Matrix Metalloproteinases Play an Active Role in Wnt1-Induced Mammary Tumorigenesis

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Abstract

The Wnt signaling transduction pathway plays a critical role in the pathogenesis of several murine and human epithelial cancers. Here, we have used mouse mammary tumor virus (MMTV)-Wnt1 transgenic mice, which develop spontaneous mammary adenocarcinoma, to examine whether matrix metalloproteinases (MMPs)—a family of extracellular proteases implicated in multiple steps of cancer progression—contributed to Wnt1-induced tumorigenesis. An analysis of the expression of several MMPs by RT-PCR and in situ hybridization revealed an increase in the expression of MMP-2, MMP-3, MMP-9, MMP-13, and MT1-MMP (MMP-14) in hyperplastic glands and in mammary tumors of MMTV-Wnt1 transgenic mice. Interestingly, whereas MMP-2, MMP-3, and MMP-9 were exclusively expressed by stromal cells in mammary tumors, MMP-13 and MT1-MMP were expressed by transformed epithelial cells in addition to the tumor stroma. To determine whether these MMPs contributed to tumorigenesis, MMTV-Wnt1 mice were crossed with transgenic mice overexpressing a MMP inhibitor—in the mammary gland. In the double MMTV-Wnt1/tissue inhibitor of metalloproteinase-2—natural MMP inhibitor—in the mammary gland. In the double MMTV-Wnt1/tissue inhibitor of metalloproteinases-2 transgenic mice, we observed an increase in tumor latency and a 26.3% reduction in tumor formation. Furthermore, these tumors grew at a slower rate, exhibited an 18% decrease in proliferative rate, and a 12.2% increase in apoptotic rate of the tumor cells in association with a deficit in angiogenesis when compared with tumors from MMTV-Wnt1 mice. Thus, for the first time, the data provides evidence for the active role of MMPs in Wnt1-induced mammary tumorigenesis. (Cancer Res 2006; 66(5): 2691-9)

Introduction

Wnts are a large family of highly conserved secreted glycoproteins implicated in cell-cell signaling in a wide variety of biological processes such as embryonic development and tissue homeostasis of adult organisms controlling cell proliferation, cell differentiation, and cell fate (1, 2). Inactivation of Wnt genes in mouse models have led to abnormal morphogenesis, and mutations of genes involved in the Wnt pathway have been shown to lead to congenital defects in humans (3). Recent studies have shown that Wnt signaling also acts as a regulator of stem cell self-renewal, raising the possibility that this could be subverted in cancer cells (4, 5). Inappropriate activation of the Wnt signaling pathway has been linked to the initiation of specific human cancers (6–8). Wnt1, the initial member of the Wnt family, has transforming abilities on mouse mammary epithelial cells, promoting epithelial to mesenchymal transition (EMT)—an early step in malignant transformation—and cell proliferation (9). Transgenic mice in which Wnt1 is overexpressed under the control of the mouse mammary tumor virus (MMTV) promoter show marked alveolar and ductal hyperplasia in the mammary gland and develop mammary adenocarcinoma within 6 months (10, 11). Tumor formation is stimulated by pregnancies as multiparous mice develop tumors more rapidly than nulliparous mice (10, 12).

Wnt proteins signal through several transduction pathways including the Wnt/β-catenin pathway. In this pathway, binding of Wnt to its transmembrane receptors frizzled (Fz) signals via disheveled (Dsh), which on activation, inhibits the glycogen synthase kinase-3β (GSK-3β). This results in the stabilization of β-catenin and its cytoplasmic accumulation due to inhibition of GSK-3β-dependent phosphorylation and subsequent degradation in the proteasome (13). As a consequence, β-catenin translocates to the nucleus and associates with the lymphoid enhancer factor–T cell factor (LEF-TCF) family of DNA-binding proteins (14). β-Catenin acts as a transactivator for the LEF-TCF transcription factor and promotes the transcription of genes containing functional TCF recognition sites, including c-myc, cyclin D1, and some matrix metalloproteinases (MMPs; refs. 15–18).

MMPs consist of a family of 25 neutral Zn2+-binding proteases that process a large variety of extracellular matrix components and other proteins including cytokines, growth factors, growth factor–binding proteins, and adhesion proteins (19, 20). These proteases have been implicated in a large variety of physiologic and pathologic conditions associated with intense tissue remodeling (21). In cancer, these enzymes have been shown to play a role in multiple steps of tumor progression in particular angiogenesis, local invasion, tumor cell intravasation and extravasation, and the formation of distant metastasis (20, 22). In many cancers, these enzymes are more abundantly expressed by stromal cells than by tumor cells in which they contribute to important changes in the tumor microenvironment (9, 23). In the extracellular milieu, the activity of MMPs is controlled by a family of four natural inhibitors known as tissue inhibitors of MMPs (TIMP). These inhibitors form stable stoichiometric 1:1 complexes with MMPs interfering with their active Zn2+ pocket (24, 25). Overexpression of some of these inhibitors like TIMP-1 in the mammary gland has been previously shown to inhibit branching morphogenesis (26) and to inhibit tumor formation in transgenic mice overexpressing a MMP transgene (27, 28).

The observation that several MMPs are transcriptionally up-regulated by the β-catenin/LEF-TCF transcriptional complex...
and the fact that Wnt1 signaling promotes the transcriptional activity of β-catenin suggest that MMPs may be downstream targets of Wnt signaling, and thus, play a critical role in Wnt1-induced tumorigenesis. To address this question, we analyzed the expression of several MMPs in the mammary gland and tumors of transgenic mice overexpressing Wnt1 under the MMTV promoter, and we examined the effect of TIMP-2 overexpression on mammary tumorigenesis and tumor growth. Our observations indicate that MMPs actively contribute to the tumorigenic activity of Wnt1.

Materials and Methods

Whole mount staining and histology. Mammary glands from wild-type mice and MMTV-Wnt1 littermates were resected and fixed overnight in 4% paraformaldehyde. Whole mount staining was done in alun carmine as described.4 For histologic examination, mammary glands and tumors were embedded in paraffin. Sections were stained with H&E, or with Masson’s trichrome to assess the collagen content in the tumor tissues.

RT-PCR. Total RNA was isolated from mammary glands and mammary tumors using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). To screen for MMP expressions, cDNAs were amplified with the following primer pairs: AAGGATGCGTCGCTTACTGATC as 5′-primer and ACCTGTGGG- TTTTCACAAAG as 3′-primer for mouse MMP-3, TTAGGGATGATACTTATTGCCCAAG as 5′-primer and CGAGAGC- CATGACCTAGAGTGT as 3′-primer for mouse MMP-7, AAGAGGCGCTT- CTGGCACACGCCTT as 5′-primer and GGTGGTATGTGGGACACA- TAGTTG as 3′-primer for mouse MMP-9, GAGATACCCCTTACCATG- CATGAGAAA as 5′-primer and AAAATGGTCCTTGGATACCTGGACG- GG as 3′-primer for mouse MMP-13, GAGATACGGGACTCATTCGAG- G as 5′-primer and CGGTAGTACTTATTGCCCAAG as 3′-primer for mouse MT1-MMP, and ACCCCGAGAGGACTGTGATG as 3′-primer and CACATT- GGAGGTTAGGAAAC as 5′-primer for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each RT-PCR was done in a thermal cycler (MJ Research, Wataertown, MA) for 35 cycles.

In situ hybridization. Paraffin sections of mammary tumors were analyzed by in situ hybridization as described previously (29). Sections were incubated overnight at 50°C in the hybridization buffer containing single-stranded riboprobes for Wnt1 and TIMP-2 transgenes; MMP-3 and MMP-9 (received from Dr. Z. Werb, University of California San Francisco, San Francisco, CA); and MT1-MMP (received from Dr. M. Seiki, University of Tokyo, Tokyo, Japan) radiolabeled with α-35S-UTP (Perkin-Elmer Life Science Products, Boston, MA). For each antisense probe used, a sense probe was also tested as negative control.

Generation of MMTV-TIMP-2 and MMTV-Wnt1/TIMP-2 transgenic mice. Full-length human TIMP-2 cDNA (1,035 bp; ref. 30) was cloned into the EcoRI site of the MMTV-long terminal repeat expression vector pMMTV3 (received from Dr. L.M. Matrisian, Vanderbilt University, Nashville, TN). A purified XhoI fragment was microinjected into B6/CBA fertilized eggs at the National Institute of Child Health and Human Resources Transgenic Mouse Development Facility at the University of Alabama at Birmingham. Three distinct founders were identified by Southern blot analysis. MMTV-Wnt1 mice in C57Bl/6 × SJL background were backcrossed in B6/CBA background for six to eight generations. Backcrossed MMTV-Wnt1 males were then bred with B6/CBA MMTV- TIMP-2 females to generate B6/CBA double transgenic animals. Mice were genotyped by PCR using forward 5′-GACATGTTCTTCTCTCTATAGCC-3′ and reverse 5′-CCACACAGCCATAGGTGTCGC-3′ primers for the Wnt1 transgene (31) and forward 5′-GCAATGCAAGCTAGTGATCAG-3′ and reverse 5′-ATCAACCTAGGTGAACACCCACTTG-3′ primers for the TIMP-2 transgene. Breeding females were monitored biweekly for the appearance of a small palpable tumor nodule in the mammary fat pads. Ages were recorded at the time of tumor discovery, and tumors were measured every 2 to 3 days with a caliper using the formula (H × L ÷ 2). All animal procedures were done in accordance with protocols approved by the Institutional Animal Care Utilization Committee at The Saban Research Institute of Children’s Hospital Los Angeles.

Northern blot. Twenty micrograms of total RNA from mammary glands were separated in a 1% agarose gel containing formaldehyde. Gels were blotted onto Hybond-N filters (Amersham Pharmacia Biotech, Buckinghamshire, England) and hybridizations were carried out according to standard procedures using α-32P-labeled cDNA fragments for human TIMP-2 as probe.

Immunohistochemistry and immunofluorescence. Mice were injected with bromodeoxyuridine (BrdUrd; 100 mg/kg) 10 minutes before sacrifice and tumors were immunostained for BrdUrd as previously described (32), and for terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) using an in situ cell death detection kit (Roche Diagnostics, Corp., Indianapolis, IN) according to the manufacturer’s instructions. The number of TUNEL-positive cells was counted in 5 to 20 fields (according to the tumor size) per tumor. Results were expressed as an average number of TUNEL-positive cells per field. Tumors were evaluated for the presence of microvessels using a rat anti-mouse PECAM/CD31 antibody (PharMingen, San Diego, CA) on frozen sections. A biotinylated goat anti-rat antibody (Vector, Rockford, IL) was used as secondary antibody. Digital pictures of whole tumor sections were analyzed with MetaMorph 6.2 software to determine the endothelial area (EA), the microvessel density (MVD), and the mean vessel size (MVS) on the tumor sections as previously described (33). To analyze the morphology of the blood vessels in tumors derived from MMTV-Wnt1 and MMTV-Wnt1/TIMP-2 mice, paraffin-embedded tumor sections were immunostained with a goat polyclonal anti-PECAM/CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as secondary antibody, as previously described (34).

Zymographies and Western blots. Mammary gland and tumor extracts were obtained after homogenization in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, and 1 mmol/L EDTA (pH 8.0)]. Aliquots of 20 μg each of protein were resolved by SDS-PAGE. For zymography analysis, gels contained 1% gelatin. For reverse gelatin zymograms (detection of TIMP), APMA-activated rabbit fibroblast collagenase was incorporated into the gel prior to polymerization (35). For Western blot analysis, gels were transferred to a nitrocellulose membrane. Blots were blocked overnight at 4°C in TBST/0.05% Tween (TBST) containing 5% dry milk. Blots were incubated with the primary antibody for 1 hour at room temperature, followed by three 5-minute washes in TBST and incubation with a horseradish peroxidase–conjugated secondary antibody. Proteins were detected using the enhanced chemiluminescence reagent (Amersham Life Science, Buckinghamshire, United Kingdom).

Gelatinase assay. Gelatinolytic activity contained in tumors from MMTV-Wnt1 and MMTV-Wnt1/TIMP-2 mice was assessed using the EnzChek gelatinase/collagenase assay kit (Molecular Probes, Inc., Eugene, OR). In brief, aliquots of tumor extracts (100 μg) were incubated in 96-well plates with 20 μg of quenched fluorescein-labeled gelatin in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 5 mmol/L CaCl₂) according to the manufacturer’s instructions. The plates were incubated at room temperature and monitored at different time points with a fluorescence microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). The increase in fluorescence emission over time represents the MMP-mediated gelatinolytic activity, which was inhibited in the presence of 10 μmol/L EDTA.

Statistical analysis. The two-sample t test was used to compare the amount of proliferative nuclei and apoptotic cells in tumors derived from MMTV-Wnt1 or MMTV-Wnt1/TIMP-2 L1 mice; and to compare EA, MVD, MVS measurements on PECAM/CD31-stained sections between tumors from MMTV-Wnt1 mice and tumors from MMTV-Wnt1/TIMP-2 L1 mice. Kaplan-Meier plots and the log-rank test were used to compare the age of

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the mice from the three experimental groups at the time of tumor detection and the probability of being tumor-free. ANOVA was used to compare the gelatinolytic activity in tumors derived from MMTV-Wnt1 or MMTV-Wnt1/TIMP-2 L1 mice.

**Results**

**MMPs are overexpressed in mammary tumors of MMTV-Wnt1 mice.** To initially determine whether MMPs could play a role in Wnt1-induced mammary tumorigenesis, we compared their expression in hyperplastic glands and tumors of MMTV-Wnt1 mice with their expression in the mammary gland of virgin wild-type females by RT-PCR. Hyperplastic mammary glands of Wnt1 transgenic mice exhibited increased branching and ductal hyperplasia as previously reported (10) and tumors consisted of islands of proliferative epithelial cells surrounded by a collagen-rich stroma (Fig. 1A). RT-PCR analysis was done for MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, and MT1-MMP (Fig. 1B). We observed a weak and variable expression of several MMPs like MMP-2, MMP-3, MMP-9, and MT1-MMP, and an absence of expression of MMP-13, in the mammary gland of wild-type mice. In the hyperplastic mammary gland of MMTV-Wnt1 mice, there was a general increase in expression of several of these MMPs, and in particular, MT1-MMP, although the expression varied among samples. However, in mammary adenocarcinoma, there was a definite expression of

![Figure 1. Up-regulation of MMP expression in mammary tumors of MMTV-Wnt1 mice. A, carmine-stained whole mount mammary glands (MGL) from wild-type (WT, a) and MMTV-Wnt1 (Wnt1, b) virgin mice. Representative H&E-stained sections of mammary glands from wild-type (c) and MMTV-Wnt1 (d) virgin mice. Representative Masson trichrome-stained section (blue) of a MMTV-Wnt1 mammary tumor (e) of the collagen content of the stromal tissue (bars, 100 μm). B, RT-PCR analysis screening for the expression of several MMPs in mammary glands from wild-type and MMTV-Wnt1 virgin mice and in MMTV-Wnt1 tumors. GAPDH was used as loading control. C, in situ hybridization on tumor sections showing stromal (asterisks) or epithelial (arrowheads) expression of the indicated MMPs. Black silver granules, hybridization signals seen under bright-field illumination. Results obtained in two independent experiments done on four different tumors for each probe (bars, 50 μm).](image-url)
MMP-13, which was not expressed in normal glands and was very weakly expressed in hyperplastic glands, as well as an increased expression of MMP-3, MMP-9, and MMP-13. We did not observe expression of MMP-7 in any mammary gland or tumor tested (data not shown).

Because this analysis by RT-PCR did not provide information on the source of expression of the different MMPs, a more detailed analysis by in situ hybridization was done on tumor sections (Fig. 1C). This analysis was informative as it revealed a strong expression of MMP-13 and MT1-MMP in epithelial cancer cells, and in the case of MT1-MMP, in particular, at the tumor-stroma interface. Interestingly, the other MMPs, including MMP-2, MMP-3, and MMP-9 were not expressed by epithelial cells, but by cells present in the stromal component of the tumor. No MMP mRNAs were detected in the presence of sense riboprobes (data not shown). The increase in expression of these MMPs in Wnt1-induced mammary adenocarcinoma raises the question of whether these MMPs actively contribute to Wnt1-induced mammary tumor formation.

**MMP/TIMP-2 transgenic mice express functional TIMP-2 in the mammary gland.** To address this question, we generated transgenic mice overexpressing a human TIMP-2 cDNA driven by the MMTV promoter to target the expression of the transgene to the mammary gland (Fig. 2A). We selected TIMP-2 among the four TIMPs because of its solubility and broad spectrum of anti-MMP activity that includes MT1-MMP (24). Three founders were identified by Southern blot analysis (Fig. 2B). DNA analysis from founders 1 and 8 revealed the presence of the 1,035 bp transgene, whereas founder 5 showed a band of much higher molecular weight, likely due to the loss of one of the two EcoRI sites located on each end of the insert. Founders 1 and 8 were used to generate MMTV/TIMP-2 line 1 (L1) and line 8 (L8) transgenic mice, respectively. Offspring were genotyped by PCR using a set of primers designed in the TIMP-2 and in the β-globin gene and located 450 bp apart (Fig. 2C). The expression level of the TIMP-2 transgene in these mice was examined by Northern blot analysis, RNA in situ hybridization, and reverse zymography. Because MMTV activity is influenced by pregnancy, we compared the expression in nulliparous and multiparous mice (Fig. 2D-F). Northern blot analysis of total RNA extracted from the mammary gland of a nulliparous MMTV/TIMP-2 L1 mouse showed a strong hybridization signal for two TIMP-2 mRNAs of 3.8 and 1.8 kb. A weaker 3.8 kb mRNA was detected in RNA from the mammary gland of a MMTV/TIMP-2 L8 mouse. This mRNA was also detected in lung RNA. These mRNAs were absent in liver RNA isolated from a wild-type mouse used as a negative control (Fig. 2D). Consistently, in situ hybridization analysis revealed the presence of the human TIMP-2 mRNA in ductal epithelial cells of nulliparous and multiparous mammary glands obtained from MMTV/TIMP-2 L1 mice and a weaker expression in the nulliparous mammary gland of MMTV/TIMP-2 L8 mice. The presence of the transgenic mRNA increased in multiparous mice. An absence of signal using a sense probe confirmed the specificity of the observed signals (Fig. 2E). There was a good correlation between the presence of human TIMP-2 mRNA in the mammary glands and the presence of a functional TIMP-2 protein as shown by reverse zymography analysis done on tissue extracts (Fig. 2F). The effect of TIMP-2 on mammary gland development was also examined. Whole mount stainings showed a delay in ductal invasion of the mammary fat pad in prepubertal MMTV-TIMP-2 L1 mice (day 40) in which the average length of the main mammary ducts was 2.7 ± 0.88 mm compared with 5.3 ± 0.74 mm in wild-type littersmates (P = 0.0002). This difference in

**Figure 2.** Generation of MMTV-TIMP-2 transgenic mice and expression of the TIMP-2 transgene. A, representation of the MMTV-TIMP-2 transgene construct. Positions of the forward (f) and reverse (r) primers used to genotype the mice by PCR (black arrows). B, Southern blot analysis of EcoRI-digested tail DNA from three different founders (F1, F5, and F8). C, PCR analysis on DNA obtained from offspring of MMTV/TIMP-2 founders 1 and 8. D, Northern blot analysis of total RNA isolated from mammary gland (MGL) and lung of virgin transgenic mice using a human TIMP-2 cDNA probe. RNA isolated from liver tissue of a wild-type mouse was used as negative control (CTL). E, localization of TIMP-2 transgene expression by in situ hybridization using a human TIMP-2 antisense (AS) probe in mammary glands from transgenic nulliparous mice from lines 1 and 8 (left), and in lactating mammary gland from line 1 (top right). A gland from transgenic virgin mouse from line 1 hybridized with a TIMP-2 sense (S) probe is shown as negative control (bottom right). Bars, 200 and 25 μm (insets). F, reverse gelatin zymography of mammary gland extracts from nulliparous (n) and multiparous (m) wild-type (WT), MMTV/TIMP-2 line 1, and MMTV/TIMP-2 line 8 mice. Recombinant TIMP-2 was used as positive control (CTL).
ductal invasion between MMTV-TIMP-2 L1 and wild-type mice was however not observed in postpubertal (day 60) mice (12.3 ± 2.91 mm in MMTV-TIMP-2 L1 versus 11.2 ± 1.86 mm in wild-type mice). Overexpression of TIMP-2 in the mammary gland had no effect on branching (data not shown).

Overexpression of TIMP-2 in the mammary gland of MMTV-Wnt1 mice inhibits tumor formation and growth. We then asked the question whether overexpression of TIMP-2 in the mammary gland could alter Wnt1-induced mammary tumor formation. Double transgenic MMTV-Wnt1/TIMP-2 L1 and MMTV-Wnt1/TIMP-2 L8 female mice and their littermate controls (MMTV-Wnt1 and MMTV-TIMP-2) were paired for breeding and monitored for the development of mammary tumors (Table 1; Fig. 3A). Among all the multiparous MMTV-TIMP-2 mice generated (>50), none developed tumors over 18 months. Among multiparous MMTV-Wnt1 mice (n = 25), all mice developed a tumor within 37 weeks, with a median time for tumor detection of 15.8 weeks. In MMTV-Wnt1/TIMP-2 L8 mice, which express little or no TIMP-2 (n = 15), all multiparous mice developed a tumor within 29 weeks, with a median time for tumor detection of 14.4 weeks which is not statistically different from MMTV-Wnt1 mice (P = 0.462). In contrast, in multiparous MMTV-Wnt1/TIMP-2 L1 mice, which overexpress TIMP-2 (n = 19), 5 mice never developed a tumor and the 14 other mice developed tumors with a median time for tumor detection of 25.2 weeks, which is significantly longer than the other groups (P = 0.0028). We also observed a significant difference in the growth rate of mammary tumors between the three groups of transgenic mice (Fig. 3B–D). Whereas tumors in MMTV-Wnt1 mice typically grew at an accelerated rate, forming large tumors of 2,000 mm³ or more within 20 days (Fig. 3B), many tumors in MMTV-Wnt1/TIMP-2 L1 transgenic mice grew at a slower rate and several remained dormant for 45 to 98 days, never forming a tumor larger than 1,000 mm³ (Fig. 3C). The growth pattern of tumors in MMTV-Wnt1/TIMP-2 L8 mice was intermediate, with a combination of faster and slower growing tumors (Fig. 3D).

Wnt1 and TIMP-2 transgenes are expressed by tumor cells and tumor derived from MMTV-Wnt1/TIMP-2 L1 mice have decreased gelatinolytic activity. Analysis of the expression of MMPs in the tumors of the three groups of transgenic mice was done on tumor extracts using gelatin zymographies (Fig. 4A) and revealed the presence of MMP-9, MMP-2, and a 75 kDa serine protease with gelatinolytic activity, likely plasmin, but there was no difference in expression among the three groups. No consistent change in the relative amounts of pro-MMP-2 and active MMP-2 was observed among the tumors derived from the three groups either. To examine the expression of TIMP-2 in these tumors, and to determine whether the TIMP-2 transgene in double transgenic mice resulted in the expression of a functional TIMP-2, we examined tissue extracts from several tumors in the three groups by gelatin reverse zymography and Western blotting (Fig. 4B). The absence of TIMP-2 was observed in tumors derived from

<table>
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<th>Genotype</th>
<th>No. Mice</th>
<th>Time to detect tumor (wk) Median (95% confidence interval)</th>
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<tr>
<td>Wnt1/TIMP-2 L1</td>
<td>19</td>
<td>25.2 (21.4-32.5)</td>
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<tr>
<td>Wnt1/TIMP-2 L8</td>
<td>15</td>
<td>14.4 (12.2-16.8)</td>
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<td>Wnt1</td>
<td>25</td>
<td>15.8 (12.4-16.8)</td>
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NOTE: Overall log-rank test between the three genotype groups and the time to develop tumor among these 59 mice (P = 0.0028). Log-rank pairwise comparisons between the three groups were: WT2L1 versus WT2L8 (P = 0.0005), WT2L1 versus Wnt1 (P = 0.0001), WT2L8 versus Wnt1 (P = 0.462).

Table 1. Occurrence of mammary tumors in MMTV-Wnt1 and MMTV-Wnt1/TIMP-2 transgenic mice.

![Figure 3](https://example.com/figure3.png)  
Mammary tumor formation and tumor growth in MMTV-Wnt1 and MMTV-Wnt1/TIMP-2 mice. A, Kaplan-Meier analysis of the percentage of tumor-free mice over time. Vertical lines, the age of mice that were alive and tumor-free at the end of our study. B–D, volume of individual tumors over time in MMTV-Wnt1 (B), MMTV-Wnt1/TIMP-2 L1 (C), and MMTV-Wnt1/TIMP-2 L8 transgenic mice (D). Numbers mark the samples selected for further analysis (see Fig. 4B).
MMTV-Wnt1 and MMTV-Wnt1/TIMP-2 L8 mice, whereas TIMP-2 was expressed in all but one tumor derived from MMTV-Wnt1/TIMP-2 L1 mice (tumor 1). Interestingly, this tumor, which was not expressing TIMP-2, grew at an accelerated rate (Figs. 3C and 4B, tumor 1). We also used in situ hybridization to verify the expression of MMPs and the TIMP-2 transgene in these tumors (Fig. 4C). Consistent with the zymography, in situ hybridization on tumor sections from the double transgenic mice showed no difference in the expression pattern of the several MMPs tested in comparison to the MMTV-Wnt1 tumors (data not shown), thus indicating that changes in growth among the three groups were not due to changes in MMP expression. Morphologic analysis on tumor sections of all groups using H&E and Masson’s trichrome stainings did not show any gross differences in the overall architecture of the tumors in the three groups (data not shown). As anticipated, tumors derived from MMTV-Wnt1/TIMP-2 L1 mice showed a very strong TIMP-2 mRNA expression in the transformed epithelial cells (Fig. 4C, a), which also expressed high levels of Wnt1 mRNA (Fig. 4C, b), whereas MMTV-Wnt1 tumors had no expression of the TIMP-2 transgene (Fig. 4C, c). To determine whether the expression of the TIMP-2 transgene resulted in a corresponding decrease in MMP activity, we tested tumor extracts for their total gelatinolytic activity in a quenched-fluorescent gelatin substrate assay. As shown in Fig. 4D, the gelatinolytic activity in MMTV-Wnt1/TIMP-2 L1 tumor extracts measured at 1, 2, 18, and 90 hours was significantly lower when compared with extracts from MMTV-Wnt1 tumors (P = 0.001). There was, however, one exception in the MMTV-Wnt1/TIMP-2 L1 group with tumor 1, which showed no expression of TIMP-2 by zymography and Western blot. The gelatinolytic activity in the extract from this tumor was of similar level (197 of fluorescence arbitrary units at 90 hours) to the ones observed in the non-TIMP2 expressing MMTV-Wnt1 tumors.

**Overexpression of TIMP-2 in Wnt1-induced mammary tumors decreases tumor cell proliferation and increases apoptosis.** Because the tumors harvested from MMTV-Wnt1/TIMP-2 L1 transgenic mice tended to be smaller than the tumors from MMTV-Wnt1 mice, we examined the proliferative and apoptotic rate of the tumor cells by analysis of BrdUrd incorporation and by TUNEL assay on tumor sections (Fig. 5). This analysis indicated a statistically significant lower percentage of BrdUrd-positive cells in MMTV-Wnt1 tumors overexpressing TIMP-2 (62.4 ± 7.9%) when compared with MMTV-Wnt1 tumors (80.4 ± 2.1%; P = 0.005). There was also a higher rate of apoptosis in tumors derived from MMTV-Wnt1/TIMP-2 L1 mice when compared with tumors derived from MMTV-Wnt1 mice (17.9 ± 12.2% versus 5.7 ± 4.4%; P = 0.018). The data indicate that the slower growth rate observed in tumors obtained from MMTV-Wnt1/TIMP-2 L1 mice is the result of a combination of decreased proliferation and increased apoptosis.

**Overexpression of TIMP-2 in Wnt1-induced mammary tumors inhibits angiogenesis.** Considering the role of MMPs in angiogenesis, we postulated that these changes in tumor cell proliferation and apoptosis could be in part secondary to changes in tumor vascularization. To test this possibility, we examined the presence of blood vessels in mammary tumors in the MMTV-Wnt1 and the MMTV-Wnt1/TIMP-2 L1 groups by immunohistochemistry for CD31/PECAM (Fig. 6). Although there was no difference in MVD between the two groups, there was a statistically significant difference in MVS and EA with smaller vessels in tumors from MMTV-Wnt1/TIMP-2 L1 mice compared with tumors obtained in MMTV-Wnt1 mice (mean MVS of 84.35 ± 38.05 pixels versus 35.43 ± 7.9 pixels; P = 0.0015). As a result, the EA of tumors from MMTV-Wnt1/TIMP-2 L1 mice was significantly smaller than the EA of tumors from MMTV-Wnt1 mice (6.9 ± 1.64% of tumor area versus 13.14 ± 4.66%; P = 0.0011).

**Discussion**

Our experiments in double transgenic MMTV-Wnt1/TIMP-2 mice point to a contributory role for MMPs in Wnt1-induced mammary tumor formation at an earlier as well as a later stage of tumor progression. We have tested a single transgenic line for its...
effect on tumorigenesis, which raises the possibility that the phenomenon observed could be the result of an effect of the integration site. We consider this unlikely for the following two reasons. First, in the MMTV-Wnt1/TIMP-2 L1 group, there was some mosaicism in TIMP-2 expression and one mouse that did not express TIMP-2 (mouse 1) developed a mammary tumor that grew at a rate similar to those in the MMTV-Wnt1 group. Second, in the MMTV-Wnt1/TIMP-2 L8 group, which did not overexpress TIMP-2, mammary tumors grew at a faster rate than in the L1 group. Our data showing a strong inverse correlation between TIMP-2 expression and tumor growth rate favors an effect of MMP inhibition on Wnt1-induced tumor formation. Interestingly, it has recently been reported that MMP inhibition could block Wnt1-mediated ErbB1 and Erk activation in cultured mammary epithelial cells, which further supports that conclusion (36). We first observed a significant delay in tumor formation in the double transgenic MMTV-Wnt1/TIMP-2 L1 group and 5 mice out of 19 in that group never developed mammary tumors. This observation suggests that the inhibition of MMP activity in the mammary gland has an inhibitory effect on Wnt1-induced malignant transformation. Previous studies using other tumor models have shown that MMPs could play a role in the early stages of cancer. For example, overexpression of MMP-3 in the mammary gland of transgenic mice results in the formation of mammary tumors, and this effect can be quenched by crossing mice with TIMP-1 transgenic mice or in the presence of exogenous TIMP-1 (27). In a colon cancer model, mice carrying a mutation in the adenomatous polyposis coli (Apc) genes (Min mice) develop spontaneous adenoma and intestinal tumors that overexpress MMP-7. The number of these tumors and their size is decreased upon treatment of mice with a synthetic MMP inhibitor (BB94) and in Min mice lacking MMP-7 (37). The apc protein is part of the complex that targets β-catenin to phosphorylation by GSK-3β and degradation by proteasome. It is also downstream of Wnt1, which through interaction with its receptor Frizzled, inhibits GSK-3β activity. Like the Apc mutation, Wnt1 signaling increases β-catenin transcriptional activity (13). Although we did not observe expression of MMP-7 in Wnt1 hyperplastic mammary glands and Wnt1 tumors, we observed an increased expression of MT1-MMP and MMP-13 in transformed epithelial cells. MT1-MMP has been shown to be up-regulated by the β-catenin/TCF complex and confers tumorigenicity when transfected in nonmalignant epithelial cells (18, 38). Whether MMP-13 is similarly up-regulated by this pathway has not yet been reported. The observation that certain MMPs, like MMP-7 and MMP-3, could cleave E-cadherin (39) suggests a possible role for these MMPs in EMT. It is thus possible that the inhibitory effect of TIMP-2 on Wnt1-induced tumor formation may have involved a specific inhibition of EMT. Another and not mutually exclusive possibility is that the inhibitory effect of TIMP-2 on angiogenesis may have created a state of dormancy in tumors that prevented them from becoming detectable.

By demonstrating that tumors in MMTV-Wnt1/TIMP-2 L1 double transgenic mice grow at a slower rate, have a decreased proliferative rate, and a decreased level of angiogenesis, we also provide evidence that MMPs contribute to the later stages of tumor progression and in particular angiogenesis in the Wnt1 mammary tumor progression. It is likely that the slow growth rate and dormancy observed in these tumors is a direct effect of inhibition of angiogenesis. MMPs have multiple effects on angiogenesis in vitro and in vivo, which include degradation of extracellular matrix proteins, release and activation of matrix-bound angiogenic factors, solubilization of membrane-bound growth factors (shed-dase activity), exposure of matrikryptic ligands, modulation of vasculogenesis, and stimulation of vessel maturation (40). Although we cannot conclude which MMPs play a more specific role, several MMPs overexpressed in Wnt1 tumors contribute to angiogenesis. MT1-MMP plays a positive role in angiogenesis in multiple xenotransplanted tumor models and is specifically inhibited by TIMP-2 and not TIMP-1 (41). MMP-9, expressed by host cells in many tumors, acts as an angiogenic switch in mice prone to develop squamous cell carcinoma and pancreatic adenocarcinoma, and promotes the recruitment of pericytes in neuroblastoma tumors (32, 42, 43). Although our data clearly documents a decrease in proteolytic activity in tumor extracts from double transgenic mice, we cannot rule out the possibility that the TIMP-2-mediated inhibition of angiogenesis may be independent of MMP inhibition. As previously reported, TIMP-2 can abrogate angiogenic factor–induced proliferation of endothelial cells in vitro and in vivo independently of MMP inhibition (44).

It is interesting to notice that in Wnt1 mammary tumors, stromal cells actively contribute to the expression of certain MMPs as documented by in situ hybridization. Whereas some MMPs like MT1-MMP and MMP-13 were also expressed by epithelial cells, others like MMP-2, MMP-3, and MMP-9 were primarily, if not exclusively, expressed by stromal cells. Such observations are consistent with what has been reported in human breast cancer (45). For example, in human breast cancer, MMP-9 is expressed by vascular pericytes (46) and stromal cells in addition to tumor cells.
(47, 48), and when cocultured with breast cancer cells, normal fibroblasts secrete MMP-9 (49). Inflammatory cells like mast cells, monocyte-macrophages, and tumor-infiltrating leukocytes have also been shown to be the major source of MMP-9 in tumors (50). Similarly, MMP-2 and MMP-3 in human breast carcinoma have been shown to be expressed by peritumoral stromal cells in addition to tumor cells (51–53). In our study, MMP-13 and MT1-MMP were identified as candidates for involvement in Wnt1-induced tumorigenesis because they are predominantly expressed by Wnt1-expressing epithelial cells. MMP-2, MMP-3, and MMP-9, which were predominantly expressed in the tumor stroma, may also have a role in stimulating endothelial cell transformation and growth, especially during the early stages of tumorigenesis.

The in vivo data presented here suggest that MMPs have an active role in Wnt1-induced mammary tumorigenesis both in the early stages of cell transformation, as shown by a delay in palpable tumor formation, and lower tumor incidence in the double transgenic mice, as well as in later stages of tumor formation and progression as shown by slower tumor growth and inhibited angiogenesis. These observations are important considering the increasingly recognized critical role played by Wnt1 signaling in cancer.

Acknowledgments

Received 8/23/2005; revised 11/15/2005; accepted 12/2/2005.

Grant support: NIH grant (Y.A. DeClerck), and the Susan G. Komen Breast Cancer Foundation (DISS2000 523), and the T.J. Martell Foundation for Leukemia, Cancer and AIDS Research (G.M. Shackelford).

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We thank Drs. David R. Shalinsky for providing the AG3340; Hongjun Peng for technical assistance with immunohistochemistry; George McNamara and the CHLA Saban Research Institute, Congressman Julian Dixon Cellular Image Core; JunQing Qian for scientific input; and Jackie Rosenberg for her help in formatting the manuscript.

References

26. Fata J, Eeco RJ, Moorehead RA, Martin DC, Kokha R. Tim-1 is important for epithelial proliferation and
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