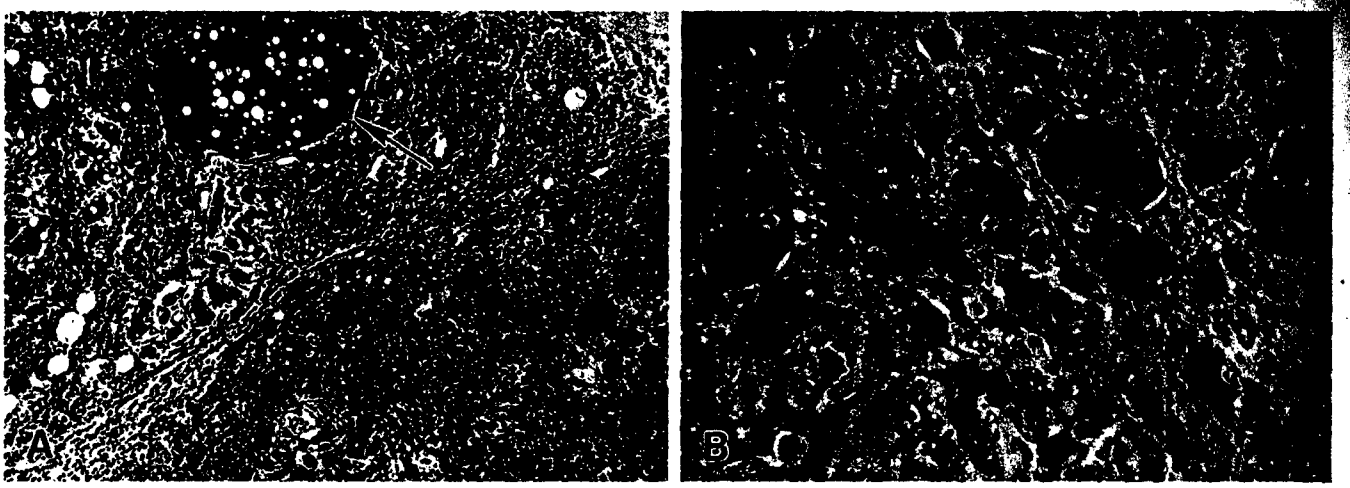

Figure 2. Photomicrographs illustrating two other tumors found in the tpr-met transgenic mouse. (*A*) A low magnification showing a small cell lymphoma which diffusely infiltrates a mammary fat pad leaving a cystically dilated duct (*arrow*) and several smaller mammary ducts (compare with 1b) (×88). (*B*) A high magnification photomicrograph illustrating a poorly differentiated giant cell tumor with osteoid formation (×220).

tional F1 females from this line, eight from line MTM2, and eight from line MTM3 were set up for forced continuous breeding (Table I). Most of the multiparous mice had mammary hyperplastic alveolar nodules (HAN) (Fig. 1, *A* and *B*) and several of them also had foci of microscopic carcinoma on whole mount examination. Seven primary mammary tumors developed in six of the MTM1 animals. In the MTM2 line, seven independent mammary tumors developed in three female animals. In the third line (MTM3), three mammary tumors were observed in two animals. The mammary tumors had one of three patterns, scirrhous, papillary, or nodular (Fig. 1, *C* and *D*), many of them resembling human mammary tumors. The nuclei were intermediate in size with delicate clumped chromatin. Histological patterns of these types have not been seen in spontaneous murine tumors (21).

Additional tumors were also found in animals from all three lines (Table I). Single animals from MTM1 and MTM3 lines developed diffuse lymphoblastic lymphomas involving the mammary gland and lymph nodes (Fig. 2 *A*) at 6–9 mo of age. One MTM3 mouse developed a thymic lymphoma. Spindle cell sarcomas were also observed in MTM2 and MTM1 mice. An unusual orbital giant cell osteosarcoma was noted in another MTM1 animal at 18 mo of age.

Northern blot analysis was performed on total RNAs extracted from various tissues and breast tumors. Only the breast tumors contained detectable tpr-met transcripts in the expected size range (Fig. 3 *A*), whereas no signal was detected in any of the normal tissues examined (liver, stomach, intestine, breast, kidney, spleen). To improve the sensitivity of detecting low levels of the tpr-met transcript, RT-PCR was performed

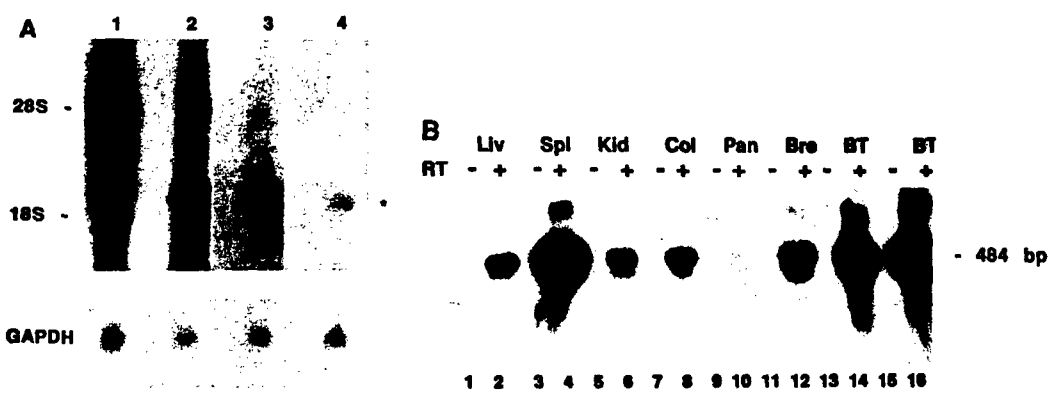

Figure 3. Analysis of tpr-met mRNA in MTM transgenic mice. (*A*) Expression of tpr-met mRNA in mammary tumors. RNAs were extracted and subjected to Northern blot analysis using a ^{32}P-labeled human c-met cDNA under stringent condition. An expected 2.5-kb RNA was detected. Some larger species of tpr-met RNA was also detected in some samples. Three mammary tumors (lanes *2*, BT4; lane *3*, BT3; lane *4*, BT2) and one lymphoma (lane *1*, Ly1) were analyzed in this blot. In contrast, tpr-met mRNA was not detected in normal tissues form both transgenic and nontransgenic litter mates (not shown). (*B*) Detection of tpr-met transcripts by reverse transcription-polymerase chain reaction (RT-PCR). Extracted RNAs from various tissues were subjected to RT-PCR as described (lanes as indicated). Control reactions without RT (−) were performed in parallel for each samples. Tissues shown include liver (*Liv*), spleen (*Spl*), kidney (*Kid*), colon (*Col*), pancreas (*Pan*), breast (*Bre*), and two brewast tumors (BT2 and 3). 10 μg of RNA was used for RT-PCR of all samples except for the mammary tumors where 1 μg was used for the reaction.

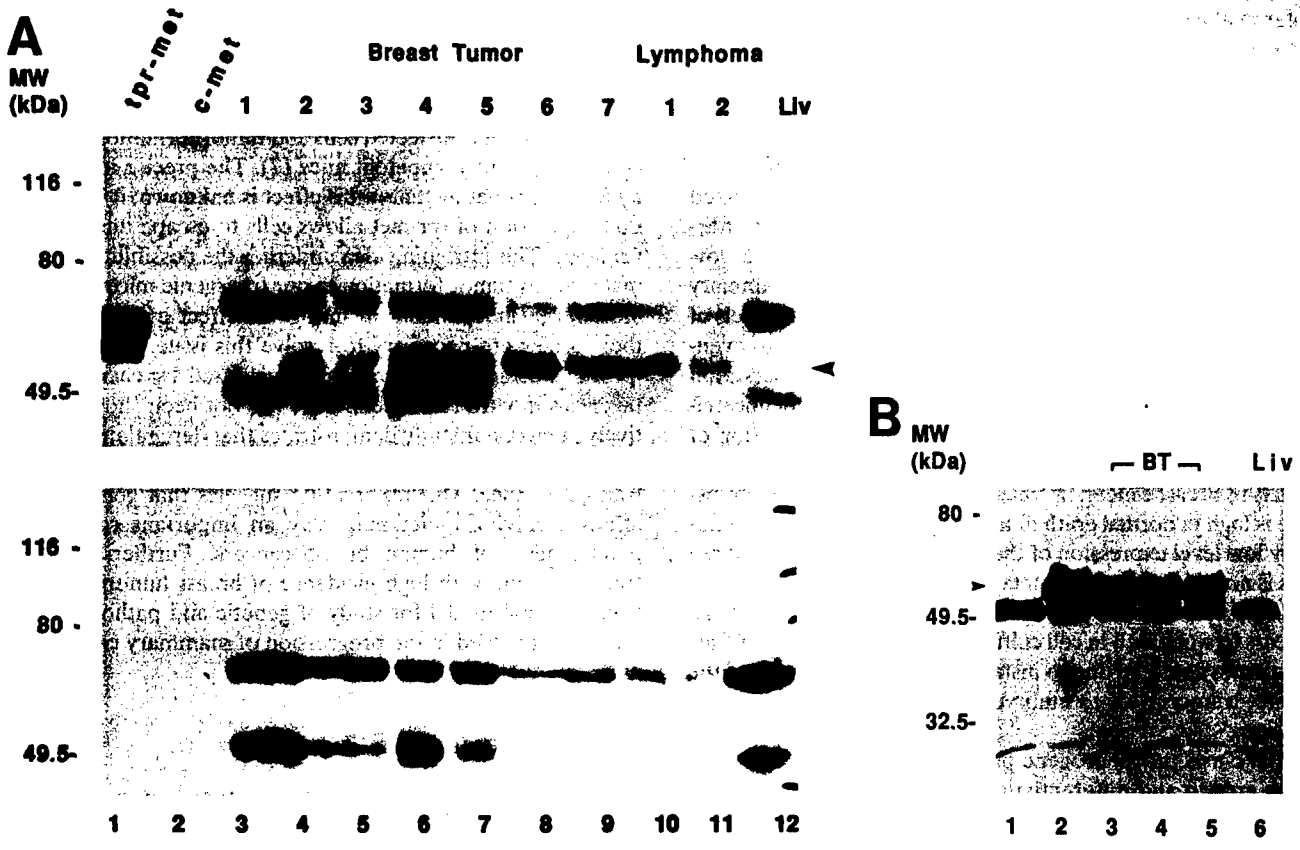

Figure 4. Analysis of tpr-met protein in tumors. (*A*) Tissues were homogenized using a Dounce homogenizer in the presence of RIPA buffer containing protease inhibitors. Cell lysates were cleared of nuclei and other cell debris by centrifugation, and 100 μg of lysates were subjected to Western blot analysis with anti-met 19S monoclonal antibody (from George Vande Woude) in 1:1000 dilution using a chemiluminescence kit (Amersham). A parallel control blot was set up using preimmune mouse serum. For positive controls (tpr-met and c-met), 20 μg of cell lysates from COS cells transfected with a tpr-met expression plasmid and HepG2 cells which expresses the 140-kD c-met protein were included. The lanes are as indicated and include data from seven breast tumors (BT1-7), two lymphomas (Ly1 and 2) and a transgenic liver. The top panel represents the blot probed with the 19S antibody and the bottom is the control. A specific 65-kD protein (*arrowhead*) was detected in most of the tumors and the tpr-met transfected cells. As expected, a 140-kD protein was detected in the HepG2 cells. (*B*) Analysis of tyrosine phosphorylation of tpr-met in tumors. 100 μg of Cell lysates (containing phosphatase inhibitors) from three breast tumors (BT5-7, lanes *3–5*) and liver (lane *6*) were immunoprecipitated with 19 S antibody, electrophoresed under reducing condition, and subjected to Western immunoblot with anti-phosphotyrosine antibody (Santa Cruz Biotechnology). As controls, 20 μg of cell lysates from untransfected COS cells (lane *1*) and COS cells transfected with tpr-met expression construct (lane *2*) were included. The 65-kD protein (*arrowhead*) was recognized specifically by the antibody since a parallel control blot using preimmune mouse serum was negative. The 50- and 25-kD bands detected on these blots are immunoglobulin heavy and light chains.

on RNAs extracted from these tissues. Using primers specific for the transgene, we were able to detect tpr-met transcripts in all the normal tissues (Fig. 3 *B*) Although the primers were designed to span the SV40 intron, we detected PCR products of both unspliced and spliced RNA (differing by 66 nt) in all the samples. This observation, however, is consistent with the previous findings that SV40 intron is not particularly efficient in splicing (22). Western Immunoblot, performed on protein lysates extracted from various tumors, showed detectable expression of tpr-met protein in most tumor tissues (Fig. 4 *A*). Analysis of normal tissues from all three lines of transgenic animals revealed no detectable tpr-met protein expression (not shown). In these tumors, the tpr-met protein also appeared to be phosphorylated on the tyrosine residue (Fig. 4 *B*). This finding is consistent with the previous observation that tpr-met protein is constitutively phosphorylated and activated through dimerization (13).

Discussion

The pattern and occurrence of mammary hyperplasia and tumors in the tpr-met transgenic strains support strongly the conclusion that these lesions are a direct effect of the transgene. Hyperplasia and tumors developed in all three independent transgenic lines (MTM1, MTM2, and MTM3). In addition, 15 FVB/n multiparous mice were followed for the same length of time (average 1.5 yr) and did not develop any tumors. This observation is consistent with other investigators' experiences with the FVB/n strain (23). Furthermore, occurrence of multiple independent mammary tumors, as observed in some of our animals, is rare in spontaneous breast tumors of normal mice.

The use of a cellular promoter (metallothionein) rather than the MMTV promoter to drive the tpr-met transgene further speaks for the uniqueness of this animal model in the study of mammary carcinogenesis. The metallothionein pro-

moter enabled us to express low levels of tpr-met in a variety of tissues, including not only mammary epithelium but also liver, spleen, kidney, and colon. Our finding that MT-tpr-met transgene mice developed a predominant breast cancer pheotype might suggest that mouse mammary epithelium is susceptible to transformation by the tpr-met oncogene. In addition, it is interesting to note that only mammary tumors expressed high-level of the tpr-met transcript and protein, whereas normal tissues including mammary epithelium expressed low level. Perhaps, during physiological hyperplasia of mammary glands as a result of pregnancy, cells expressing higher levels of the oncoprotein were selected and clonally expanded to eventually form foci of mammary malignancy. This observation suggests that in this transgenic model, mammary adenocarcinoma developed as a direct effect of high-level expression of tpr-met. This is in contrast to some of the other transgenic models of breast cancer, in which basal expression of the transgene is high in normal epithelium (23). The reason for the relatively low level expression of the tpr-met transgene in all three lines is not clear. Since the metallothionein promoter is considered a relatively strong promoter and the transgene construct appears to work well in cell culture, the low RNA levels in various tissues could be due to instability and short half-life of the tpr-met transcript or to embryonic lethality of high level expression.

The c-met protooncogene product plays an important role in the growth and differentiation of epithelial cells in various organs. Recent studies have demonstrated a wide distribution of this protein in normal tissues, including breast, intestine, stomach, liver, pancreas, and kidney, and more interestingly, increased levels of met RNA and protein in a number of human tumors, particularly thyroid, gastric, hepatic, intestinal, and soft tissue tumors (15, 16, 24). It is interesting to note that several mesenchymal tumors (osteosarcoma, spindle cell sarcoma) were detected in the MTM mice. This observation is consistent with previous findings that tpr-met efficiently transform NIH-3T3 fibroblasts and that met overexpression occurs frequently in human sarcoma (8, 16). Examination of liver tissues in the MTM transgenic animals also revealed "nuclear unrest" of hepatocytes with increased mitotic activities (not shown). This observation is interesting in light of the potent mitogenic effect of HGF on hepatocytes (6).

A recent study showed that the c-met locus on chromosome 7 (7q21-22) was deleted in 41% of 245 patients with primary breast cancer (25). In addition, patients with loss of heterozygosity on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival, suggesting that this region of chromosome 7, possibly the c-met gene, may be the site of a breast tumor or metastasis suppressor gene. Although our study suggests that tpr-met is a dominant oncogene in mammary carcinogenesis, it is possible that wild-type c-met may play a different role in growth and proliferation of mammary epithelium. Recent studies on the function of HGF-met activation suggested a potential regulatory role for c-met in the morphogenesis of mammary epithelium (26). The significance of genetic alterations involving c-met in human mammary carcinogenesis thus requires further study.

Co-expression of human c-met and its ligand HGF/SF has been shown to confer increased invasiveness and metastasis to NIH 3T3 cells in transfection studies (7). This phenomenon is probably mediated through an autocrine activation of c-met. In these experiments, transfection of the tpr-met oncogene alone appears to be equally potent as the co-expression of met-HGF/SF, consistent with the observation that the tpr-met is constitutively activated. Furthermore, it is interesting to note that expression of tpr-met, but not of met-HGF/SF, appears to allow growth of transfected cells and tumor formation in heterogeneic immune competent mice (7). The precise mechanism by which tpr-met mediates this effect is unknown; it is possible that expression of tpr-met allows cells to escape immune surveillance. This intriguing idea underlies the possibility that tpr-met induces tumor formation in our transgenic mice through a mechanism independent of mitogenic effect of tpr-met. Further studies are necessary to resolve this issue. Although the tpr-met oncogene was originally generated by chemical mutagenesis in vitro and its existence has not been shown definitively in vivo, our study demonstrates that deregulated met expression can lead to development of mammary tumors in transgenic mice. This observation suggests that genetic alterations involving c-met may play an important role in the pathogenesis of human breast cancers. Furthermore, this transgenic line with high incidence of breast tumors can be a useful animal model for study of genetic and pathological alterations involved in the progression of mammary carcinogenesis.

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