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Research Article

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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-

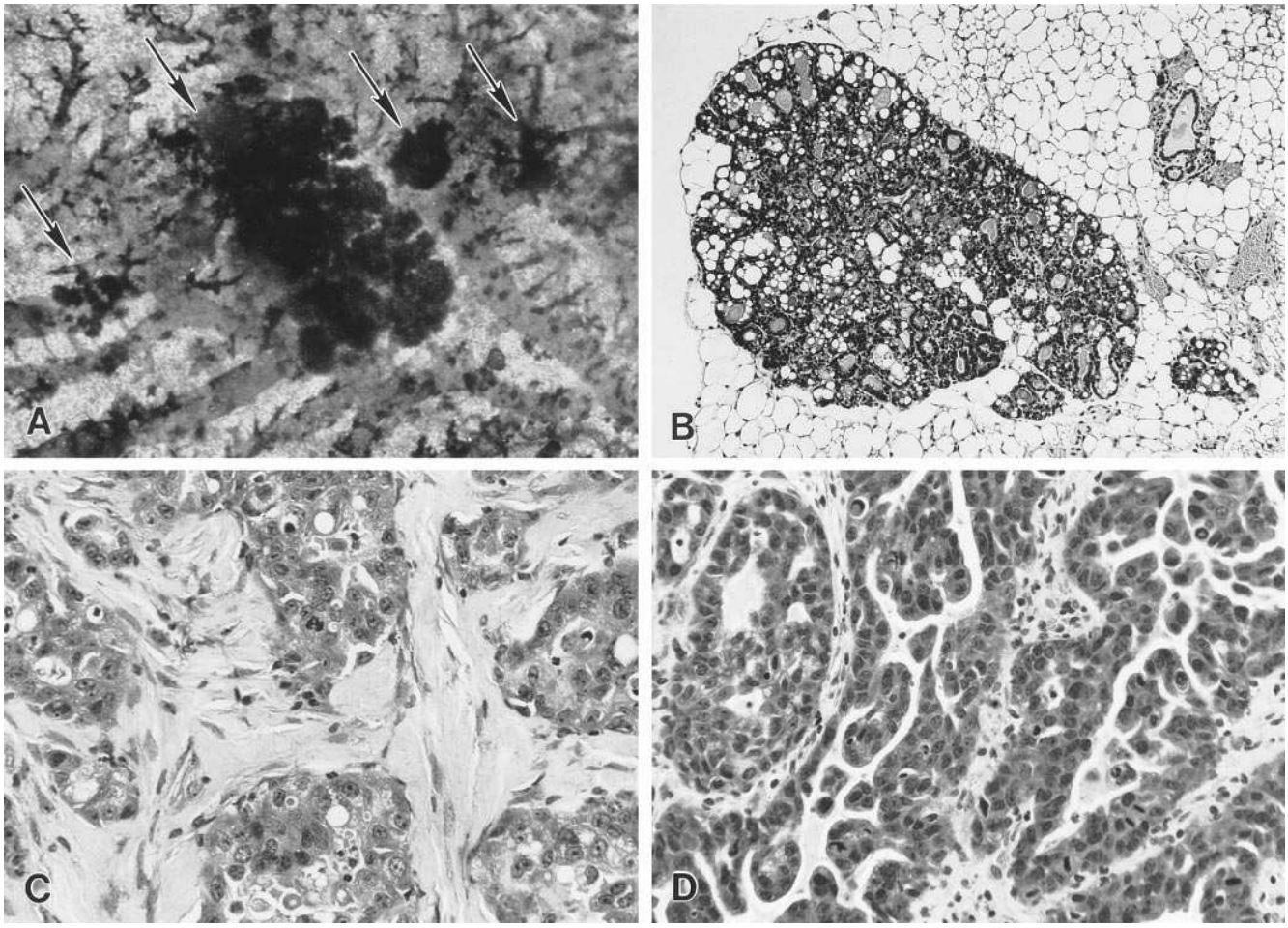

Figure 1. Photomicrographs illustrating the progression from mammary hyperplasia to malignancy in the tpr-met transgenic mouse. (*A*) A fat-cleared, hematoxylin-stained mammary gland whole mount preparation showing large foci of mammary hyperplasia which stand out from the background as dark lobular 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).

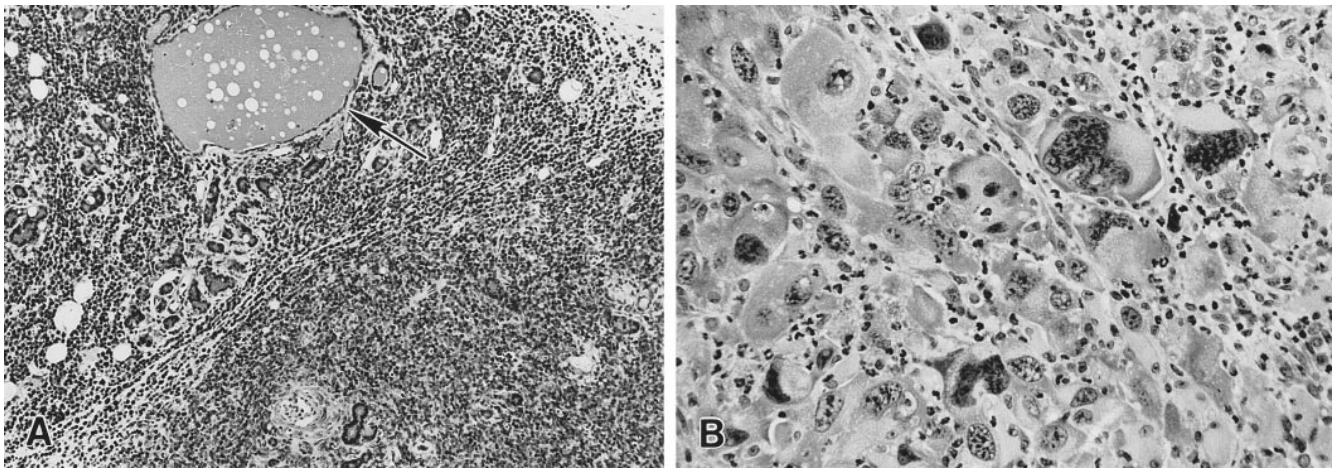

Figure 2. Photomicrographs illustrating two other tumors found in the tpr-met transgenic mouse. (*A*) A low magnification showing a small cell lymphoma which diffusely infiltrates a mammary fat pad leaving a cystically dilated duct (*arrow*) and several smaller mammary ducts (compare with 1b) (×88).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

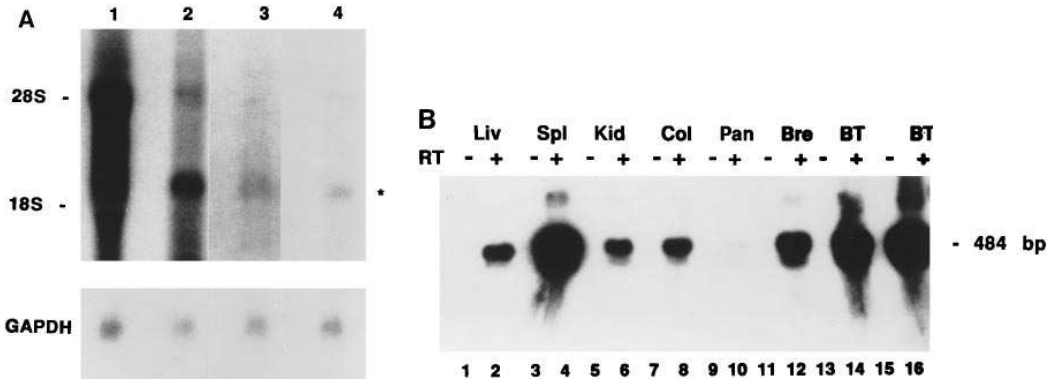

Figure 3. Analysis of tpr-met mRNA in MTM transgenic mice. (*A*) Expression of tpr-met mRNA in mammary tumors. RNAs were extracted and subjected to Northern blot analysis using a ^{32}P-labeled human c-met cDNA under stringent condition. An expected 2.5-kb RNA was detected. Some larger species of tpr-met 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.

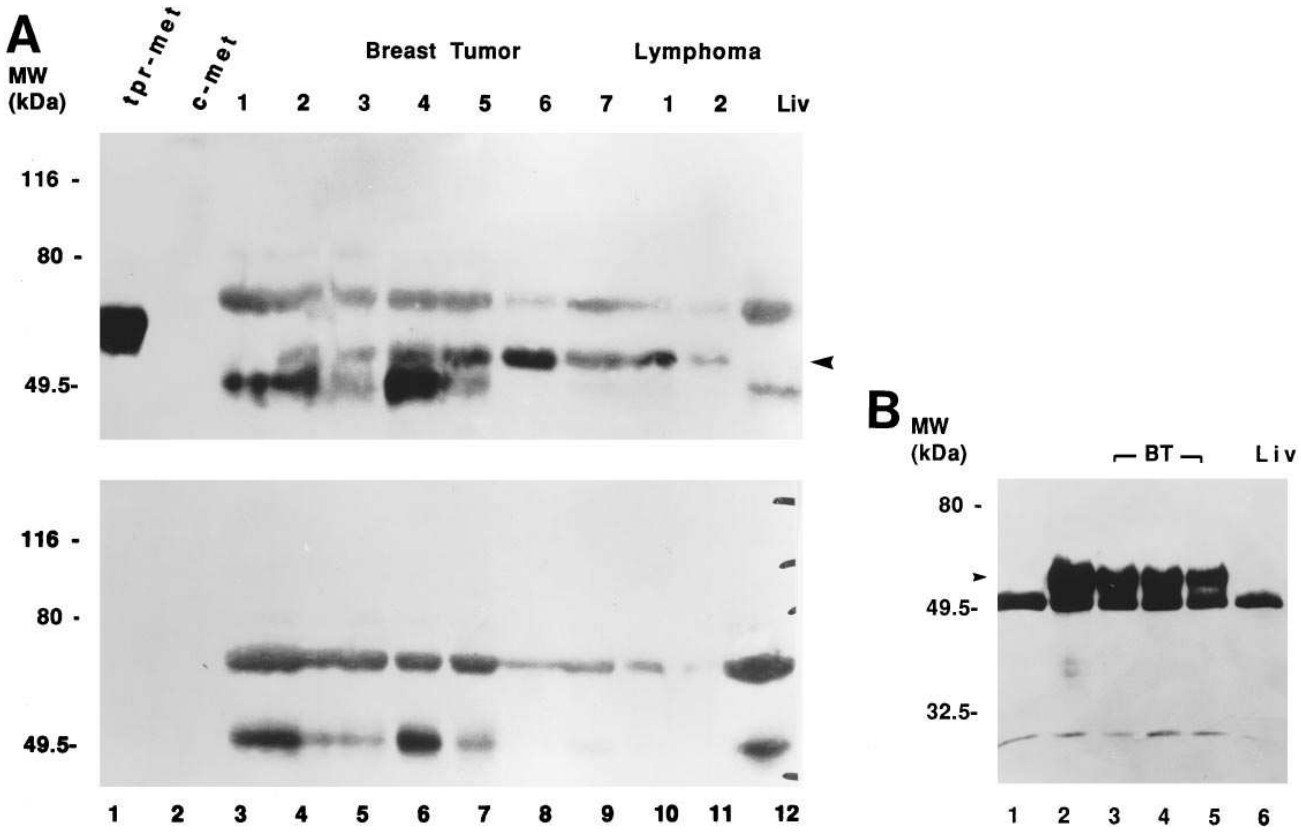

Figure 4. Analysis of tpr-met protein in tumors. (*A*) Tissues were homogenized using a Dounce homogenizer in the presence of RIPA buffer containing protease inhibitors. Cell lysates were cleared of nuclei and other cell debris by centrifugation, and 100 μg of lysates were subjected to Western blot analysis 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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