Cystatin M: A Novel Candidate Tumor Suppressor for Breast Cancer

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ABSTRACT

The contribution of pericellular proteolysis to tumor progression is well documented. To better understand protease biology and facilitate clinical translation, specific proteolytic systems need to be better defined. In particular, the precise role of endogenous protease inhibitors still needs to be deciphered. We reported previously that cystatin M, a potent endogenous inhibitor of lysosomal cysteine proteases, significantly suppressed in vitro cell proliferation, migration, and Matrigel invasion. Here, we show that scid mice orthotopically implanted with breast cancer cells expressing cystatin M show significantly delayed primary tumor growth and lower metastatic burden in the lungs and liver when compared with mice implanted with mock controls. The incidence of metastasis, however, appeared to be unaltered between the cystatin M group and the control group. Experimental metastasis assays suggest that cystatin M suppressed tumor cell proliferation at the secondary site. By using laser capture microdissection and quantitative reverse transcription-polymerase chain reaction, we found consistent expression of cystatin M in normal human breast epithelial cells, whereas expression was decreased by 86% in invasive ductal carcinoma (IDC) cells of stage I to IV patients. Complete loss of expression of cystatin M was observed in two of three IDCs from stage IV patients. Immunohistochemical studies confirmed that expression of cystatin M in IDCs was partially or completely lost. We propose cystatin M as a novel candidate tumor suppressor gene for breast cancer.

INTRODUCTION

Cystatins are endogenous inhibitors of endosomal/lysosomal cysteine proteases (1, 2), whose impaired regulation of expression and activity has been implicated in cancer progression (3, 4). Cystatins control catalytic function of target proteases by forming reversible, high-affinity complexes (5, 6). In addition to their ability to act as cysteine protease inhibitors, cystatins have been shown to exhibit a plethora of other functions: they can stimulate nitric oxide release from interferon-γ-primed macrophages (7); modulate interleukin production and T-cell proliferation (8, 9); stimulate DNA synthesis and proliferation of fibroblasts, mesangial cells, and neuronal stem cells (10–12); inhibit respiratory burst and phagocytosis in neutrophils (13); inhibit viral replication (14); and inhibit bone resorption (15). Cystatin M [also cloned as cystatin E (16)] is a low molecular mass protein that is secreted in both a glycosylated (17 kDa) and an unglycosylated (14 kDa) form (16, 17). In a study of established human breast epithelial cell lines with various tumorigenic and metastatic potentials, 10 of 12 carcinoma cell lines showed little or no cystatin M mRNA expression, whereas normal and premalignant cells expressed abundant levels of the transcript (17). These in vitro data suggested that loss of expression of cystatin M might be associated with the progression of human breast cancer.

In a previous study, we stably transfected the highly tumorigenic and metastatic human breast cancer cell line MDA-MB-435S with a cystatin M expression vector and studied the effects of cystatin M expression on the malignant properties of the cells. In this system, cystatin M was found to suppress in vitro cell proliferation, migration, matrix invasion, and tumor-endothelial cell adhesion (18). The aim of the present study was to explore the in vivo function of cystatin M and test whether this cell-secreted product had bona fide tumor- and/or metastasis-suppressing function. Using the previously established transfectants, we show for the first time that cystatin M expression delayed tumor growth in the mammary fat pads (MFPs) and lowered the spontaneous metastatic burden in the lungs and liver of scid mice. Consistent with the data on cell lines, we also show that both cystatin M mRNA and protein expression are partially or completely lost in human breast cancer specimens. Thus, the effect of cystatin M expression on tumor cell proliferation in vitro and tumor growth in vivo, together with loss of cystatin M expression in tumor cells, classify cystatin M as a novel candidate tumor suppressor for breast cancer.

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma MDA-MB-435S cells were stably transfected with either an empty vector or a cystatin M expression vector and characterized as described previously (18).

Animals. Outbred, female ICR scid (severe combined immunodeficient) mice were used (Taconic, Germantown, NY). All mice were handled according to standard procedures and according to an animal protocol approved previously by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Human Breast Tissues. This study included 15 archival human breast tissues. Three specimens were diagnosed as normal breast tissue; one additional normal specimen was obtained from breast reduction mammoplasty; three specimens each were from stage I, II, and IV patients with invasive ductal carcinoma (IDC); and two specimens were from stage III patients with IDC. The clinical and pathological information regarding tumor size, lymph node, and remote metastasis status [tumor-node-metastasis (TNM)] was based on the original clinical reports. Handling of human tissues and protection of patient privacy followed strict guidelines of the Louisiana State University Health Sciences Center Institutional Review Board.

Immunoblot Analysis. Serum-free media conditioned by cell clones (parental, mock-1, mock-2, cysM-12, cysM-13, and cysM-17) and the corresponding cell lysates were prepared and analyzed by SDS-PAGE, followed by immunoblotting as described previously (18). Membranes were probed with rabbit polyclonal antibodies raised against human cystatin M and monoclonal antibodies against β-actin (clone C4; ICN, Aurora, OH) and α-tubulin (clone B-5-1-2; Sigma, St. Louis, MO). For detection, horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) and West Pico SuperSignal chemiluminescence (Pierce, Rockford, IL) were used in conjunction with Kodak BioMax MR films (Eastman Kodak Co., Rochester, NY).

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NOTE: R. Shridhar and Q. Dai contributed equally to this work.

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Analysis of Primary Tumor Growth after Orthotopic Injection. Transfected MDA-MB-435S cell clones were injected into MFPs of female scid mice. A total of 10 and 11 mice received 200 μL of sub-nipple injections of 1.0 × 10^6 cells/mL PBS of cysM-13 and mock-1, respectively. Starting at day 14 after implantation, the two perpendicular diameters A and B (where A ≈ B) of each palpable tumor were measured every 4 to 5 days using a metric caliper (FACOM Tools, Chicago, IL). The tumor volume (expressed in mm^3) was calculated using the following formula: V = A × B^2/2. At day 55, when there was no significant difference in mean tumor volume between the mock-transfected group and the cystatin M-treated group, mice were sacrificed. Tumors from the MFP areas were excised and weighed. Lungs and livers were removed and stored immediately in liquid nitrogen for further analysis.

Experimental Lung Metastasis Assay. For this assay, 0.5 × 10^6 cells of the parental (nontransfected), cystatin M-transfected, and mock-transfected clones were injected into the blood circulation via the tail vein. Mice were sacrificed after 5 and 11 weeks. Lungs and livers were removed and stored immediately in liquid nitrogen for further analysis.

DNA Extraction and Quantification. Genomic DNA was extracted from a piece of liver and the whole right lung of scid mice and from human MDA-MB-435S cells using a DNA extraction kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. DNA concentration and purity were determined fluorometrically using the PicoGreen double-stranded DNA Quantification Kit (Molecular Probes, Eugene, OR).

Determination of the Number of Metastatic Tumor Cells in the Liver and Lungs. Detection of human breast cancer cells in mouse lung and liver was based on the specific polymerase chain reaction (PCR) amplification of a 480-bp α-satellite (SAT) DNA (19, 20), which is present only on human chromosomes, in particular, on chromosome 17. Amplification of a 489-bp fragment from the genes of human and mouse 18S ribosomal DNA (18S) served as an internal control for equal sampling of total genomic DNA (21). The oligonucleotide sequences of the PCR primers were as follows: SAT forward primer, 5'-GGGATTATCTGCGTACTAAACAG-3'; SAT reverse primer, 5'-AAAGCTTACATGGATGACGTAG-3'; reverse primer, 5'-GGAACAGAAGATGCTGAGG-3'; and 18S reverse primer, 5'-GGA-CATCTAAGGCGTAC-3'.

Conventional Polymerase Chain Reaction and Gel Electrophoresis. The PCR reaction mix consisted of iQ Supermix (Bio-Rad, Hercules, CA), selected primers (200 mmol/L), and 0.5 μg of genomic DNA as template in a final volume of 50 μL. The conditions for PCR amplification were as follows: hot start at 95°C for 3 minutes; followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and terminated by extension for 7 minutes at 72°C and hold at 4°C. PCR products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The thermal cycling conditions for real-time PCR were as described above for conventional PCR, except that iQ SYBR Green Supermix (Bio-Rad) and 40 cycles were used. Fluorescence intensity of the SYBR Green-labeled PCR products was measured continuously during the reaction using the iCycler iQ Optical Detection System (Bio-Rad). The cycle threshold number (Ct) was determined for each amplification reaction in the exponential phase. The measured Ct values were plotted against the logarithm (log 10 ) of the number of human cells. In addition, to the electrophoretic analysis of PCR products (see above), a melting curve was performed on completion of amplification to monitor the quality of primers and amplification reactions. To calculate the actual number of metastatic cells present in the lung, a calibration curve was generated as follows. Genomic DNA isolated from a known number of human MDA-MB-435S cells was mixed with total genomic DNA extracted from the lungs of healthy, tumor-free scid mice. PCR DNA extractions were then performed with 0.5 μg of total DNA as described above, and ΔCt values were plotted against the logarithm (log_10) of the number of human cells. All experiments were performed in triplicate, and results represent the mean ± SE.

Laser Capture Microdissection and Total RNA Extraction. Frozen archival human breast tissues (see above) were thawed at room temperature and immediately embedded into TissueTek OCT medium (VWR Scientific Products Corp., San Diego, CA). Semi-thin sections (8 μm) were prepared in a Leica CM3050S cryostat and mounted onto laser capture microdissection (LCM) slides, which were provided with the Histogene LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA). Slides were processed according to the kit protocol. After they had been fixed, stained, and air dried, sections were laser microdissected with a PicCell-II LCM system (Arcturus). About 1,500 to 2,000 breast epithelial cells were laser-captured from each tissue specimen. According to the manufacturer’s instructions, total RNA was extracted using the PicoPure RNA Isolation Kit (Arcturus) and contaminating DNA was eliminated using RNase-free DNase I (Qiagen, Valencia, CA).

Reverse Transcription Followed by Conventional or Real-Time Polymerase Chain Reaction. For analysis of cystatin M mRNA expression, total RNA isolated from microdissected breast epithelial cells of different histopathologies was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). One tenth of the reaction volume (2 μL) was immediately used for conventional or real-time PCR in a final volume of 50 μL as described above. Template was replaced by nuclease-free water in nontemplate controls, and reverse transcription reactions performed in the absence of iScript served as reverse transcription-negative controls. The oligonucleotide sequences of the cystatin M primers were as follows: CM forward primer, 5'-GTACTTCCGTAGGCGATTG-3'; and CM reverse primer, 5'-TAGGAGCCTGGAGGAGTTGC-3'. The cycling conditions were as follows: hot start at 95°C for 3 minutes; followed by 45 cycles at 93°C for 30 seconds, 53°C for 30 seconds, and 72°C for 55 seconds; and terminated by extension for 7 minutes at 72°C. Electrophoresis and detection of PCR products were as described above. For quantitative comparison, the relative abundance of the cystatin M mRNA was normalized against that of the 18S rRNA as described before. Reverse transcription-polymerase chain reaction reactions were performed in triplicate. The relative mean cystatin M mRNA expression level in breast epithelial cells of normal patients (n = 4) was defined as 100%. The levels of cystatin M mRNA in IDC cells of stage I to IV patients were determined as 2^-ΔΔCt × 100%, where ΔΔCt = ΔCt_tumor – ΔCt_normal for stage i = stage I to IV.

Immunohistochemistry. Archival paraffin-embedded human breast tissues (the samples used for LCM) were cut in 4- to 5-μm sections, deparaffinized, and rehydrated according to routine techniques. Sections were treated for antigen retrieval, preincubated in 0.3% H2 O2, and then incubated in 20% normal horse serum. Polyclonal rabbit anti-cystatin M antibodies (see above) or the corresponding rabbit preimmune serum were both diluted 1:500 and incubated with sections for 30 minutes. Immunohistochemical staining proceeded according to the protocol provided with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA).

Statistical Analyses. The Kolmogorov-Smirnov test was used to evaluate goodness of fit before performing parametric tests. Student’s t test was performed for comparisons of primary tumor growth, spontaneous metastatic burden, and cystatin M mRNA expression levels in microdissected human breast epithelial cells. One-way analysis of variance analysis followed by Fisher’s Least Significant Difference post hoc test was used for comparison of log numbers of experimental lung colony-forming cells. Differences with P < 0.05 were considered statistically significant. All results are expressed as mean ± SE.

RESULTS

Effect of Cystatin M Expression on Tumor Growth. In previous studies, we demonstrated that cystatin M suppresses in vitro proliferation of human breast cancer MDA-MB-435S cells (18). To test the in vivo effect of cystatin M expression, we selected one cystatin M-overexpressing clone (Fig. 1A, CysM 13) and one mock-transfected clone, the latter of which does not express cystatin M (Fig. 1A, Mock 1). These clones were orthotopically injected into MFPs of scid mice. As shown in Fig. 1B, mice implanted with the cystatin M-expressing clone had significantly (P = 0.01) delayed tumor growth and developed much smaller tumors during the first 6 weeks after implantation when compared with mock-transfected cells. At day 45 after implantation, the tumor size in the cystatin M group was still significantly smaller when compared with mock-transfected cells. At day 55 after implantation, no statis-
Fig. 1. Effect of cystatin M expression on primary tumor growth. A, comparison of cystatin M expression in MDA-MB-435S cell lines. Several clones were analyzed by immunoblotting for constitutive secretion of cystatin M (top panel). Tubulin and actin in the corresponding cell lysates (bottom panel) were used as a control of equal cell numbers for the preparation of cell-conditioned secretions. No cystatin M was detected in the various cell lysates (data not shown). The clones mock-1 and cysM-13 were used in all subsequent studies. B, comparison of primary tumor growth between scid mice orthotopically implanted with cystatin M and mock-transfected MDA-MB-435S cells. Ten or 11 mice received MFP injections of $1.0 \times 10^7$ cystatin M- or mock-transfected cells, respectively, in 200 µL of PBS. Every 4 to 5 days, the tumor volume ($V = \frac{A \times B^2}{2}$, where $A$ is the length of the tumor, and $B$ is the width of the tumor) was measured using calipers. Compared with the tumors of the mock group, those of the cystatin M group were significantly smaller up to day 45 as marked by asterisks ($P = 0.003$, 0.01, 0.002, 0.001, 0.007, 0.009, 0.036 for days 14 to 45). Beyond day 45, there was no difference in tumor volumes. C, at the day of sacrifice (day 14), there was no difference in the mean wet weight of tumors. D, illustration of a representative MFP tumor at day 55. Primary tumors were carefully examined to confirm proper implantation in the MFPs and surgically removed for further analysis.

Quantification of Human Cells in Mouse Liver and Lungs. Human breast carcinoma MDA-MB-435S cells have been shown previously to spontaneously metastasize to the lungs and liver after orthotopic implantation into MFPs (22). To assess the effect of cystatin M expression on spontaneous metastasis, we chose to perform the comparison of the mock group and the cystatin M group at a time point when the animals have a similar primary tumor burden. At day 55, all mice were sacrificed, and the tumors were surgically exposed at the primary implantation site and, together with the surrounding tissue, examined carefully. As shown in Fig. 1D, the mammary tumors were firmly attached to and surrounded by the yellowish MFP tissue. The tumors did not attach to neighboring tissues (skin and muscle), and the peritoneum was intact and smooth. The primary tumors were carefully removed and weighed. There was no statistical difference between the mean tumor weights of the cystatin M group and the mock group (Fig. 1C). Thus, at day 55, we harvested the liver and lungs for the study of spontaneous metastases.

Accurate and quantitative determination of the metastatic load in a given tissue, we used a previously published PCR approach (20). In Fig. 2, DNA amounts that corresponded to known numbers of human MDA-MB-435S cells were mixed with a constant amount of mouse lung DNA (equivalent to 1 million mouse lung cells). Fig. 2A shows that both amplification reactions were clean and yielded a single PCR product of the expected size, i.e., 480 bp for SAT17A and 489 bp for 18S. A clear dose-response was observed after 35 cycles of conventional PCR followed by agarose gel electrophoresis (Fig. 2A). Mouse lung DNA in the absence of any human DNA gave no detectable SAT band but showed a clear 18S band (Fig. 2A, Lane M). Conversely, 100% of human DNA gave clear bands for both SAT and 18S (Fig. 2A, Lane H). Conventional PCR allowed the detection of as little as 1 human MDA-MB-435S cell in 1 million mouse lung cells. To quantitatively determine the number of human cells present in a mouse lung, we established a calibration curve using real-time PCR. This way, as few as 20 human cells can be detected in a whole mouse lung (Fig. 2B). The inset in Fig. 2B shows real-time amplification of the 18S control over the whole range of calibrations. The tight curves of the 18S amplifications and the low coefficient of variation of the Ct numbers suggest equal sampling of total DNA ($n = 13$; mean Ct $\pm$ SD $= 12.7 \pm 0.5$; coefficient of variation $= 3.9$). Nonetheless, we normalized Ct values of SAT against 18S ($\Delta$Ct $= CT_{SAT} - CT_{18S}$) to calculate the relative abundance of SAT DNA versus 18S internal control. When $\Delta$Ct is plotted against the logarithm of the number of human cells, a calibration curve is obtained that fits a straight line (Fig. 2C and D, $R^2 = 0.9911$). From this linear calibration curve, an equation can be drawn that gives the actual number of cells ($N$) from a measured $\Delta$Ct ($Log_{10}N = -0.2961 \times \Delta$Ct $+5.5328$). SAT DNA was readily detected and could be quantified over a wide range extending from 20 to $>200,000$ cells per lung. Liver metastases were quantified in a similar way.

Effect of Cystatin M Expression on Spontaneous Lung and Liver Metastasis. With the quantitative method described above, we were able to deduce the actual number of human MDA-MB-435S cells that had spontaneously metastasized to the mouse lungs (Fig. 3A). In parallel to real-time PCR, we performed conventional PCR using 20 amplification cycles followed by electrophoretic analysis of the PCR products (Fig. 3B). As can be seen from Fig. 3A and B, there was a very good correlation between the actual number of metastatic cells and the fluorescence intensity of the SAT band. From statistical analysis of the $\Delta$Ct values, the cystatin M group was found to exhibit 2.4-fold fewer metastatic cells in the lungs than the mock group (Fig. 3C; $P < 0.05$). After converting $\Delta$Ct values into logarithm of actual number of cells, cystatin M expression in MDA-MB-435S cells was found to decrease spontaneous metastatic burden in the lungs by 57% (Fig. 3D). Similar to the lungs, the metastatic burden in the liver was also significantly reduced in the cystatin M group (Fig. 3E; $P < 0.05$). In fact, the cystatin M group was found to exhibit...
5.1-fold fewer metastatic cells in the liver than the mock group (Fig. 3F).

**Histologic Examination and Counting of Spontaneous Lung Metastases.** Because the number of metastatic lesions could determine surgical success and thus could potentially determine the survival rate, given proper treatment, we determined whether cystatin M affected the number of spontaneous metastatic lesions in the lungs regardless of the size of the lesions. There seemed to be no site preference within the lung for metastasis because lesions occurred everywhere, e.g., intravascular, bronchocentric, angiocentric, pleural, and alveolar regions (data not shown). There was no significant difference in the number of metastatic lesions between the mock group and the cystatin M group. This may reflect the small number of animals tested in this study and/or the high variability in lesion number in the cystatin M group when compared with the mock group (data not shown). Thus, we cannot exclude that cystatin M expression might have a weak effect on metastasis to the lungs.

**Effect of Cystatin M Expression on Experimental Lung Colony Formation.** In the spontaneous metastasis assay above, all mice had metastases in the lungs (Fig. 3A and B), and the number of lesions did not differ between the mock group and the cystatin M group. This suggested that cystatin M expression might primarily influence the growth and survival of metastatic cells at the secondary site. To test this hypothesis, we performed experimental lung metastasis assays (23) and compared overall colony burden at 5 and 11 weeks after tail vein injection of tumor cell clones. There was no difference in colony burden at 5 weeks (Fig. 4A and B). At 11 weeks (i.e., 6 weeks later), the overall colony burden in the parental and mock groups had dramatically increased from about 257 and 457 to 18,621 and 23,988 cells/lung, respectively. The metastatic burden of the cystatin M group in comparison increased only from 347 to 1,820 cells/lung over the same time period (Fig. 4C and D). This corresponds to a delay similar to the one observed at the primary site and translates into a statistically significant decreased burden at 11 weeks in the cystatin M group when compared with the parental and mock groups (Fig. 4C and D). This provides additional evidence that cystatin M expression has an antiproliferative effect at the secondary site.

**Analysis of Cystatin M Expression in Microdissected Human Breast Epithelial Cells.** To further evaluate the putative role of cystatin M as a tumor suppressor for breast cancer, we analyzed cystatin M mRNA expression in human breast tissue biopsies. Because of the heterogeneity of the disease (24, 25), mRNA expression was analyzed in laser capture-microdissected epithelial cells of defined stages of human primary breast cancer progression. Due to limited patient material, we compared in this pilot study cystatin M expression levels in breast epithelial cells from normal tissue and IDC of stage I to IV patients. IDC was used as the representative histopathological type in this study because it accounts for 60% to 80% of all breast cancer occurrences.

Fig. 5A is an illustration of the microdissection procedure. Normal breast epithelial cells were captured from frozen sections of four normal tissue specimens. LCM allowed us to selectively capture the...
single layer of normal breast epithelial cells while leaving the underlying layer of myoepithelial cells on the glass slide (Fig. 5A, top panels, arrowheads). IDC cells were collected from stage I to IV patients (Fig. 5A, bottom panels). The cystatin M transcript was consistently expressed in normal breast epithelial cells (Fig. 5B/H11002/D).

In contrast, expression of cystatin M mRNA in IDC cells from breast cancer patients with different stages of disease was much lower compared with that in normal epithelial cells (Fig. 5B/H11002/D). Complete loss of expression was observed in IDC cells from two of three stage IV patients (Fig. 5B). Because there were only two to three cases per stage, cystatin M expression levels in IDC from stage I to IV patients were pooled for comparison with normal cases. Our data show that there is a >80% decrease in cystatin M mRNA expression in IDC cells when compared with normal breast epithelial cells (Fig. 5E and F; P < 0.05), which in some instances leads to the complete loss of expression at advanced stages.

**Immunohistochemical Analysis of Cystatin M in Breast Tissue Specimens.** The expression of cystatin M was further examined at the protein level using paraffin-embedded tissues from the same patients as described above and immunohistochemical staining. The characterization of the rabbit polyclonal antibody directed against human cystatin M will be described elsewhere.9 Briefly, this antibody does not cross-react with human cystatin C, which we have previously shown to be secreted by all cell clones analyzed, including the parental clone, the two mock clones, and the three cystatin M clones (Fig. 1A; ref. 18). In addition, in breast speci-
mccs that included some normal human skin, we found identical immunostaining for cystatin M in the skin as this has been reported by others using a different antibody (26). Immunolocalization of cystatin M in normal human breast and IDC tissue is shown in Fig. 6A. Epithelial cells and some myoepithelial cells of normal breast glands showed strong immunostaining for cystatin M; otherwise little or no background staining of stromal components or fat tissue was seen (Fig. 6A, Normal 1). For comparison, it can be seen from Fig. 6A that on consecutive sections, the preimmune serum and secondary antibody alone, respectively, gave little or no background staining. In contrast to the immunostaining of normal breast epithelial cells, IDC cells overall showed little or no immunostaining for cystatin M (Fig. 6A, IDC 1). At higher magnification, immunostaining for cystatin M in the normal human breast epithelium was found to be primarily cytoplasmic (Fig. 6B, Normal 2). We also noticed strong staining of the residual normal glands near IDC (Fig. 6B, IDC 3). Discrete areas in some IDC cases showed weak immunostaining that, however, was also cytoplasmic (Fig. 6C, IDC 4). The heterogeneity of the cystatin M staining in some IDC cases could explain the fluctuations seen before at the mRNA level (Fig. 5C and D).

**DISCUSSION**

Loss of expression of certain genes in tumor cells has often been linked to the potential of their gene products to act as tumor or metastasis suppressors (27, 28). Initial observations from Sotiropoulos et al. (17) showed that 10 of 12 established human breast cancer cell lines lacked expression of cystatin M, whereas normal and premalignant cells expressed the 0.6-kb transcript. Previously, we have addressed the potential biological significance of the loss of expression of cystatin M by analyzing the malignant behavior in culture of human breast carcinoma MDA-MB-435S cells constitutively expressing the protein (18). In the present study, we have addressed the potential in vivo significance of the loss of expression of cystatin M.

During the first 45 days, scid mice bearing orthotopically injected cystatin M-expressing MDA-MB-435S cells developed significantly smaller tumors than mock-injected controls. At day 45 after injection, the mean size of the tumors in the cystatin M group corresponded to that of the tumors in the control group at day 26, which corresponds to a delay of nearly 3 weeks. After day 45, however, a “catch up” mechanism seemed to alleviate or neutralize the effect of cystatin M on primary tumor growth. The in vivo situation was thus quite reminiscent of the in vitro situation, in which cystatin M also appeared to...
neutralize cystatin M. In preliminary studies, we did indeed find one (cysM-17) of three cystatin M clones that had completely lost expression of cystatin M after in vivo passage. In addition, another clone (cysM-12), which still expressed the transgene, was resuming growth like the mock controls after several passages in vitro (data not shown). Altogether, our data show that cystatin M is unfavorable for tumor cell growth and that tumor cells eventually find ways to escape the growth-inhibitory pressure of cystatin M.

To analyze the effect of cystatin M expression on spontaneous metastasis, we sought to exclude the possibility that differences in metastasis could arise from unequal tumor loads. Day 55 after orthotopic implantation of tumor cells seemed to be a suitable time point to perform such analyses because, at this time point, there was no difference in the mean tumor size and weight between the mock group and the cystatin M group. Thus, at day 55 after MFP inoculation, there were on average 17,378 human cells present in the lungs of the mock group compared with 7,413 cells for the cystatin M group. This represents a 57% reduction in the metastatic burden of the lungs. Metastasis was further confirmed by routine histologic examination of lung sections and determination of the number of metastatic lesions. Although we did find a slightly reduced number of metastatic lesions in the lungs of the cystatin M group when compared with the mock group, this decrease did not reach statistical significance.

In the liver, we observed a more pronounced effect of cystatin M expression on the spontaneous metastatic burden when compared with the lungs. Similar to the lungs however, the incidence of metastasis was again 100%. The metastatic cascade is such that cells first metastasize to the lung from the primary MFP site, and then metastasis of metastases is responsible for developing secondary metastases in the liver (29). Therefore, the unequal load of cells in the lungs could explain why the spontaneous metastatic burden in the liver was reduced by 5-fold on cystatin M expression. Our findings with cystatin M are in striking contrast to results obtained by others with cystatin F (leukocystatin; refs. 30 and 31). Indeed, cystatin F (or CMAP) can be conceived as a potent mediator of liver metastasis (32, 33), whereas our data suggest that cystatin M may have the opposite function. One may ask how it is possible that two inhibitors with the same supposed function could produce such different results. There are several answers to this question, which illustrate the complexity one has to face in clinical trials when dealing with protease inhibitors: First, cystatin F and cystatin M share only about 22% sequence homology at the protein level (30). Second, it is well established that individual cystatins show affinities for different target proteases that can vary more than 1,000-fold (1). Therefore, a biological response may depend largely on the local concentration of inhibitor (34). Third, different cystatins or even the same cystatin may have different expression levels and/or activities in different tumors (18, 35–37). Fourth, the activity of a cystatin might depend on whether it is produced by epithelial cells (like cystatin M) or rather by infiltrating host cells (like cystatin F; ref. 30). Fifth, cystatins, like tissue inhibitors of metalloproteinases (38) and plasminogen activator inhibitors (39), are a family of multifunctional proteins known to possess a broad range of biological activities (8, 10, 11).

Because of the effect of cystatin M on tumor cell proliferation and because there was no difference in the overall incidence of metastasis between the mock group and the cystatin M group, we further hypothesized that cystatin M expression might primarily influence the growth and survival of the metastatic cells at the secondary site. Using the experimental lung metastasis assay (40), we were able to confirm that cystatin M expression, rather than being unfavorable for seeding and survival of the cells at the secondary site, was in fact not favorable for expansion of already established lung colonies. Given our previous data on tumor cell proliferation in vitro (18), subcutaneous tumor

![Fig. 6. Immunolocalization of cystatin M in normal human breast and IDC tissue.](image-url)
growth (data not shown), and orthotopic tumor growth (this study), cystatin M appears to have primarily a tumor-suppressing function.

To further probe the tumor suppressor-like characteristics of cystatin M in human breast cancer, we evaluated the relationship between cystatin M expression level and clinical stage. This established for the first time that cystatin M is expressed at both the mRNA and protein levels in the normal human breast epithelium. In addition, IDC cases showed a statistically significant and >80% decrease from normal expression levels. For a better evaluation of the clinical relevance of cystatin M expression, future studies will need to evaluate more patients and stages covering the whole spectrum of breast cancer progression. A careful pathological grading should also be considered because expression levels of a gene product could vary greatly between low- and high-grade tumors (41).

To address whether cystatin M functions in vivo through protease-dependent and/or protease-independent mechanisms might appear trivial at first glance, but in reality, it is not. Some of the obstacles that will have to be overcome to answer this question are as follows. (a) The physiologic target of cystatin M among the 12 human lysosomal cysteine proteases remains to be clearly defined. (b) There are no enzyme substrates or inhibitors available that would allow specific and quantitative assessment of each protease. (c) Cystatin M is a double-headed inhibitor, blocking the action of both papain- and legumain-type lysosomal cysteine proteases (5). (d) Cystatin variants with engineered point mutations may lose inhibitory capacity for one cathepsin but not for another (42). (e) Engineered cystatin variants might lose inhibitory capacity because of incorrect folding and thus yield data that are difficult to interpret. (f) The same region that binds a target protease might be involved in the binding of some other protein(s). Future endeavors will therefore need to address this question using a combination of different approaches.

In conclusion, this study shows for the first time that cystatin M significantly delayed breast tumor growth and lowered the metastatic burden at secondary sites. More importantly, the observed decrease in cystatin M expression in human breast cancers strongly suggests that cystatin M may represent a novel candidate tumor suppressor gene for this type of cancer.

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Cystatin M: A Novel Candidate Tumor Suppressor Gene for Breast Cancer

Jun Zhang, Ravi Shridhar, Qun Dai, et al.

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