The role of tumor-derived matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) in cancer cell dissemination was analyzed by employing two variants of human HT-1080 fibrosarcoma, HT-hi/diss and HT-lo/diss, which differ by 50–100-fold in their ability to intravasate and metastasize in the chick embryo. HT-hi/diss and HT-lo/diss were compared by quantitative reverse transcription-PCR and Western blot analyses for mRNA and protein expression of nine MMPs (MMP-1, -2, -3, -7, -8, -9, -10, -13, and -14) and three TIMPs (TIMP-1, -2, and -3) in cultured cells in vitro and in primary tumors in vivo. MMP-1 and MMP-9 were more abundant in the HT-hi/diss variant, both in cultures and in tumors, whereas the HT-lo/diss variant consistently expressed higher levels of MMP-2, TIMP-1, and TIMP-2. Small interfering RNA-mediated down-regulation of MMP-2 and TIMP-2 increased intravasation of HT-lo/diss cells. Coordinate disruption of the developing HT-hi/diss tumors with recombinant TIMP-1 and TIMP-2 significantly reduced HT-hi/diss cell intravasation. However, a substantial increase of HT-hi/diss dissemination was observed upon small interfering RNA-mediated down-regulation of three secreted MMPs, including the interstitial collagenase MMP-1 and the two gelatinases, MMP-2 and MMP-9, but not the membrane-tethered MMP-14. The addition of recombinant pro-MMP-9 protein to the HT-hi/diss tumors reversed the increased intravasation of HT-hi/diss cells, in which MMP-9 was stably down-regulated by short hairpin RNA interference. This rescue did not occur if the pro-MMP-9 was stoichiometrically complexed with TIMP-1, pointing to a direct role of the MMP-9 enzyme in regulation of HT-hi/diss intravasation. Collectively, these findings demonstrate that tumor-derived MMPs may have protective functions in cancer cell intravasation, i.e. not promoting but rather catalytically interfering with the early stages of cancer dissemination.

Proteolysis of extracellular matrix (ECM)3 proteins and proteolytic modifications of cell surface receptors by matrix metalloproteinases (MMPs) are critically important for tumor cell dissemination. Functional activity of MMPs is tightly regulated through transcriptional and translational expression, proenzyme activation, and inhibition of activated enzymes by natural tissue inhibitors of metalloproteinases (TIMPs). There is much evidence that up-regulated expression of many MMPs and, paradoxically, TIMPs is correlated with tumor aggressiveness in various cancers. The pivotal role of the MMP/TIMP system in tumor metastasis has been clearly demonstrated for some of the initial steps of the metastatic cascade, including tumor invasion and angiogenesis (1–8). However, depending on tissue context, individual MMPs and TIMPs have specific effects on the cell environment, either promoting or inhibiting various steps in tumor progression.

Early events in tumor dissemination include intravasation, the process whereby cancer cells enter the vasculature. Although it appears to be a rate-limiting step of the metastatic cascade, studies of MMPs and TIMPs in intravasation are very limited. This limitation stems largely from the lack of quantitative experimental models to analyze intravasation in mammals. Most intravasation experimental systems are based on intravital imaging in mice and rats at the site of tumor development, requiring sophisticated equipment and software but not allowing for a straightforward quantitation of intravasation events or direct pharmacological intervention (9–11). Therefore, intravasation studies remain mainly observational albeit insightful, and the limited experimental manipulations have included a collection of invasive, but not intravasated, cells within primary tumors (12, 13).

A rapid and highly versatile alternative model for examining intravasation is the human tumor/chick embryo model, which
recapitulates all stages of spontaneous metastasis. This model system, employing grafting of human cancer cells on the chorioallantoic membrane (CAM) of the chick embryo, has been repeatedly and successfully utilized to examine early steps of the metastatic cascade, including tumor cell invasion, vasculotropism, and intravasation (14–25). Within 4–7 days following grafting on the CAM, several types of human cancer cells develop aggressive primary tumors, from which tumor cells escape and enter the CAM vasculature. These escaped tumor cells can be detected in the distal portions of the CAM, which serves as a unique repository for intravasated cells. Subsequently, the disseminated tumor cells can be traced to the internal organs of the embryo such as liver, lungs, and bone marrow. The actual numbers of human tumor cells present in the chick embryo tissues can be determined by a quantitative PCR (qPCR) detecting Alu repeats unique for a primate genome (17, 19, 20).

To analyze the role of MMPs and TIMPs in tumor cell intravasation, we employed two variants of human HT-1080 fibrosarcoma selected in vivo for their differential ability to disseminate in the human tumor/chick embryo model (21). These two cell variants are characterized by high and low dissemination and therefore have been named HT-hi/diss and HT-lo/diss, respectively. They differ by 50–100-fold in their ability to intravasate to the CAM vasculature and metastasize to the internal organs such as liver and lungs. This tumor cell dissemination is partially MMP-dependent because the broad scale MMP inhibitor GM6001 (Ilomastat) effectively, but not completely, inhibits HT-hi/diss intravasation and metastasis (21), thus implicating tumor-derived and/or stroma-derived MMPs in the process of intravasation.

Gene expression analysis in normal cells and tumor cell lines of different tissue origin, including the HT-1080 fibrosarcoma, demonstrated that high levels of several tumor MMPs and TIMPs were positively associated with high invasion ability and metastatic potential (26–33). The parental HT-1080 cell line was shown to express a wide repertoire of MMPs, including MMP-1, -2, -3, -7, -8, -9, -10, -13, and -14 and three TIMPs, including TIMP-1, -2, and -3 (26, 29, 34). Therefore, a comparison of two HT-1080 intravasation variants, HT-hi/diss and HT-lo/diss, would be highly instructive for examining the possible role of individual tumor MMPs and TIMPs in the intravasation step of the metastatic cascade. In recent studies, activity-based protein profiling of HT-hi/diss and HT-lo/diss serine hydrolases has already implicated the ability of HT-hi/diss cells to activate uPA as one of the key determinants of their efficient intravasation (35).

In this study, we performed an extensive MMP and TIMP gene and protein expression analysis of HT-hi/diss and HT-lo/diss, both in cell cultures and primary tumors. We identified significant differences between the two intravasation variants in the expression levels of several MMPs, i.e. MMP-1, MMP-2, and MMP-9, and two TIMPs, i.e. TIMP-1 and TIMP-2. These results determined the overall strategy of a functional analysis for individual MMPs and TIMPs, i.e. using siRNA- and shRNA-mediated down-regulation of those proteins that were differentially overexpressed in the corresponding intravasation variant. Although down-regulation of TIMP-2 in HT-lo/diss significantly increased its dissemination, down-regulation of secreted tumor MMPs, namely interstitial collagenase MMP-1 and gelatinases MMP-2 and MMP-9, repeatedly enhanced intravasation and metastasis of HT-hi/diss. Collectively, the findings of this study highlight pleiotropic and contrasting functions of the tumor MMP/TIMP system, including its ability to negatively regulate tumor cell intravasation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—HT-hi/diss and HT-lo/diss cell variants were selected in vivo from HT-1080 human fibrosarcoma (ATCC, Manassas, VA) for a 50–100-fold differential in their ability to intravasate in the chick embryo model (21). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 10 μg/ml gentamicin (D-10) in a humidified incubator in the presence of 5% CO₂ at 37 °C. On day 10 of incubation the CAM was dropped, and 0.4–1 × 10⁶ cells were grafted on the CAM in a 25-μl volume. Following an additional 4–6 days of incubation, the embryos were sacrificed. The primary tumors were excised, weighed, and snap-frozen in liquid nitrogen for gene and protein analyses or fixed in Zn-formalin (Fisher) and embedded in O.C.T. compound (Sakura Finetek, Inc., Torrance, CA) for histological examination. Portions of the distal CAM and liver were harvested, frozen on dry ice, and analyzed by quantitative real time PCR (qPCR) to determine numbers of intravasated and metastasized human cells.

**Real Time PCR for Quantitative Detection of Human Tumor Cells**—The number of human cells within chick embryo tissues was determined by qPCR of the Alu repeats (Alu-qPCR) as described (21). Briefly, genomic DNA was extracted from harvested tissues using the Puregene kit (Genta Systems, Minneapolis, MN). Human Alu sequences were amplified by qPCR using 60 ng of genomic DNA as template in a 10-μl reaction, containing 2 mM MgCl₂, 200 μM dNTP, 0.4 units of Platinum Tag polymerase (Invitrogen), 1:10⁶ dilution of SYBR®Green dye (Molecular Probes, Eugene, OR), and 0.4 μM of each Alu-sense (5’-ACGCTCTGAATCCCAGACTT-3′) and Alu-antisense (5’-TGGCCGAGCTGGATGCA-3′) primers. Alu-qPCR was carried out at 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. Each assay included negative controls (water and samples of tissues from the embryos that have not received any human cells), a positive control (human genomic DNA), and experimental samples in duplicate. The number of tumor cells in each tissue sample was determined using a standard curve generated by a serial (10-fold) dilution of HT-1080 cells within a constant number (10⁶) of chick embryo fibroblasts.
**TABLE 1**

| Gene name (other name) | Primer sequence used in real time RT-PCR (forward and reverse) | Antibody used in Western blotting (WB) and immunohistochemistry (IH) |
|------------------------|---------------------------------------------------------------|---------------------------------------------------------------------|
| MMP-1 (fibroblast collagenase) | 5′-ACAGGCCAGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 36665 (R & D Systems) |
| MMP-2 (gelatinase A) | 5′-GGGCTGAAGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 42-SD11 (Calbiochem) |
| MMP-3 (stromelysin-1) | 5′-CCCAAGGAGATCTATTTCCCTCCTGGA-3′ | IH, mouse mAb VB3 (Calbiochem) |
| MMP-7 (matrilysin) | 5′-GGGCTGAAGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 905 and goat Ab AF513 (R & D Systems) |
| MMP-8 (neutrophil collagenase) | 5′-GGGCTGAAGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb ID2 (Calbiochem) |
| MMP-9 (gelatinase B) | 5′-GCCGCAGATCTATTTCCCTCCTGGA-3′ | WB, rabbit Ab RPI-MMP-8 (Triple Point Biologics) |
| MMP-10 (stromelysin-2) | 5′-ATGGTCTAAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 7-11C (72) |
| MMP-13 (collagenase-3) | 5′-CTGTCATTCAATCTACATCCTGGA-3′ | WB, mouse mAb 8-3H (72) |
| MMP-14 (MT1-MMP) | 5′-AACGCTGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb IVC5 (Lab Vision Corp.) |
| TIMP-1 | 5′-ACCTACCGAGATCTATTTCCCTCCTGGA-3′ | WB, rabbit Ab RP2-MMP-13 (Triple Point Biologics) |
| TIMP-2 | 5′-GGGCTGAAGAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 114-IF2 (Calbiochem) |
| TIMP-3 | 5′-GGGCTGAAGAGATCTATTTCCCTCCTGGA-3′ | IH, rabbit Ab M 927 against anti-hinge region (Sigma) |
| GAPDH | 5′-GCCACAGATCTATTTCCCTCCTGGA-3′ | WB, mouse mAb 7-6C1 (Calbiochem) |

Quantitative Real Time RT-PCR (qRT-PCR)—Total RNA was harvested from cell cultures or CAM tumors in 2–3 independent experiments and isolated with RNAqueous 4PCR (Ambion, Foster City, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), and the resulting cDNA library was diluted 100-fold. Specific primers for individual human MMPs and TIMPs (Table 1) were designed using the on-line Primer 3 software and synthesized by Integrated DNA Technologies (Coralville, IA). The sequence for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the amount of total human cDNA. qRT-PCR was performed using an I-cycler real time PCR machine (Bio-Rad). Each reaction (a total volume of 25 μl) contained 5 μl of the diluted cDNA library as template, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 0.4 μM each of forward and reverse primers. PCR conditions included heating for 2 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and then 30 s at 72 °C. A melt curve analysis was performed to ensure specific amplification. For each target gene, relative levels of expression were normalized using the GAPDH signal (ΔCt = Ct_GAPDH - Ct_Target)). Difference in the expression levels between the two HT-1080 variants was determined according to the following formula: 2^ΔCt, where ΔCt = |ΔCt_HT-1080/diss - ΔCt_HT-1080/to/diss|. The differences from the qPCR of individual RNA preparations were statistically analyzed to get means ± S.E.

Immunohistochemistry—Primary tumors were excised from the CAM of chick embryos at days 4–5 and were fixed in Zn-formalin and paraffin-embedded or frozen in the O.C.T. compound. Paraffin-embedded sections were used in staining with anti-MMP-9 and MMP-14 antibodies (Table 1) after antigen retrieval with the antigen unmasking solution (Vector Laboratories, Burlingame, CA). Frozen sections were fixed with cold methanol and used for MMP-2 and TIMP-2 staining with corresponding antibodies (Table 1). Tissue sections were treated with 0.3% hydrogen peroxide and blocked with PBS supplemented with 2% bovine serum albumin and 5% normal goat serum. Sections were incubated with individual primary antibodies (10 μg/ml blocking solution) overnight at 4 °C, then with corresponding secondary biotinylated antibodies for 1 h, followed by incubation with avidin–horseradish peroxidase conjugate (Vector Laboratories) for 30 min, and development with a diaminobenzidine chromogenic substrate. Sections were counterstained with Mayer’s hematoxylin. Digital images were captured using the Olympus BX60 microscope equipped with a digital DVC video camera and processed with Adobe Photoshop 6.0 software.

Generation and Purification of Recombinant Pro-MMP-9 TIMP Complex—Human recombinant pro-MMP-9 (rMMP-9) was mixed with a 5 M excess of recombinant TIMP-1 (both a generous gift from Dr. R. Fridman, Wayne State University, School of Medicine, Detroit, MI), and incubated for 1 h at room temperature. The formed rMMP-9–TIMP-1 complexes were purified and separated from the unbound excess TIMP-1 by gelatin-Sepharose chromatography. Control preparation of rMMP-9 was prepared in an identical way except that TIMP-1 was omitted during preincubation. The proteins bound to gelatin-Sepharose were eluted with 10% Me2SO and analyzed by Western blotting and zymography.

Western Blot Analysis and Zymography—Cell cultures were initiated in D-10; after reaching confluence, culture medium was changed for SF-DMEM and conditioned medium was harvested 18 or 48 h later. The cells were washed with PBS and lysed in modified RIPA (mRIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and protease inhibitors). To extract proteins from the primary CAM tumors, samples were snap-frozen in liquid nitrogen, ground, and lysed.
TABLE 2
Target sequences for MMP and TIMP siRNA and shRNA constructs

| Gene   | Target sequence | Ref./Source |
|--------|----------------|-------------|
| MMP-1  | 5'-AAATGATGACCTTTAGTTGACAGA-3' | 51 |
| MMP-2  | 5'-AAGACCAAGATGATGACAGA-3' | 73 |
| MMP-9  | 5'-AAATCACCATTAGTTGACAGA-3' | 51 |
| MMP-14 | 5'-AACGCAACCCTGAGTA-3' | 51 |
| TIMP-1 | 5'-AAGATGTTATAAGGCTAGTTACCA-3' | HP GenomeWide, Qiagen, catalog number SI00745318 |
| TIMP-2 | 5'-AGACCAATATATTGAAAACCA-3' | HP GenomeWide, Qiagen, catalog number SI03071285 |
| Nonsilencing | 5'-AAATCTTCCTGACAGTACGTTAC-3' | Qiagen, catalog number 1022076 |
| Nonspecific | 5'-AAATCTTCCTGACAGTACGTTAC-3' | 74 |
| Scrambled | 5'-AAATCGATGATGACTAGAGA-3' | 51 |
| GFP    | 5'-CGGCAAGCTGACCCCTGAACTTCAT-3' | Qiagen catalog number 1022064 |

in mRIPA buffer. Equal amounts of proteins were separated on SDS-polyacrylamide pre-cast gels (Bio-Rad) and transferred onto Immobilon-P polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk and incubated overnight at 4 °C with 1 μg/ml antibodies specific for human MMPs and TIMPs (Table 1). The blots were incubated with corresponding secondary horseradish peroxidase-conjugated antibodies (Pierce), and the proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce).

To verify the presence of MMP-9 and TIMP-1 in the rMMP-9-TIMP-1 complexes eluted from gelatin-Sepharose, the membrane with the transblotted proteins was cut at approximately a 40-kDa level, and the upper portion was probed with MMP-9-specific mAb 7-11C, whereas the lower portion was probed with mAb against TIMP-1.

For zymography, equal amounts of proteins eluted from gelatin-Sepharose or proteins from conditioned media, lysed cells, or primary tumors were separated on 8 or 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100, incubated overnight at 37 °C in 40 mM Tris buffer, pH 7.6, supplemented with 200 mM NaCl and 10 mM CaCl₂. The gels were stained with Coomassie Blue (G-250) and destained in distilled water to visualize bands of gelatinolytic activity.

**MMP and TIMP Down-regulation by RNA Interference (RNAi)—Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) targeting individual MMPs and TIMPs were synthesized (Qiagen, Valencia, CA) against the human sequences indicated in Table 2. Negative control siRNA constructs with no known homology to mammalian genes included nonsilencing, nonspecific, and GFP sequences (Table 2). Transfections were performed according to the manufacturer’s instructions with 10 or 100 nM of individual siRNAs using HiPerFect (Qiagen) or Lipofectamine 2000 (Invitrogen), respectively. The siRNA-treated cells were harvested 18–24 h later, washed, and resuspended in SF-DMEM, and used in further experiments.

Stable suppression of MMP-9 was achieved by infection of HT-hi/diss cells with a lentiviral construct. MMP-9 shRNA target sequence (forward 5’-TAACTACATATTGATGACAGA-3’ and reverse 5’-CTGGATGACAGA-3’), with the loop sequences (underlined), was inserted into the pLentiLox 3.7 plasmid containing enhanced green fluorescent protein (GFP) (pLL3.7-GFP) to express double-stranded short hairpin RNA. Control shRNA vector was generated by using a scrambled sequence (Table 2). The lentiviral stock was prepared by calcium chloride transfection of 293T cells with four plasmids as follows: the pL3L.7-GFP, pMDLg-pRRE, pRSV-rev, and pVSV-G according to Ref. 36. Following a 48-h incubation, the viral supernatant was concentrated by ultracentrifugation, and the pellet was resuspended in SF-DMEM. Vector titers were determined by infection of HT-hi/diss cells with serial dilutions of the vector stocks followed by FACS analysis for GFP-positive cells. HT-hi/diss cells were infected with the MMP-9 and control lentiviral vector constructs, and GFP-positive cells FACS sorted to generate stable, 85–100% positive cell populations.

To analyze the in vivo effects of RNAi-mediated down-regulation of MMPs and TIMPs on intravasation, a total of 0.4–1 × 10⁶ siRNA-transfected or shRNA-transduced cells were grafted in 25 μl onto the CAM of chick embryos. Following incubation for 4–5 days, the embryos were sacrificed, tumors weighed, and portions of distal CAM analyzed by Alu-qPCR for numbers of intravasated cells. Down-regulation of MMPs and TIMPs in the siRNA- and shRNA-treated cells was verified by Western blotting and gelatin zymography in each independent experiment. Where indicated, HT-hi/diss tumors developing from cells with stably downregulated MMP-9 were treated twice (100 ng on day 1 and 200 ng on day 3 per individual tumor) with the rMMP, kindly provided by Dr. R. Fridman (Wayne State University, Detroit, MI).

**Data Analysis and Statistics—**Data processing and statistical analysis were performed using GraphPad Prism® (GraphPad Software, Inc., San Diego). All experiments were performed at least twice, and the total number of experiments, animals, and samples is indicated in the text or figure legends. Data are presented as means ± S.E. calculated from pooled fold differences as the ratios of numerical values for individual embryos over a mean of the control in the corresponding experiment. Student’s t test (p < 0.05) was used to compare differences between the sets of experimental data.

**RESULTS**

MMP and TIMP Expression in HT-1080 Cell Variants in Vitro—HT-hi/diss and HT-lo/diss cells were analyzed by qRT-PCR for transcription of genes encoding selected MMPs and their natural inhibitors, TIMPs. (MMP and TIMP indicate proteins, and MMP and TIMP indicate human genes.) In both cell variants, high levels of gene expression (ΔCt ≤ 8) were indicated for two MMP genes, MMP-2 and MMP-14, and for three TIMP genes, TIMP-1, TIMP-2, and TIMP-3 (Fig. 1A, top). MMP-1, -3,
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FIGURE 1. Gene (top) and protein (bottom) expression analysis of individual MMPs and TIMPs in HT-lo/diss and HT-hi/diss cell cultures (A) and CAM tumors (B). A, HT-lo/diss and HT-hi/diss cells were plated in D-10 and after an overnight incubation were washed, detached, and snap-frozen in liquid nitrogen. Gene expression analysis was performed on mRNA extracted from the cells as described under “Experimental Procedures.” In parallel cultures, D-10 was exchanged for serum-free DMEM, and conditioned medium was collected 24 h later for protein expression analysis by Western blotting (WB). B, HT-lo/diss and HT-hi/diss cells were seeded on the CAM of chick embryos. Four days later, the developed tumors were excised and snap-frozen for gene and protein expression analyses. Levels of gene expression in the tumors were determined by RT-qPCR as described under “Experimental Procedures.” Primer sequences and source of specific antibodies are presented in Table 1. Gene expression is referred to as low, moderate, and high when expression values over the mean value of HT-lo/diss expression. The data are from two independent experiments.

and -9 were expressed at moderate levels (ΔCt between 9 and 14), whereas low levels of expression were characteristic of MMP-7, MMP-8, MMP-10, and MMP-13 (ΔCt > 14). Among tested MMP genes, relative expression of MMP-1, -3, -7, -9, -10, and -13, was 2–10-fold higher in HT-hi/diss compared with HT-lo/diss (2.4 ± 0.1, 9.6 ± 2.7, 4.8 ± 1.3, 4.0 ± 0.6, 4.1 ± 0.3, and 2.6 ± 1.4-fold, respectively). MMP-8 gene expression was found 3.8 ± 0.3-fold higher in HT-lo/diss cells than in HT-hi/diss cells, which is in agreement with reported elevated expression of MMP-8 in less aggressive cancers (37, 38). Unexpectedly, mRNA for MMP-2 (gelatinase A) and MMP-14 (MT1-MMP) was more abundant in HT-lo/diss than in HT-hi/diss (3.2 ± 0.2 and 2.5 ± 0.4-fold, respectively). Among TIMP genes, expression levels of TIMP-1 and TIMP-2 were consistently higher in HT-lo/diss as compared with HT-hi/diss (1.4 ± 0.4 and 2.0 ± 0.4-fold, respectively) (Fig. 1A, top).

Differential expression of MMP and TIMP genes was further analyzed on the protein level (Fig. 1A, bottom). MMP-7, -8, -10, and -13 proteins were not detected by Western blotting in cultured cells or conditioned medium from both cell variants, consistent with the relatively low levels of gene expression of these specific MMPs (ΔCt ≥ 14). Despite relatively high mRNA levels, TIMP-3 protein was not detected either in conditioned medium or cell lysates, likely because it could be tightly bound to the ECM deposited by the cells during cultivation in serum-containing medium (39). The protein levels of other MMPs and TIMPs in HT-hi/diss relative to HT-lo/diss corresponded closely to the mRNA pattern. Thus, MMP-1 and MMP-9 were detected at higher levels in HT-hi/diss cells (2.4 ± 0.4, 4.2 ± 1.9-fold, respectively), whereas MMP-2, TIMP-1, and TIMP-2 proteins were more abundant in the HT-lo/diss variant (3.4 ± 1.2, 2.1 ± 0.2, and 2.8 ± 1.1-fold, respectively). Expression of MMP-3 and MMP-14 proteins did not significantly differ between the two cell variants (1.3-fold difference) (Fig. 1A, bottom).

Human MMPs and TIMPs in HT-hi/diss and HT-lo/diss CAM Tumors in Vivo—The gene and protein analysis of developing tumors in vivo may be more indicative for the role of MMPs and TIMPs during the actual intravasation process. Expression levels of selected MMP and TIMP genes were thus analyzed in several individual HT-hi/diss and HT-lo/diss tumors (Fig. 1B, top). The MMPs with low levels of expression...
in vitro (i.e. MMP-7, -8, -10, and -13) were not detected in the mRNA pool from the tumor tissue. For the identified MMPs, the overall pattern of gene expression in vivo resembled that found in cultured cells in vitro. Thus, MMP-1, -3, and -9 were overexpressed in HT-hi/diss tumors (10.8 ± 5.0-, 10.6 ± 6.0-, and 2.8 ± 0.6-fold, respectively), whereas expression of MMP-2 and -14 remained higher in HT-lo/diss tumors (9.5 ± 4.8- and 1.7 ± 0.4-fold, respectively). Interestingly, the difference in mRNA levels of TIMP-1 and especially TIMP-2 between HT-hi/diss and HT-lo/diss tumors increased compared with in vitro conditions and corresponded to 3.2 ± 0.9- and 6.6 ± 2.2-fold, respectively.

Western blot analysis was performed on five HT-lo/diss and HT-hi/diss tumors (Fig. 1B, bottom, and Fig. 2). Relative expression of MMP-1, -2, -9, and -14 proteins in CAM tumors was quite similar to that demonstrated for cultured cells. Specifically, levels of MMP-1 and MMP-9 in tumors were higher in HT-hi/diss than in HT-lo/diss (6.7 ± 0.4 and 1.5 ± 0.2-fold, respectively), whereas MMP-2 levels were higher in HT-lo/diss compared with HT-hi/diss (4.8 ± 0.4). It is noteworthy that in contrast to cultured cells, tumors displayed higher levels of activated MMPs: 45 kDa MMP-1, 62 kDa MMP-2, and 82 kDa MMP-9 (Fig. 2). Similar to in vitro conditions, TIMP-2 was significantly higher and TIMP-1 was slightly higher in HT-lo/diss tumors than in HT-hi/diss tumors (3.9 ± 0.3 and 1.4 ± 0.2-fold, respectively), whereas TIMP-3 protein was not detectable.

To visualize the spatial distribution of individual MMPs and TIMPs in vivo, HT-lo/diss and HT-hi/diss tumors were immunohistochemically stained for human MMPs and TIMPs. Because of avian cross-reactivity with many MMP and TIMP antibodies raised against human proteins, successful staining allowing clear discrimination between host and tumor cells was achieved only for human MMP-2, MMP-9, MMP-14, and TIMP-2. Primary HT-lo/diss tumors exhibited higher levels of staining for MMP-2 and TIMP-2 proteins, whereas more MMP-9 protein was detected in HT-hi/diss tumors (Fig. 3). MMP-9 was more intensively stained at the CAM/tumor border, especially in HT-hi/diss cells that appeared to be invading the surrounding stroma. MMP-14 protein was clearly localized to both cytoplasm and plasma membrane of human tumor cells and appeared more abundant in HT-hi/diss than in HT-lo/diss...
tumors, which may be attributed to accumulation of MMP-14 degradation products as indicated by Western blotting (Fig. 2). Thus, the in vivo expression levels of individual MMPs and TIMPs observed by immunohistochemistry in the primary tumors were consistent with those indicated by gene and protein expression analyses and provided a confirmatory validation of their in vivo differential expression in the two intravasation variants.

Functional Role of TIMPs in Tumor Cell Intravasation—The gene and protein analysis in vivo indicated that MMP-2, TIMP-1, and TIMP-2 were expressed at higher levels in HT-lo/diss tumors, whereas HT-hi/diss tumors were more abundant in MMP-1 and MMP-9. To delineate possible functional roles for these members of the MMP/TIMP family in the intravasation process, we suppressed expression of individual MMPs and TIMPs by RNA interference.

To verify whether elevated expression of TIMP-1 and TIMP-2 plays an inhibitory role in tumor cell intravasation, HT-lo/diss cells were treated with TIMP-1 and TIMP-2 siRNAs and then grafted on the CAM of the chick embryos (Fig. 4). Western blot analysis of conditioned media confirmed substantial down-regulation of TIMP-1 (by 79% at day 3) and TIMP-2 (by 88% at day 3) (Fig. 4, A and B). HT-lo/diss cells with down-regulated TIMP-1 and TIMP-2 gave rise to CAM tumors similar in size to those generated by cells treated with control siRNA (Fig. 4D, top). Although intravasation rates of TIMP-1 siRNA-treated cells remained unchanged, intravasation of TIMP-2 siRNA-treated cells increased 1.6-fold compared with a negative control (p = 0.018) (Fig. 4D, bottom). Correspondingly, there also was a 2-fold increase (2.1 ± 0.5, p = 0.001) in liver metastasis by HT-lo/diss cells with reduced expression of TIMP-2 (data not shown). Analogous results were observed for HT-lo/diss cells treated with MMP-2 siRNA; down-regulation of MMP-2 protein expression by 70.6 ± 3.7% (Fig. 4C) was accompanied by a 3-fold increase (3.0 ± 0.7, p = 0.014) in intravasation (Fig. 4D, bottom).

However, despite a significant increase in intravasation of HT-lo/diss cells, their absolute levels of dissemination never reached those characteristic of HT-hi/diss.

These results indicate that down-regulation of TIMP-2 and also MMP-2, both expressed at higher levels in HT-lo/diss than in HT-hi/diss, enhances tumor cell dissemination, therefore

FIGURE 3. Immunohistochemical analysis of individual human MMPs and TIMP-2 in HT-lo/diss and HT-hi/diss tumors. HT-lo/diss and HT-hi/diss cells were grafted on the CAM of the chick embryos. Following 4–5 days of development, the embryos were sacrificed, and the tumors were excised and processed for immunohistochemical staining as described under “Experimental Procedures.” Specificity of staining is indicated on the left of the panels. Staining for human MMP-2 and TIMP-2 was performed on frozen sections, whereas paraffin-embedded sections were stained for human MMP-9 and MMP-14. Positive staining is associated with brown-colored substrate localized to the cytoplasm and plasma membrane of tumor cells. Arrows point to the endoderm layer of the CAM; arrowheads point to blood vessels in the mesoderm of the CAM. White dotted lines delineate the tumor/CAM border. Original magnification, ×200. Bar, 50 μm.
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FIGURE 4. Functional role of TIMPs in tumor cell intravasation. A–C, HT-lo/diss cells, transfected with control GFP, TIMP-1, TIMP-2, and MMP-2 siRNA constructs, were detached after an overnight incubation, washed, and resuspended in serum-free DMEM. Aliquots of the cells were replated in D-10, which was exchanged for serum-free medium 24 h later. Samples of conditioned medium were collected within 18–24 h and analyzed by Western blotting for expression levels of TIMP-1 (A), TIMP-2 (B), and MMP-2 (C). D, HT-lo/diss cells transfected with siRNA constructs were plated on the CAM at 5 × 10^4 cells per embryo to assess the effects of specific siRNA down-regulation on tumor growth (top) and CAM intravasation (bottom). Tumors were excised and weighed 5 days following cell grafting. The portions of distal CAM were harvested and analyzed by quantitative Alu-qPCR to determine numbers of human cells within the chick embryo tissue. Data pooled from three independent experiments employing a total of 7–19 embryos per variant are presented as means ± S.E. of fold differences over control determined for individual embryos. The average tumor weight in the control group was 250 ± 29 mg (n = 49). The average number of HT-lo/diss cells calculated as intravasated in control embryos was 145 ± 23 cell equivalents per 60 ng of a total DNA extracted from the CAM tissue. Fold differences were determined as ratios of individual variables over a mean of control siRNA group and analyzed by Student’s t test (p < 0.05) for significance (*). E, HT-hi/diss tumors developing on the CAM of chick embryos were treated with recombinant TIMP-1 (TIMP-1) or TIMP-2 (0.5 μg in 0.1 ml of PBS, 5% MeSO_4 per embryo) or MMP inhibitor GM6001 (0.1 ml of 1 mM solution in PBS, 5% MeSO_4 per embryo) on day 2, 3, and 4 after cell grafting. On day 5, the embryos were sacrificed, tumors excised and weighed (top), and the portions of distal CAM analyzed for numbers of intravasated cells (bottom). The average tumor weight in the control group was 192 ± 10 mg (n = 15). The average number of HT-hi/diss cells calculated as intravasated in control embryos was 8958 ± 2751 cell equivalents per 60 ng of total DNA extracted from the CAM tissue. Data are presented as means ± S.E. of pooled fold differences determined from each individual embryo over a mean of the control in each experiment. A total of three experiments for individual MMP treatments and five experiments for GM6001 treatment were performed. *p < 0.05 in one-tailed Student t test; **p < 0.05 in a two-tailed Student t test.

not only indicating their partial involvement in HT-lo/diss dissemination but also suggesting that the individual tumor-derived MMPs and TIMPs can exhibit not contrasting but similar protective roles in cancer cell intravasation.

Because HT-hi/diss cells express lower levels of TIMP-1 and TIMP-2 as compared with HT-lo/diss, we supplemented HT-hi/diss tumors developing in the live embryos with natural and synthetic MMP inhibitors, i.e. TIMPs and GM6001. Direct topical treatment with recombinant human TIMP-1 and TIMP-2 proteins resulted in a respective 48.8 ± 9.8% and 42.5 ± 11.9% decrease in HT-hi/diss cell intravasation, without affecting tumor size. This diminishment of intravasation was comparable with a 57.2 ± 8.8% decrease in HT-hi/diss intravasation caused by a topical application of the broad range MMP inhibitor Ilomastat (GM6001) on HT-hi/diss tumors (Fig. 4E), thereby directly indicating the involvement of as yet undefined MMPs in early steps of tumor cell dissemination.

Functional Roles of Tumor-derived MMPs in Intravasation and Metastasis of HT-hi/diss Cells—Our previous findings have indicated that down-regulation of MMP-9 in HT-hi/diss surprisingly did not decrease but rather significantly increased levels of cell dissemination from the primary CAM tumors (21). To analyze the roles of the tumor MMP/TIMP system in intravasation in more detail, we extended the RNAi down-regulation approach and performed in vivo experiments employing HT-hi/diss cells treated with specific siRNAs and also shRNAs targeting several individual MMPs (Table 2).

MMP-1 was down-regulated in the high disseminating HT-1080 variant because it is a true collagenase, previously implicated in dissemination of tumor cells (3, 32, 40); and furthermore, MMP-1 has been demonstrated to be more abundant in HT-hi/diss cells (Figs. 1 and 2). MMP-1 protein levels were significantly decreased in HT-hi/diss cells 72–84 h after treatment with a specific siRNA (by 60.4 ± 12.8%, n = 5) (Fig. 5A, top), whereas levels of MMP-2 and MMP-9 remained unchanged (Fig. 5A, bottom), confirming the absence of specific off-target effects on the gelatinases. Surprisingly, down-regulation of MMP-1 in HT-hi/diss cells was accompanied by a substantial increase in CAM intravasation (2.3 ± 0.3-fold, p < 0.001) and liver metastasis (2.1 ± 0.3-fold, p = 0.003) in five independent experiments (Fig. 5E, middle and bottom). The specificity of this intravasation enhancement is further indicated by the absence of any effect of MMP-1 down-regulation on primary tumor growth (Fig. 5E, top).

We next verified the effects of siRNA-mediated down-regulation of MMP-2 on the dissemination of HT-hi/diss cells. Protein expression analyses performed 72 h after siRNA treatment confirmed efficient down-regulation of MMP-2: 69.7 ± 3.1% (n = 10) by zymography and 54.4 ± 9.4% (n = 4) by Western blotting (Fig. 5B). It is noteworthy that down-regulation of MMP-2 also did not induce significant off-target effects as indicated by comparable levels of MMP-9 detected as the 92-kDa gelatinolytic band (Fig. 5B, zymograph). When MMP-2 siRNA-treated HT-hi/diss cells were grafted on the CAM (six independent experiments), no significant effects on tumor growth were observed (Fig. 5E, top). However, down-regulation of MMP-2 in HT-hi/diss resulted in a 2.5-fold increase in intravasation (2.5 ± 0.3, p < 0.001) and a 2-fold increase in metastasis (Fig. 5E, middle and bottom).

In HT-hi/diss cells transfected with MMP-9 siRNA, Western blot analysis and zymography confirmed that MMP-9 production was significantly reduced even 72 h later (Fig.

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A

Western blot

MMP-9

MMP-2

MMP-2

siRNA: Control

B

Western blot

MMP-1

MMP-1

MMP-2

siRNA: Control

C

Western blot

MMP-1

MMP-9

MMP-2

siRNA: Control

D

Western blot

MMP-14

siRNA: Control

E

Tumor Growth

Fold difference

siRNA: Control

CAM Intravasation

Fold difference

siRNA: Control

Liver Metastasis

Fold difference

siRNA: Control

FIGURE 5. Down-regulation of MMPs in HT-hi/diss cells and its effect on tumorigenesis and tumor cell dissemination. HT-hi/diss cells were plated at 7 × 10^3 cells per 60-mm Petri dish and 18 h later transfected with siRNA constructs: control and specific for MMP-9 (A), MMP-2 (B), MMP-1 (C), and MMP-14 (D) as described under “Experimental Procedures.” After an overnight incubation, the cells were detached, washed, and resuspended in SF-DMEM. Cell suspensions were grafted on the CAM to analyze for dissemination in vitro. After an overnight incubation, the cells were detached, washed, and resuspended in SF-DMEM. Cell suspensions were grafted on the CAM to analyze for dissemination in vivo and also cultured in vitro to confirm down-regulation of a corresponding MMP. A–D, aliquots of the cells were re-plated in D-10 at 0.5–1.0 × 10^5 cells per well of a 6-well cluster. The following day, D-10 was exchanged for SF-DMEM, and 24–48 h later conditioned medium (A–C) or cell lysates (D) were collected and analyzed by Western blotting (top) and zymography (bottom) for expression levels of indicated MMPs. Levels of MMPs unrelated to a targeted MMP are shown in zymographs of conditioned media to illustrate the lack of significant off-target effects of specific siRNA compared with a control siRNA as follows: MMP-2 in cells treated with MMP-9 siRNA and scrambled siRNA (A), MMP-9 in cells treated with MMP-2 siRNA and scrambled siRNA (B), MMP-2 and MMP-9 in cells treated with MMP-1 siRNA and GFP siRNA (C), MMP-9 in cells treated with MMP-14 siRNA and scrambled siRNA (D). E, siRNA transfected cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate to allow for activation of MMP-2 proenzyme by MMP-14. Therefore, the lack of active forms of MMP-2 in MMP-14 siRNA-treated cells indicates efficient down-regulation of MMP-14 activity. E, to analyze the effects of MMP down-regulation on tumor cell tumorigenesis (top), CAM intravasation (middle), and liver metastasis (bottom), siRNA-treated cells were grafted on the dropped CAM at 0.4–1.0 × 10^5 cells per chick embryo. Five days after cell grafting, the embryos were sacrificed, and the tumors were excised and weighed. The portions of the distal CAM and liver were analyzed by quantitative Alu-qPCR to determine the numbers of human cells within chick tissue samples. The average tumor weight in the control group was 162 ± 5 mg (n = 149). The average number of HT-hi/diss cells calculated as intravasated to the CAM and metastasized to the liver in control embryos was 3958 ± 398 and 1452 ± 199 cell equivalents per 60 ng of total DNA extracted from the corresponding tissue. The data are means ± S.E. of fold differences determined from the ratios of individual embryos over a mean of a corresponding control determined for each independent experiment. Statistical analysis was performed by using two-tailed Student’s t test to compare fold differences pooled from 5 to 12 independent experiments employing a total of 36–73 embryos for each siRNA treatment. *p < 0.05.
individual tumor MMPs, we have generated HT-{-hi/diss} cells in which MMP-9 is stably suppressed by a lentiviral construct expressing MMP-9 shRNA. Zymographic and Western blot analyses confirmed substantial down-regulation of MMP-9 in the transduced cells in vitro (Fig. 6A, left panels). When grafted in the chick embryo, the HT-{-hi/diss} cells, in which MMP-9 gene expression was stably silenced with the shRNA virus construct, formed CAM tumors expressing significantly low amounts of MMP-9 protein as compared with their control counterparts as indicated by zymography and Western blotting (Fig. 6A, right panels). MMP-9 suppression with the shRNA vector did not significantly affect the development of HT-{-hi/diss} tumors (Fig. 6B, top). However, intravasation of HT-{-hi/diss} cells infected with the MMP-9 shRNA virus vector was dramatically increased by 3.98 ± 0.96-fold \((p < 0.005)\) (Fig. 6B, bottom). This finding affirmed that the substantial increase in tumor cell dissemination upon MMP down-regulation in the CAM model is not dependent on the method of RNAi delivery or the nature of the RNAi construct.

A rescue approach was then applied to verify whether it is a diminishment in MMP-9 protein that indeed causally enhances tumor cell invasion. The HT-{-hi/diss} cells in which MMP-9 was stably down-regulated by the MMP-9 shRNA construct were grafted on the CAM, and 24 h later, the CAM area with the developing tumor was treated with either buffer or 100 ng of human recombinant pro-MMP-9 (rMMP-9). The treatment was repeated 48 h later by applying 200 ng of rMMP-9 onto the growing primary tumor. Within 24–48 h after the second application, the primary tumors were excised, weighed, and assessed for reversal of enhanced tumor cell dissemination. The primary tumors developed from HT-{-hi/diss} cells transduced with recombinant MMP-9 or MMP-9 shRNA vectors were treated with recombinant MMP-9 at 100 and 200 ng per embryo, respectively, or buffer alone (0.1% bovine serum albumin, 5% Me₂SO in PBS). On day 4, tumors were excised and weighed. The proteins were extracted from the individual tumors with mRIPA buffer, and 50 µg of total protein from individual tumors were separated by SDS-PAGE under nonreducing conditions and analyzed by zymography (right bottom). Vertical lines have been placed over the digital image of the same zymograph to separate the different groups of individual tumors that were analyzed simultaneously to ensure quantitative comparison of protein expression between variants. B, MMP-9 levels are inversely related to the invvasation ability of HT-{-hi/diss} cells. The growth of individual primary tumors developed from HT-{-hi/diss} cells transduced with control and MMP-9 shRNA constructs and treated and nontreated with rMMP-9 was compared as a fold difference in tumor weight against the mean of control shRNA group (top). The average tumor weight in control was 229 ± 13 mg \((n = 30)\). The portions of distal CAM were harvested and analyzed by quantitative Alu-qPCR to determine numbers of intravasated human cells (bottom). The average number of intravasated HT-{-hi/diss} cells transduced with control shRNA vector was 763 ± 135 \((n = 30)\) per 60 ng of total DNA extracted from the CAM tissue. C, reversal of enhanced intravasation by HT-{-hi/diss} cells transduced with MMP-9 shRNA requires the activity of exogenously added rMMP-9. Individual tumors developing from HT-{-hi/diss} cells with stably down-regulated MMP-9 were treated with TIMP-free rMMP-9 (rMMP-9/PBS) or MMP-9 stoichiometrically complexed with TIMP-1 (rMMP-9/TIMP-1) and purified by gelatin-Sepharose chromatography. The presence of MMP-9 and TIMP-1 in corresponding eluate fractions was verified by zymography and Western blotting (insets above the respective bars). Treatments with the rMMP-9/PBS and rMMP-9/TIMP-1 fractions were performed on day 1 (100 ng per tumor) and day 3 (150 ng per tumor) after cell grafting. The following day, the samples of distal CAM were harvested and analyzed for the numbers of intravasated cells (bar graph). Data are means ± S.E. of fold differences determined as ratios of numerical values for each individual embryo over control. Fold differences were pooled from four independent experiments employing a total of 8–33 embryos per variant. *\(p < 0.05\) for shMMP-9 groups treated and nontreated with rMMP-9.
processing and accumulation of low molecular weight MMP-9 species (Fig. 6A, right). The increase in the total levels of MMP-9 protein in the primary tumors was accompanied by a ~67% decrease in intravasation as compared with the levels observed in the animals treated with buffer alone \( (p < 0.05) \) (Fig. 6B, bottom), reversing the substantially enhanced intravasation back to near control levels. These findings indicate that the amount of human MMP-9, tumor-derived or exogenously added, is inversely associated with the extent of HT-hi/diss intravasation.

Finally, we took advantage of the fact that MMP-9 complexed with TIMP-1 undergoes much less activation and exhibits reduced enzymatic activity as compared with unencumbered, TIMP-free MMP-9 (41–43). This feature allowed us to address whether the proteolytic activity of exogenously added rMMP-9 proenzyme is involved in the apparent modulation of tumor cell intravasation in the rescue experiments. The stoichiometric MMP-9–TIMP-1 complex was purified by incubating the rMMP-9 proenzyme with a 5-fold molar excess of rTIMP-1, followed by separation of the generated 1:1 rMMP-9–rTIMP-1 complexes by gelatin-Sepharose chromatography. For the control preparation of rMMP-9, rTIMP-1 was omitted from the preincubation allowing for elution of rMMP-9 incubated in PBS alone (rMMP-9/PBS). The presence of MMP-9 and TIMP-1 in the corresponding gelatin-Sepharose fractions was verified by zymography and Western blotting (Fig. 6C, insets). In the absence of TIMP-1, eluted rMMP-9/PBS caused a greater than 60% decrease in tumor cell intravasation (Fig. 6C), which is consistent with the effect of exogenous, TIMP-free rMMP-9 (Fig. 6B). In contrast, the rMMP-9–TIMP-1 complex, although containing equal levels of MMP-9 protein and gelatinolytic activity (see corresponding insets in Fig. 6C), failed to diminish the enhanced intravasation of HT-hi/diss cells in which MMP-9 had been stably suppressed by shRNAi.

Altogether, these findings indicate that the proteolytic activity of MMP-9 protein or the products generated by this activity are responsible for a decrease in tumor cell intravasation, and therefore they point to direct protective functions of the MMP-9 enzyme in early stages of tumor cell dissemination.

**DISCUSSION**

The goal of this study was to link the MMP/TIMP system to tumor cell intravasation and to analyze how MMPs and their natural inhibitors are possibly involved in this very early step of metastasis. Up to now, no MMPs have been examined in the context of the intravasation step in the metastatic cascade. Taking advantage of the available two intravasation cell variants generated from the same parental HT-1080 fibrosarcoma (21), we extensively profiled MMP and TIMP gene and protein expression in HT-hi/diss and HT-lo/diss cultures in vitro and in tumors in vivo (Figs. 1–3) in search for tumor-derived MMPs and TIMPs that might be critical in the onset of cancer cell dissemination. Comparison of mRNA and protein levels from both cell cultures and primary tumors indicated several candidates that were differentially expressed between HT-hi/diss and HT-lo/diss. Human MMP-1 and MMP-9 were more abundant in the HT-hi/diss than in the HT-lo/diss both in vitro and in vivo. In contrast, MMP-2, TIMP-1, and TIMP-2 were consistently expressed at higher levels in the HT-lo/diss cultures and CAM tumors. MMP-14 protein analysis demonstrated more degradation species in the HT-hi/diss in contrast to more proenzyme/full-length enzyme species in the HT-lo/diss, possibly pointing to higher enzymatic/self-degrading activity of MMP-14 in the high intravasating variant.

The importance of the MMP/TIMP system in HT-hi/diss intravasation and metastasis is indicated by a significant reduction of intravasation and metastasis by treatment with a broad range MMP inhibitor GM6001 (Ilomastat) as well as with recombinant TIMP-1 and TIMP-2. Supplementing the HT-hi/diss developing tumors with TIMP-1 and TIMP-2 proteins resulted in the anticipated decrease of HT-hi/diss intravasation. These findings clearly implicated MMPs in tumor cell intravasation and prompted us to extensively analyze the role of individual tumor MMPs during the early stages of tumor cell dissemination.

To examine the functional role of tumor-derived MMPs in the intravasation process, we utilized RNAi gene silencing to specifically down-regulate those individual MMPs and TIMPs, which demonstrated a significant differential between HT-lo/diss and HT-hi/diss. Because of their higher expression at both mRNA and protein levels, MMP-2, TIMP-1, and TIMP-2 were individually down-regulated in HT-lo/diss. Although diminishment of TIMP-1 did not affect HT-lo/diss dissemination, down-regulation of MMP-2 and TIMP-2 resulted in a significant increase of HT-lo/diss intravasation (Fig. 4). However, dissemination of HT-lo/diss never reached the levels of intravasation or metastasis characteristic of HT-hi/diss, indicating that elevated expression of MMP-2 and TIMP-2 is unlikely to play the major role in the impaired dissemination of HT-lo/diss cells. In this regard, when active uPA, which plays a substantial role in HT-hi/diss intravasation and is completely absent in HT-lo/diss, was added to HT-lo/diss tumors, it also only moderately enhanced cell intravasation, however, not bringing it to the level of HT-hi/diss (35). In conjunction with the results herein, these findings suggest that the deficiency in a single protease or proteolytic pathway is unlikely to determine the intravasation deficiency of individual tumor cells.

Our comparative analysis of HT-hi/diss and HT-lo/diss cells showed that HT-hi/diss cells had considerably higher levels of MMP-1 and MMP-9 than HT-lo/diss cells, suggesting a pro-metastatic function of these tumor-derived MMPs. However, down-regulation of MMP-1 in HT-hi/diss cells resulted in a greater than 2-fold increase in tumor cell intravasation, pointing to an unexpected function of this MMP. It is interesting that shRNA-mediated down-regulation of MMP-1 demonstrated a lack of its contribution to the frequency of tumor formation in a breast carcinoma xenograft model (44). Therefore, it appears that the mechanisms by which MMP-1 is involved in tumor cell dissemination are not entirely clear and may highlight both pro-metastatic and anti-metastatic attributes of this collagenase depending on the stage of progression and type of tumor.

Down-regulation of another MMP, i.e. MMP-9 differentially up-regulated in HT-hi/diss, demonstrated overall a 3–4-fold increase of HT-hi/diss intravasation in the chick embryo model. In mammalian model systems, RNAi targeting of MMP-9 partially reduced experimental lung metastasis of rat.
sarcoma (45) or decreased xenograft growth of DU145 human prostate cells in mice (46). It is noteworthy that efficient attenuation of tumor angiogenesis and invasion in other murine models by silencing of tumor MMP-9 required its down-regulation synergistically with cathepsin B, or uPA receptor, or uPA receptor plus uPA (47–50). These findings indicate that MMP-9 can be a potentially difficult molecule to safely target without inducing contrasting effects on cancer progression (8). This contention was extended in our model system not only to MMP-9 but to yet another gelatinase, MMP-2, whose down-regulation in HT-hi/diss also generated a significant 2.5-fold increase in intravasation despite the fact that, unlike MMP-9 and MMP-1, its expression in HT-hi/diss was lower than in HT-lo/diss. Altogether, these findings allow us to add interstitial collagenase, MMP-1, and the two gelatinases, MMP-2 and MMP-9, to the list of tumor MMPs with likely protective functions in HT-hi/diss dissemination at least in our model system.

Consistent with our previous observation on MMP-14 in HT-hi/diss cells (21), but still in contrast with results from another group employing parental HT-1080 cells (51), down-regulation of membrane-bound MMP-14 did not affect HT-hi/diss intravasation or metastasis (Fig. 5). Despite efficient siRNA down-regulation of MMP-14, we were not able to decrease intravasation and metastasis of the parental HT-1080 cells as well as dissemination of another cancer type, i.e. human epidermoid carcinoma HEp3 (data not shown). MMP-14 has been shown to play a crucial role in tumor cell invasion and angiogenesis, especially in neovessel formation ex vivo (3, 34, 51–53). The angiogenic aspect may not play a significant role in tumor cell intravasation in the chick embryo model, because the CAM is a highly vascularized tissue with a dense network of capillaries readily available for intravasation. It is also possible that tumor cells placed on the CAM are in such close proximity to the vascular bed that it may limit the role of MMP-14 expressed on tumor cells in making a pathway toward angiogenic blood vessels. Therefore, it is likely that the major effects of MMP-14 are poised on tumor cell migration and invasion but not ultimately on vascular dissemination.

Although the molecular analyses combined with our functional examination in vivo clearly point to an important role of the MMP/TIMP system in tumor cell intravasation, the contribution of tumor-derived MMPs and TIMPs could not be straightforwardly interpreted by MMP-mediated ECM degradation or release of active pro-angiogenic factors. Mounting evidence indicates that certain MMPs may actually negatively regulate certain aspects of tumor progression. Specifically, MMP-9 has been shown to play a protective role in tumor development through the release of the angiogenic inhibitor tumstatin (54). Thus, decreased levels of tumstatin in MMP-9-deficient mice were associated with increased angiogenesis and accelerated growth of Lewis lung carcinoma implants (55). MMP-mediated production of yet another angiogenic inhibitor, endostatin, also implicates MMP-9, among other MMPs, in suppression of tumor progression (56, 57). In addition, increased levels of angiostatin, produced in α1-null mice over-expressing MMP-9, were attributed to the decreased tumor angiogenesis and reduced tumorigenesis (58, 59). There are also findings demonstrating that other MMPs, such as MMP-7, MMP-8, MMP-11, and MMP-12, indeed exhibit protective roles in certain types of tumors and that their tumor-suppressive functions may also be independent of the production of known anti-angiogenic factors (37, 38, 60–63).

The actual molecular and/or catalytic mechanisms of how certain MMPs serve as protectors or negative regulators in specific stages of tumor progression have not been elucidated. Similarly, in our model system the mechanisms as to how down-regulation of specifically tumor-derived MMP-9, MMP-2, and MMP-1 leads to enhanced intravasation are not understood. From the rescue experiments involving purified recombinant pro-MMP-9 added to the tumors developing from the cells with down-regulated MMP-9 (Fig. 6), it is clear that the enhanced intravasation of HT-hi/diss cells is because of diminished levels of MMP-9 protein expression and not off-target effects of the RNAi treatments. Furthermore, the failure to reverse the enhanced intravasation with the rMMP-9-TIMP-1 complex, previously shown to be impaired in both proenzyme activation and its activity (41–43), suggests mechanistically that the pro-MMP-9 needs to be activated and catalytically active to modulate tumor cell intravasation. These findings suggest that MMP-9 may proteolytically generate one or more negative regulators of intravasation and that upon down-regulating MMP-9 protein the putative negative regulation is dampened. The added recombinant MMP-9 and apparently its proteolytic activity may restore the generation of the negative regulators or suppressors, thereby bringing the HT-hi/diss intravasation back to the levels characteristic of the untreated cells.

Speculating on the nature of the relevant protein substrates cleaved by active MMP-9, generating not positive effectors of tumor progression but putative negative regulators of intravasation, might be warranted in light of our paradoxical results. The most well established in vivo substrates for MMP-9 are the ECM-derived, unfolded fibrillar collagens and native type IV collagen, although a significant number of other proteins, not normally associated with the ECM, have been shown to be cleaved by MMP-9 in vitro (64). Cleavage of most of these ECM and non-ECM proteinaceous substrates by tumor-derived MMP-9 generally has been positively associated either with creating pathways for tumor cell invasion or generating/releasing bioactive factors such as vascular endothelial growth factor and transforming growth factor-β enhancing tumor angiogenesis and progression (65, 66). In contrast, our results force us to speculate that a specific inhibitor or dampener of intravasation is generated as the result of cleavage by the MMP-9 enzyme. Such an inhibitor could be a previously undefined fragment of those MMP-9 susceptible collagens that are present in our model system. Conversely, the intravasation suppressor could be a known fragment of ECM collagen that might have been shown previously to be inhibitory for other tumor-related processes such as tumor angiogenesis but not yet molecularly tested for the effects on intravasation. Tumstatin, derived from type IV collagen via MMP-9 cleavage (54), would be an example of a possible intravasation suppressor that would need to be tested.

It should be considered that an alternative to the MMP-9-generated negative regulator of intravasation might exist in the model. It is possible that an enhancer of intravasation is pro-
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duced in the tumor tissue, which is susceptible to catalytic degradation or inactivation by MMP-9. Consequently, down-regulation of MMP-9 would up-regulate the levels of such an enhancer and therefore increase the levels of intravasation. However, because tumor cell intravasation itself is so poorly understood, specific enhancers of intravasation are yet undefined and speculation on how and where MMP-9 might catalytically inactivate such molecules is premature at this time. Thus far, limited proteomic and degradomic analyses have not yet elucidated specific MMP-9-mediated cleavage reactions occurring in the HT-hi/diss CAM tumor that might result in the production of a distinct negative regulator or suppressor.

Taken together, the results of this study indicate that a delicate balance exists between the contrasting roles of the MMPs in tumor cell intravasation. It is also likely that MMPs of tumor and stroma/host origin play multiple and probably opposing roles in different stages of tumor cell metastasis. In this regard, stroma-derived MMPs such as MMP-9, brought to the site of primary tumor formation by inflammatory cells, have been demonstrated to play a critical role in tumor progression (4, 67–71). Down-regulation of these stroma-derived MMPs, by exogenous addition of the broad range synthetic and specific natural MMP inhibitors, might be responsible for the substantial but not complete reduction of HT-hi/diss intravasation and metastasis in our experimental model. In contrast, down-regulation of these similar but tumor-derived MMPs led to a paradoxical enhancement of intravasation and metastasis. Studies in our laboratory are currently underway to delineate the factors and molecular mechanisms by which some tumor-derived MMPs negatively regulate tumor cell intravasation and might be catalytically distinct from stroma-derived MMPs.

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