# Regulation of Cell Invasion and Morphogenesis in a Three-dimensional Type I Collagen Matrix by Membrane-type Matrix Metalloproteinases 1, 2, and 3

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*Abstract.* During tissue-invasive events, migrating cells penetrate type I collagen-rich interstitial tissues by mobilizing undefined proteolytic enzymes. To screen for members of the matrix metalloproteinase (MMP) family that mediate collagen-invasive activity, an in vitro model system was developed wherein MDCK cells were stably transfected to overexpress each of ten different MMPs that have been linked to matrix remodeling states. MDCK cells were then stimulated with scatter factor/hepatocyte growth factor (SF/HGF) to initiate invasion and tubulogenesis atop either type I collagen or interstitial stroma to determine the ability of MMPs to accelerate, modify, or disrupt morphogenic responses. Neither secreted collagenases (MMP-1 and MMP-13), gelatinases (gelatinase A or B), stromelysins (MMP-3 and MMP-11), or matrilysin (MMP-7) affected SF/HGF-induced responses. By contrast, the membrane-anchored metalloproteinases, membrane-type 1 MMP, membrane-type 2 MMP, and membrane-type 3 MMP (MT1-, MT2-, and MT3-MMP) each modified the morphogenic program. Of the three MT-MMPs tested, only MT1-MMP and MT2-MMP were able to directly confer invasion-incompetent cells with the ability to penetrate type I collagen matrices. MT-MMP–dependent invasion proceeded independently of proMMP-2 activation, but required the enzymes to be membrane-anchored to the cell surface. These findings demonstrate that MT-MMP–expressing cells can penetrate and remodel type I collagen-rich tissues by using membrane-anchored metalloproteinases as pericellular collagenases.

Key words: collagen • metalloproteinases • scatter factor/hepatocyte growth factor • mock • tubulogenesis

## Introduction

During (patho)physiologic events ranging from growth and development to tumor invasion and metastasis, normal or neoplastic cells traverse tissue barriers comprised largely of type I collagen, the major structural component of the extracellular matrix (Liu et al., 1995; Pulcher et al., 1997; Benbow et al., 1999; Murphy and Gavrilovic, 1999). Whereas the collagen matrix is presumably remodeled during cell ingress, the triple-helical structure of type I collagen renders the molecule resistant to almost all forms of proteolytic attack save, in large part, to members of the matrix metalloproteinase (MMP)[1] gene family (Birkedal-Hansen, 1987; Nagase and Woessner, 1999). Interestingly, though several MMPs have been linked to the degradation of type I collagen during tissue-resorptive events, the identity of those proteinases that allow migrating cells to invade or remodel type I collagen barriers remains undefined (Benbow et al., 1999).

Beyond the identification of the specific MMPs that promote collagen degradation, additional constraints complicate our ability to understand the manner in which these systems are mobilized during invasive processes. First, MMPs are uniformly synthesized as inactive zymogens with latency maintained by the enzymes' prodomains. Hence, the expression of degradative activity usually requires a bimolecular proteolytic processing event wherein the MMP prodomain is removed (Nagase and Woessner, 1999). Secondly, the invading cell must either position the type I collagenase activity on its surface in a manner that allows tissue-invasive signals to be interpreted accurately or induce collagenolytic activity in surrounding cell populations in a spatially regulated manner (Werb, 1997; Mur-

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[1] *Abbreviations used in the paper:* MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; SBTI, soybean trypsin inhibitor; SF/HGF, scatter factor/hepatocyte growth factor; TIMP, tissue inhibitor of metalloproteinases.

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phy and Gavrilovic, 1999). Thirdly, both the processing of the MMP zymogen to its mature form and the retention of the enzyme's collagenolytic activity must take place in tissues that are normally bathed in high concentrations of circulating antiproteinases (Weiss, 1989).

Whereas solitary cells can display invasive phenotypes, branching morphogenesis requires cells to invade and remodel the surrounding collagen matrix in a coordinated fashion to ultimately establish a defined structure (Gumbiner, 1992; Birchmeier and Birchmeier, 1993). As collagen-degrading MMPs would be predicted to either enhance or modify such tubulogenic programs, we reasoned that an in vitro model that recapitulates a morphogenic process would provide a sensitive platform for identifying the subset of proteinases that participate in the remodeling of the type I collagen matrix. MDCK cells are highly differentiated epithelial cells that can be induced to undergo branching morphogenesis in type I collagen matrices in response to the morphogen, scatter factor/hepatocyte growth factor (SF/HGF; Montesano et al., 1991a,b, 1999). As such, we stably transfected MDCK cells to overexpress each of ten different MMPs that previously have been linked to invasive/morphogenic states (Nagase and Woessner, 1999), and examined the ability of these cells to interpret proinvasive, as well as tubulogenic signals. Surprisingly, seven of the ten secreted MMPs tested, which included the type I collagenases: MMP-1 (collagenase 1), MMP-13 (collagenase 3), and MMP-2 (gelatinase A), failed to modulate invasion or morphogenic responses. However, three MMPs were identified that either accelerated, disrupted, or modified branching tubulogenesis. Interestingly, this subset of proteinases all belong to the membrane-anchored family of MMPs (i.e., the membrane-type MMPs [MT-MMPs]; Murphy and Gavrilovic, 1999; Nagase and Woessner, 1999). The ability of membrane-anchored, rather than secreted, MMPs to regulate the SF/HGF-dependent tubulogenic program suggests that this class of enzymes has been specifically engineered to mediate pericellular proteolytic processes associated with cell invasion and morphogenesis through type I collagen-rich barriers.

# Materials and Methods

## Cell Culture

All cell lines were obtained from ATCC. MDCK and COS-1 cell lines were routinely maintained in complete medium: DME supplemented with 10% FCS (Atlanta Biologicals), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), all from Life Technologies. Stably transfected MDCK cell lines were maintained in normal medium containing 700 μg/ml geneticin (G418; Life Technologies). All cells were cultured at 37°C in a 5% CO$_2$/95% air atmosphere.

## Expression Vector Construction and Transfection

Full-length cDNAs were generated using the following primer sets: MT1-MMP, forward TCT CGG ACC ATG TCT CCC GCC CCA, reverse CTA TCA GAC CTT GTC CAG CAG GGA; MT2-MMP, forward GGG CGC CGA GAG CAT GGG CAG; reverse CTA TCA GAC CCA CTC CTG CAG CGA; MT3-MMP, forward GCA CAG TTC ACT ATG ATC TTA CTC, reverse CTA CTA CAC CCA CTC TTG CAT AGA; MMP-9 (gelatinase B), forward CTC ACC ATG AGC CTC TGG CAG CCC, reverse TAG TCC TCA GGG CAC TGC AGG ATG; MMP-2 (gelatinase A), forward GGG AGC GCT ACG ATG GAG GCG CTA, reverse CCA GGG CCA GCT CAG CAG CCT AGC; MMP-3 (stromelysin-1), forward GTA AAG CCA GTG GAA ATG AAG AGT, reverse CTA TCA ACA ATT AAG CCA GCT GTT. The resulting full-length PCR products were cloned into pCR3.1 Uni vector (Invitrogen) according to the manufacturer's instructions. Full-length MMP-1 (collagenase-1) cDNA was obtained by assembling a 5′ PCR fragment (using a primer set consisting of: forward, GGA AGC TTA CCT TGC ACT GAG AAA G, reverse TCC AAT ACC TGG GCC TGG T) and an XbaI fragment containing the 3′ end of human MMP-1 isolated from p35-1 (ATCC). Full-length human MMP-13 (collagenase-3; provided by Peter Mitchell, Pfizer, Groton, CT) was subcloned as a HindIII fragment in pCR3.1 Uni. Full-length human MMP-7 (matrilysin) cDNA was provided by Lynn Matrisian (Vanderbilt University, Nashville, TN) and subcloned in pCR3.1 Uni as an EcoRI fragment. Full-length MMP-11 cDNA (Pei et al., 1994) was subcloned into the HindIII/XbaI sites of pCR 3.1 Uni from pREP9. Soluble MT1-MMP (ΔMT1-MMP; Met$^1$-Gly$^{535}$), which lacks the COOH-terminal transmembrane and cytosolic domains of the wild-type proteinase, and a cytosolic tail-truncated mutant MT1-MMP (MT1-MMP$_{ct;}$ Met$^1$ to Phe$^{563}$) were generated as described (Ohuchi et al., 1997; Hiraoka et al., 1998). To generate soluble MT2-MMP (ΔMT2-MMP; Met$^1$-Asn$^{625}$), a BstEII–XhoI fragment was excised from a full-length MT2-MMP expression plasmid and replaced with a PCR fragment synthesized using a primer set consisting of: forward, TGT CTT TTT CAA AGG TGA CCG CTA CTG GCT CT, reverse, AGA CTC GAG CGG CCA ATT TCA GTT CAC CGT CCG TGC CAC CTC CTC CA. Epitope-tagged MT1-, MT2-, and MT3-MMP constructs were generated by inserting primers encoding the FLAG epitope (5′-GAC TAC AAG GAC GAC GAT GAC AAG-3′) after the COOH-terminal valine of each molecule and cloned into pCR3.1 Uni.

Stably transfected MDCK cell lines were generated using lipofectamine (Life Technologies) according to the manufacturer's instructions. After transfection, single colonies were isolated after 2–3 wk of G418 selection. For stable transfectants, results shown are representative of three or more clones tested. COS-1 cells were transiently transfected using lipofectamine in the same manner, but without G418 selection.

## Cell Proliferation, Scattering, and Migration Assays

To measure cell proliferation rates, $5 \times 10^4$ cells were plated into each well of a 12-well tissue culture plate (Costar, Corning Inc.) and cultured for 3, 5, or 7 d in complete medium as described above. At each time point, the cells were harvested by trypsinization and counted using a hemacytometer. Cell scattering was assessed by seeding $2 \times 10^5$ cells/well in 6-well tissue culture plates (Costar, Corning Inc.). After 18–24 h, the culture medium was replaced with fresh culture medium either alone or containing 50 ng/ml SF/HGF (Genentech, Inc.). After an additional 24 h, the cultures were examined using phase-contrast optics and photographed. Cell migration rates were assessed by seeding $2–5 \times 10^5$ cells atop collagen gels with surfaces partially covered by glass coverslips (1 × 2 mm; Corning Inc.). When the cells reached confluence, the glass fragments were removed, leaving a well-demarcated, cell-free area on the gel. At this time, SF/HGF (50 ng/ml) was added to all cultures in the absence or presence of the synthetic MMP inhibitor, BB-94 (5 μM; gift of British Biotechnology) and the distances migrated across the gels were measured 24 h later using an ocular micrometer on a Nikon inverted microscope.

## Invasion and Tubulogenesis Assays

*Collagen.* Type I collagen was prepared from rat tail tendons as described (Elsdale and Bard, 1972) and dissolved in 0.2% acetic acid to a final concentration of 2.7 mg/ml. To induce gelling, collagen was mixed with 10× DME and 0.34 N NaOH in an 8:1:1 ratio at 4°C, and 1 ml of this mixture was added to the upper well of a 24-mm Transwell dish (3-μm pore size; Corning, Inc.). After gelling was complete (45 min at 37°C), $2–5 \times 10^5$ cells in complete medium were added to the upper well. After an additional 24-h incubation period, SF/HGF was added to the lower compartment of the Transwell chambers at a final concentration of 50 ng/ml. In indicated experiments, protease inhibitors were added to both the upper and lower wells at the following final concentrations: 5 μM BB-94 (0.1% DMSO; final concentration); 200 ng/ml recombinant tissue inhibitor of metalloproteinase-1 (TIMP-1; Oncogene Research Products); 200 ng/ml recombinant TIMP-2 (gift of Amgen, Thousand Oaks, CA); 200 μg/ml aprotinin; 10 μM bestatin; 100 μg/ml soybean trypsin inhibitor (SBTI); 100 μM E64 (0.1% ethanol; final concentration); and 50 μM pepstatin (0.1% methanol; final concentration; all from Sigma-Aldrich). None of the solvents used affected MDCK or COS-1 cell behavior when tested alone. FCS was depleted of plasminogen or gelatinase, respectively, by lysine-sepharose or gelatin-sepharose (both from Amersham Pharmacia Biotech) affinity chromatography (Rosenthal et al., 1998). All media, including SF/HGF and inhibitors, were replaced every 2 d. MDCK and COS-1 invasion assays were routinely terminated after 12 and 5 d, respectively. Invasive foci were counted in randomly selected fields at 20× on a phase-contrast microscope. Invasion depths were measured from digitally captured images of hematoxylin and eosin-stained cross-sections.

*Matrigel.* Cell invasion into a reconstituted basement membrane extract was assessed by adding 800 μl of Matrigel (Becton Dickinson) to the upper well of 24-mm Transwell dishes. After gelling at 37°C for 30 min, $2–5 \times 10^5$ cells were added to the upper compartment. After a 24-h incubation period, the medium was exchanged and SF/HGF (50 ng/ml) was added to the lower chamber. Media and SF/HGF were replaced every 2 d in the course of a 12-d incubation period.

*Peritoneum.* Peritoneum-derived cultures were prepared by gently stretching fat-free portions of rat gut mesentery (resected from adult male rats anesthetized and then killed) over the opening of a 12-mm Transwell from which the polycarbonate membrane had been removed. The mesentery was tied onto the well with surgical silk thread, and the outer edge of the mesentery below the thread was cut away with a scalpel. Mesothelial cells were lysed by immersion in 1 N ammonium hydroxide for 1 h at room temperature. The wells were then rinsed repeatedly in culture medium and placed inverted in a 15-cm petri dish. A small bolus of type I collagen (~100 μl) was applied to the bottom of the peritoneum and was gelled for 45 min at 37°C. After gelling, the wells were inverted and placed in clean 12-well dishes and $1–2 \times 10^5$ cells were added to the upper wells onto the surface of the peritoneum in complete medium. After 24 h, the medium was exchanged and SF/HGF (50 ng/ml) was added to the lower compartments in the absence or presence of BB-94 as described above.

### Western Blots and Zymography

MMP protein expression by stably transfected clones was assessed by Western blotting as described (Pei and Weiss, 1995, 1996), with the exception of MT3-MMP (see below). For secreted MMPs (MMP-1, -2, -3, -7, -9, -11, and -13), as well as soluble ΔMT1-MMP and ΔMT2-MMP, MDCK clones were incubated for 24 h in serum-free medium and supernatants were collected in the presence of proteinase inhibitor cocktail (Calbiochem). MT-MMP proteins were solubilized from Triton X-114–extracted cells as described by Toth et al. (1997). Supernatants and detergent extracts were resolved by PAGE under reducing conditions and the proteins transferred to nitrocellulose membranes. Immunoblot analyses were performed with one of the following antibodies: MMP-1 rabbit polyclonal (gift of H. Birkedal-Hansen, National Institute of Dental and Craniofacial Research, Bethesda, MD) at 1:2,000; MMP-2 mouse monoclonal (Molecular Oncology, Inc.) at 1:2,000; MMP-3 mouse monoclonal (Calbiochem # IM236L) at 1:200; MMP-7 rabbit polyclonal (gift of H. Welgus, Parke-Davis, Ann Arbor, MI) at 1:500; MMP-9 rabbit polyclonal (gift of H. Welgus) at 1:5,000; MMP-11 rabbit polyclonal (Pei et al., 1994) at 1:1,000; MMP-13 rabbit polyclonal (gift of Peter Mitchell, Pfizer, Groton, CT) at 1:2,000; MT1-MMP mouse monoclonal (Calbiochem # IM39L) at 1:500; MT2-MMP mouse monoclonal (Calbiochem # IM48L) at 1:500; and mouse monoclonal M2 against a FLAG epitope (Sigma-Aldrich) at 1:1,000. After incubation with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody, protein bands were visualized using an ECL kit according to the manufacturer's instructions (Pierce Chemical Co.).

The ability of cells to activate MMP-2 was determined by gelatin zymography (Rosenthal et al., 1998). In brief, cells were incubated with serum-free medium containing recombinant proMMP-2. After a 24-h incubation at 37°C, the supernatants were collected and resolved under nonreducing conditions on 10% polyacrylamide gels impregnated with 2 mg/ml gelatin (Sigma-Aldrich). Gels were rinsed once in 2.5% Triton X-100 for 30 min at room temperature and then incubated in developing solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.6) for 8–16 h. Gels were stained with Coomassie blue and areas of gelatinolytic activity were detected as transparent bands.

### Northern Blot

As commercially available MT3-MMP antibodies lacked sensitivity (i.e., epitope-tagged MT3-MMP expressed in transiently transfected cells was detectable with antiepitope, but not anti-MT3-MMP antibodies; see below), stable clones were identified by Northern blot analysis. Total RNA was isolated from MT3-MMP–transfected and control MDCK cells using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA (10 μg/lane) was electrophoretically separated in a 1% agarose-formaldehyde gel, transferred to a nylon filter (Hybond-N; Amersham Pharmacia Biotech) and hybridized with $^{32}P$-labeled cDNA probes for human MT3-MMP and 36B4. Bands were visualized by autoradiography as described (Rosenthal et al., 1998).

### Immunofluorescence, Light, and Electron Microscopy

E-cadherin immunofluorescence was performed as described (Lochter et al., 1997a) using a mouse monoclonal E-cadherin antibody (Transduction Laboratories; # C20820) at 1:100 and Texas red-conjugated anti-mouse IgG (Vector Laboratories, Inc.). Fluorescent images were captured using a Spot digital camera (Diagnostic Instruments, Inc.) through a Leica upright microscope.

Collagen and Matrigel cultures were prepared for light microscopy after fixation in 4% paraformaldehyde in PBS. Fixed gels were then removed from the Transwell, dehydrated in a graded ethanol series, and embedded in paraffin. Sections (5–7-μm thick) were cut and stained with hematoxylin and eosin. For electron microscopy, gels were fixed in 2% glutaraldehyde/1.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Sections (500-nm thick) were stained with toluidine blue before examining by transmission electron microscopy (TEM) as described (Hiraoka et al., 1998). Peritoneum cultures were prepared as for TEM, and bright-field images of toluidine blue stained sections were captured digitally before TEM examination.

## Results

### Role of Secreted MMPs in Modulating SF/HGF-induced Invasion/Tubulogenesis

When cultured atop type I collagen matrices, MDCK cells formed confluent monolayers that remained confined to the surface of the underlying gel for the entire culture period (i.e., 12 d; Fig. 1 A). In the presence of SF/HGF, however, the epithelial cells rapidly invaded the underlying collagen matrix and assembled into complex branching tubular networks (Fig. 1 B). This SF/HGF-induced invasion/tubulogenesis program was unaffected either by inhibitors directed against serine proteinases (SBTI or aprotinin), cysteine proteinases (E-64), aspartate proteinases (pepstatin), aminopeptidases (bestatin), or by culturing cells under plasminogen-depleted conditions (Fig. 1 C). However, both the synthetic MMP inhibitor, BB-94, and the recombinant MMP inhibitor, TIMP-2, completely blocked the MDCK invasion program (Fig. 1, C and D).

Coincident with invasive programs in vivo, epithelial cells alone or in collaboration with surrounding stromal cells express a mix of collagenolytic and gelatinolytic MMPs that are secreted into the extracellular milieu (Werb, 1997; Murphy and Gavrilovic, 1999; Nagase and Woessner, 1999). To determine whether secreted enzymes that either: directly degrade type I collagen (i.e., MMP-1, MMP-13, or MMP-2); cooperate in collagenolytic processes (i.e., MMP-3, MMP-7, or MMP-9); or display patterns of expression that are tightly associated with invasive/morphogenic processes (i.e., MMP-11), can perturb invasion and/or branching morphogenesis, stably transfected MDCK clones overexpressing each of the respective MMPs were cultured atop type I collagen matrices and stimulated with SF/HGF. Surprisingly, despite the fact that the synthesis of each of the respective MMPs was increased significantly over wild-type cells (Fig. 2 A), none of the overexpressed proteinases altered the kinetics of

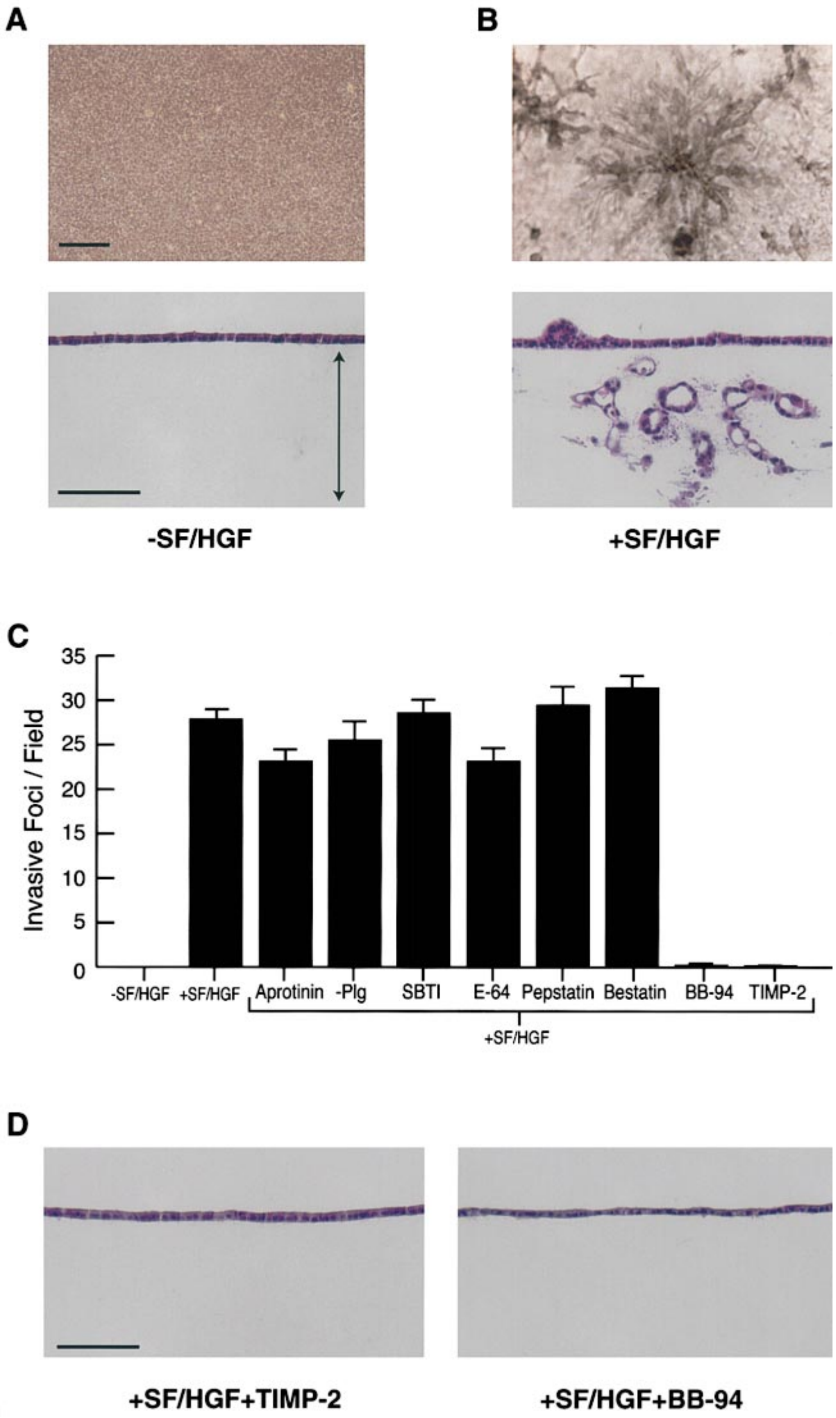


*Figure 1.* Invasive and tubulogenic properties of resting and SF/HGF-stimulated MDCK cells cultured atop a type I collagen matrix. A, Unstimulated MDCK cells on a collagen gel form a monolayer with cells confined to the gel surface (shown en face) in the top panel and as an H and E stained cross-section in the bottom panel. Bar, 100 μm. The double-headed arrow in the cross-section marks the boundaries of the collagen gel. B, After stimulation for 12 d with SF/HGF (50 ng/ml), MDCK cells invaded the collagen gel and formed a complex meshwork of branching tubular structures. C, MDCK invasion by SF/HGF-stimulated cells was unaffected by inhibitors of serine proteinases (200 μg/ml aprotinin, 100 μg/ml SBTI), cysteine proteinases (100 μM E-64), aspartate proteinases (50 μM pepstatin), aminopeptidases (10 μM bestatin), or serum plasminogen depletion (−Plg) in the course of a 12-d culture period. In contrast, BB-94 (5 μM) or recombinant TIMP-2 (200 ng/ml) completely blocked invasion. Results are shown as the mean number of invasive foci ± 1 SD in ten randomly selected fields in a single representative experiment of four performed. D, Representative cross-sections of MDCK cells stimulated with SF/HGF in the presence of either BB-94 or TIMP-2 for 12 d. Bar, 100 μm.

the invasive process or the characteristics of the tubulogenic program (Fig. 2, B–I). In data not shown, neither invasion nor tubulogenesis was enhanced when control or transfected clones were stimulated with SF/HGF under serum-free conditions in the absence or presence of 20 μg/ml plasminogen.

### *MT1-MMP Accelerates Invasion and Disrupts Branching Tubulogenesis*

Whereas secreted MMPs were unable to modify SF/HGF-induced type I collagen invasion, membrane-anchored MMPs could potentially allow cells to more productively focus collagenolytic activity to the pericellular milieu (Nakahara et al., 1997; Hiraoka et al., 1998; Murphy and Gavrilovic, 1999). After stable transfection with MT1-MMP cDNA, overexpressing MDCK cells proliferated at rates comparable to control transfectants (Fig. 3 A) and displayed normal epithelial morphology, as well as E-cadherin staining (Fig. 3 B). Furthermore, MT1-MMP transfectants scattered normally across a plastic 2-dimensional substratum in response to SF/HGF (Fig. 3 B) and migrated across the surface of type I collagen gels at rates comparable to control transfectants (i.e., 550 ± 23 μm/24 h versus

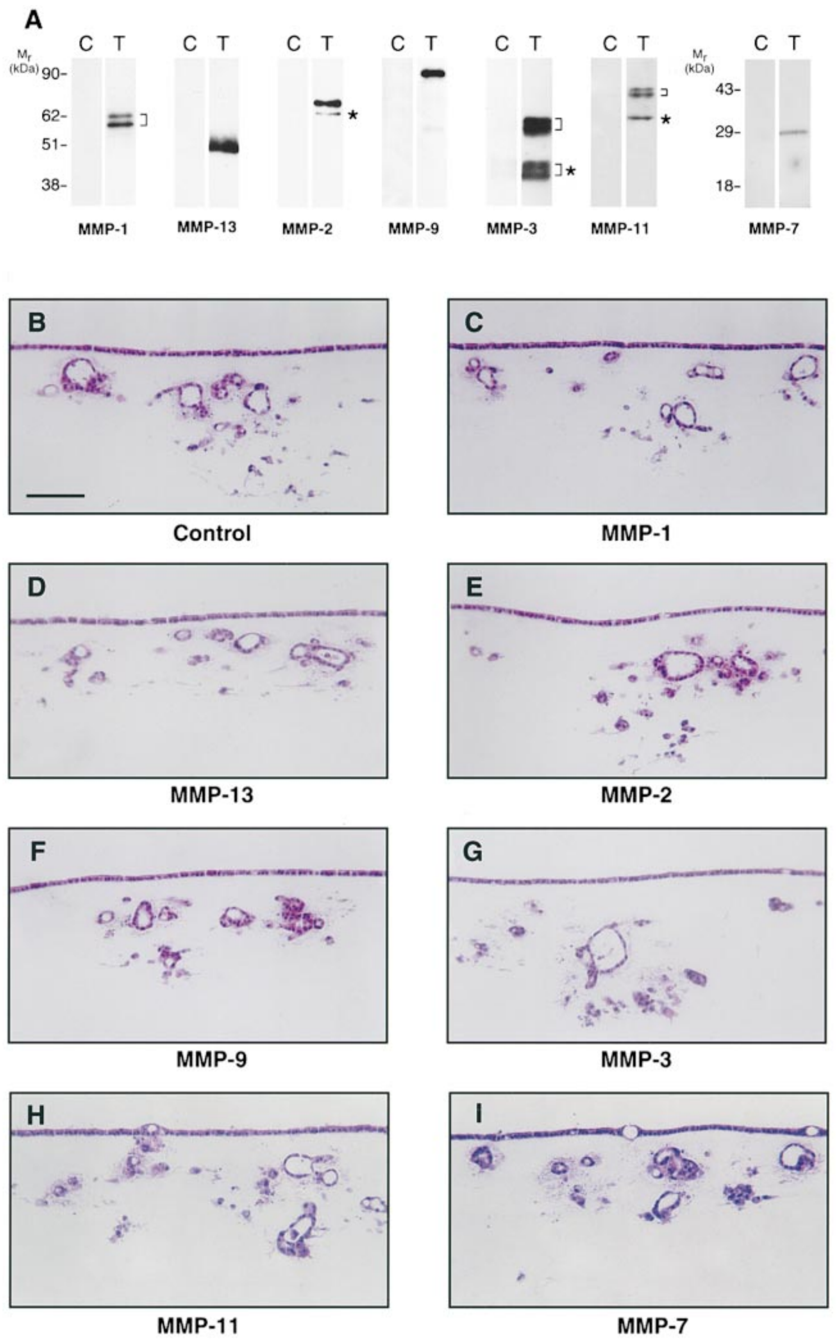


*Figure 2.* MDCK cells overexpressing secreted MMPs display normal invasive and morphogenic activities. A, Western blots showing the relative expression levels of the identified MMPs in control (C) and transfected (T) MDCK cells. Brackets indicate the positions of the glycosylated and nonglycosylated forms of proMMP-1, proMMP-3, and proMMP-11, respectively, whereas asterisks indicate active glycosylated and nonglycosylated forms of MMP-3, or active forms of MMP-2 and MMP-11. B–I, After stimulation with SF/HGF for 12 d, the invasion patterns of control transfectants (B) and the clones overexpressing each of the indicated MMPs (C–I) were indistinguishable. Neither control nor MMP transfectants displayed invasive activity in the absence of SF/HGF (data not shown). Bar, 100 μm.

563 ± 40 μm/24 h, $n = 3$, respectively). However, when MT1-MMP transfectants were cultured atop type I collagen gels and triggered with SF/HGF, invasion was markedly accelerated as early as day three (Fig. 3 C). By day 12, MT1-MMP transfectants cultured in the absence of SF/HGF also expressed a degradative activity that resulted in the formation of well-circumscribed pits. In the presence of SF/HGF, MDCK cells overexpressing MT1-MMP lost the ability to form a branching network and instead, completely degraded the underlying collagen substratum via a BB-94– or TIMP-2–sensitive process (Fig. 3 D). The addition of TIMP-1, which, in contrast to TIMP-2 does not effectively inhibit membrane-anchored metalloproteinases (Nagase and Woessner, 1999), had no effect on the heightened collagenolytic activity of the MT1-MMP transfectants (Fig. 3 D).

To identify structural features of the MT1-MMP molecule critical to the invasive process, stable transfectants were generated that expressed either a cytosolic tail-deleted form of the proteinase (MT1-$MMP_{ct}$) or a soluble, transmembrane-deleted mutant (ΔMT1-MMP). Whereas the enhanced degradative potential of MT1-MMP transfectants was not affected by deleting the cytosolic tail of the proteinase, membrane anchoring proved essential as the tubulogenic response of the wild-type phenotype was recovered when the metalloproteinase was expressed as a soluble mutant (Fig. 4, A–D). Because the transmembrane deletion mutant is secreted as a fully active enzyme

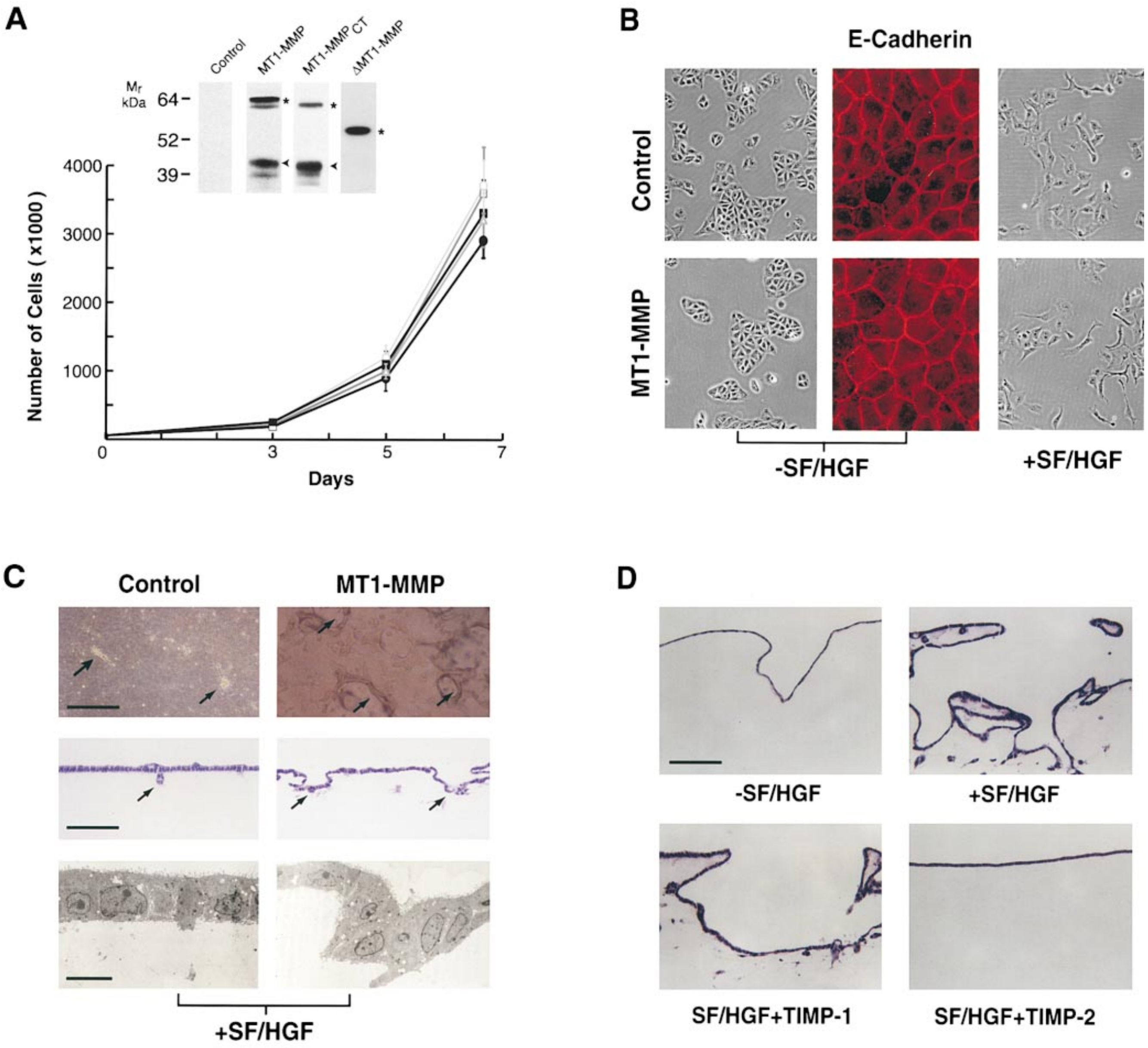


*Figure 3.* Characterization of MT1-MMP–overexpressing MDCK cells. A, Proliferative response of wild-type (■), vector control-transfected (●), full-length MT1-MMP (□), cytosolic tail-deleted MT1-MMP (MT1-MMP$_{ct}$; △), and soluble MT1-MMP (ΔMT1-MMP; ○) during a 7-d culture period. Cell number at each time point was measured in triplicate and the error bars represent the SD of a single representative experiment of three performed. The inset shows Western blots of MT1-MMP expression in either control or MT-MMP transfectants as indicated. Asterisks (*) mark the position of active MT1-MMP species whereas the arrowheads mark the position of a membrane-anchored, catalytically inactive fragment of MT1-MMP ($M_r$ ~43 kD; Lehti et al., 1998). MT1-MMP stable transfectants did not express detectable levels of MT2-MMP or MT3-MMP, as assessed by Western or Northern blot analysis, respectively. B, Epithelial morphology, E-cadherin staining, and SF/HGF-induced scattering responses of control or MT1-MMP–transfected cells cultured as described in Materials and Methods in either the absence or presence of SF/HGF. C, In the presence of SF/HGF, control vector-transfected MDCK cells cultured atop type I collagen gels for 3 d display widespread, but shallow foci of invasion (arrows) as viewed en face (top; bar, 100 μm), in cross-section (middle; bar, 100 μm), or by TEM (bottom; bar, 5 μm). In contrast, MDCK cells overexpressing MT1-MMP generated numerous large pits in the collagen matrix (arrows) extending well below the surface monolayer as viewed en face (top), in cross-section by light microscopy (middle) or by TEM (bottom). D, After 12 d, MT1-MMP overexpressing cells cultured in the absence of SF/HGF formed pits in the underlying collagen gel whereas SF/HGF-stimulated cells generated interconnecting cyst-like structures extending to the surface of the underlying polycarbonate membrane (compare to Fig. 1, A–D). Invasion by MT1-MMP transfectants was inhibited completely by TIMP-2, but not TIMP-1. Bar, 100 μm.

(Pei and Weiss, 1996; Ohuchi et al., 1997), these results indicate that MT1-MMP must be anchored to the cell surface to affect invasion and/or branching morphogenesis.

MT1-MMP not only is able to degrade a range of target substrates directly, but also indirectly as a function of its ability to process proMMP-2 or -13 to their active forms (Knauper et al., 1996; Pei and Weiss, 1996; D'Ortho et al., 1997; Ohuchi et al., 1997). Thus, we sought to determine

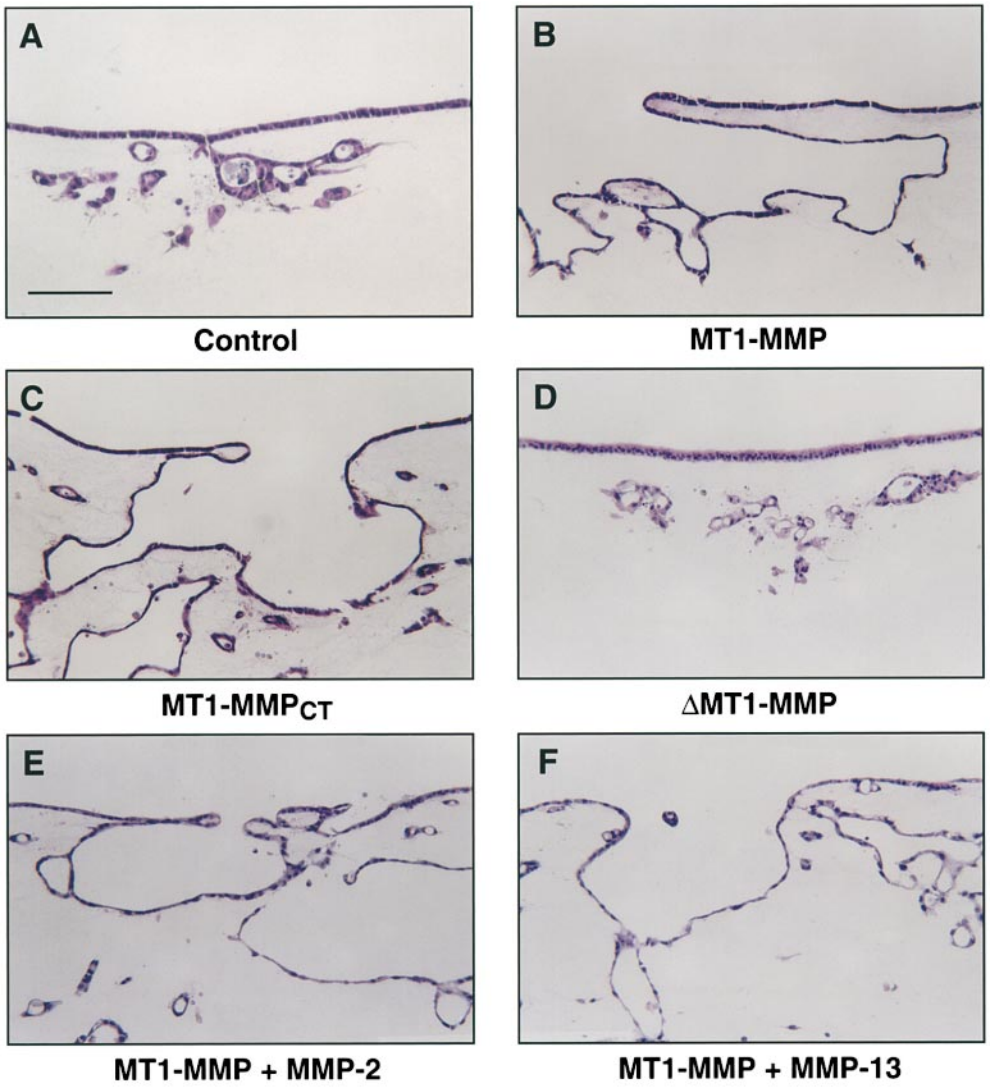
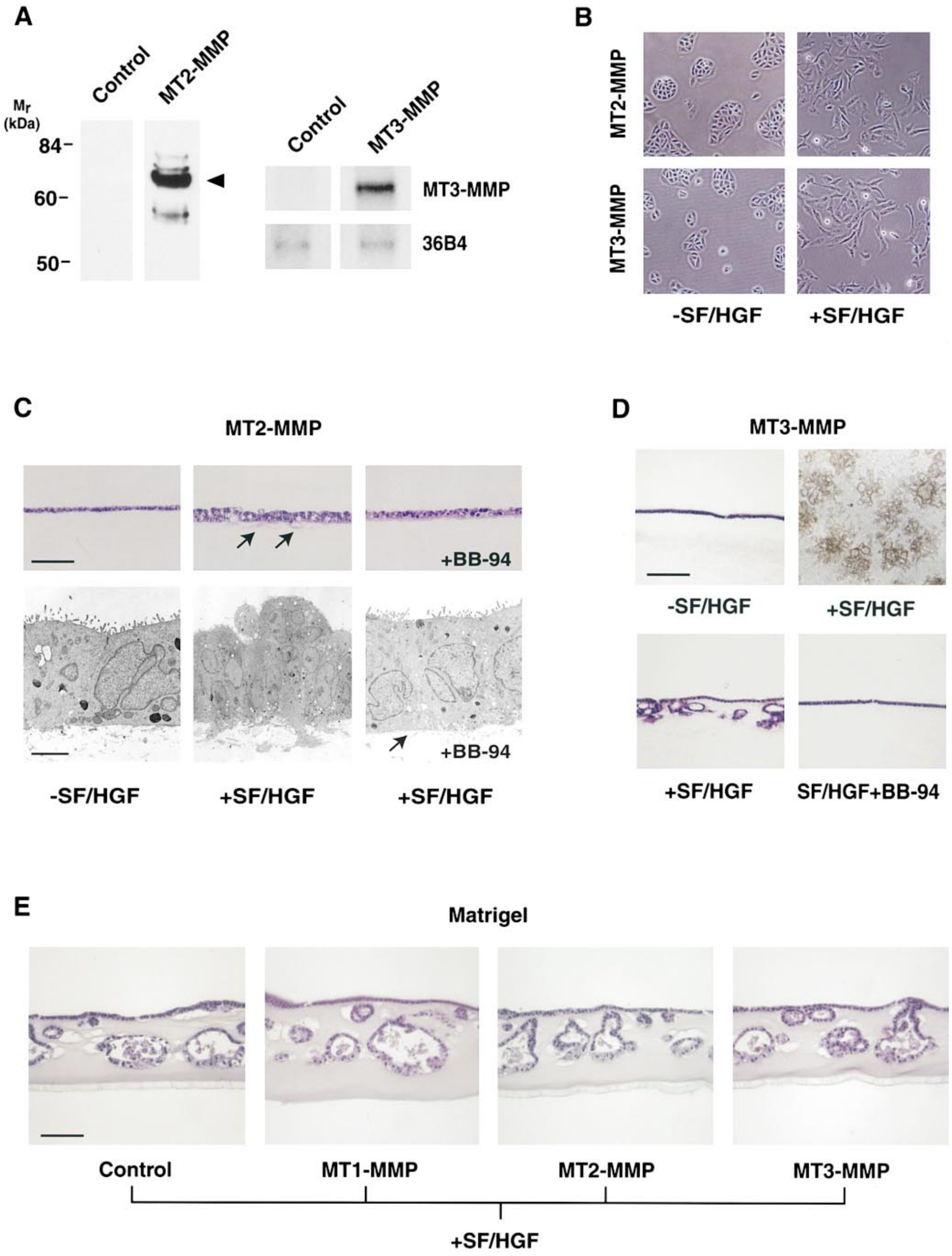


*Figure 4.* Regulation of MT1-MMP–dependent invasion and tubulogenesis. MDCK cell clones transfected with a control vector (A), MT1-MMP (B), MT1-MMP lacking the cytosolic domain (MT1-MMP$_{ct}$; C) or soluble MT1-MMP (ΔMT1-MMP; D) were cultured atop type I collagen gels for 12 d in complete medium with SF/HGF. Invasive activity of MT1-MMP overexpressing cells was not altered when cocultured in a 1:1 ratio with MDCK clones overexpressing MMP-2 (E) or MMP-13 (F). Bar, 100 μm.

whether either of these MMPs, though inactive in the morphogenic screen when overexpressed alone, would further modify the MT1-MMP–mediated invasion program. However, when MT1-MMP transfectants were cultured together at a 1:1 ratio with MDCK cells overexpressing either MMP-2 or MMP-13, neither invasion nor tubulogenesis was further enhanced (Fig. 4, E and F). Likewise, when MT1-MMP overexpressing cells (which express undetectable levels of endogenous MMP-2; Hiraoka et al., 1998) were cultured in FCS depleted of MMP-2, morphogenic responses were unaltered (data not shown).

### *MT-MMPs as Morphogenic Regulators*

MT1-MMP is structurally related to at least two other membrane-anchored MMPs, MT2-MMP and MT3-MMP (Nagase and Woessner, 1999). To determine whether invasion and/or branching morphogenesis could be similarly modulated by these proteinases, we isolated and examined stable MDCK clones that overexpressed either MT2-MMP or MT3-MMP alone (Fig. 5 A). As shown in Fig. 5, MT2-MMP and MT3-MMP transfectants displayed growth patterns (data not shown) and morphologic characteristics similar to control-transfected clones (Fig. 5 B). In addition, scattering responses across a plastic substratum (Fig. 5 B), as well as migratory rates across the surface of collagen gels, were unaffected (563 ± 40 μm/24 h, 533 ± 38 μm/24 h, and 575 ± 37 μm/24 h, respectively, and for control, MT2-MMP, and MT3-MMP transfectants; $n = 3$). Unexpectedly, however, MT2-MMP transfectants stimulated with SF/HGF lost almost all invasive and morphogenic activity in the three-dimensional collagen gel system (Fig. 5 C). Instead, MT2-MMP–overexpressing cells formed a disorganized multilayered structure atop the collagen matrix. While not frankly invasive, TEM analysis demonstrated that the SF/HGF-stimulated cells extended shallow, invasive processes into the collagen gel via a BB-94– or TIMP-2–sensitive mechanism (Fig. 5 C). Membrane anchoring of MT2-MMP was required for this effect as MDCK transfectants overexpressing soluble MT2-MMP displayed invasive and tubulogenic properties indistinguishable from control transfectants (data not shown). As epithelial cells that overexpress active MMPs have been reported to undergo an epithelial-to-mesenchymal cell transformation (Lochter et al., 1997b), the absence of a morphogenic response raised the possibility that MT2-MMP


A
Control
MT2-MMP
Mr
(kDa)
84–
60–
50–
Control
MT3-MMP
MT3-MMP
36B4
B
MT2-MMP
MT3-MMP
-SF/HGF
+SF/HGF
C
MT2-MMP
+BB-94
+BB-94
-SF/HGF
+SF/HGF
+SF/HGF
D
MT3-MMP
-SF/HGF
+SF/HGF
+SF/HGF
SF/HGF+BB-94
E
Matrigel
Control
MT1-MMP
MT2-MMP
MT3-MMP
+SF/HGF

transfectants had lost their epithelial cell-like characteristics. However, MT2-MMP overexpressing cells expressed a normal tubulogenic program when triggered with SF/HGF atop thick matrices of Matrigel (Fig. 5 E).

In contrast to the characteristics displayed by either MT1-MMP or MT2-MMP transfectants, MDCK clones overexpressing MT3-MMP mounted a nearly normal invasive and tubulogenic program in response to SF/HGF (Fig. 5 D). Cells overexpressing MT3-MMP invaded the collagen substratum at rates comparable to control cells after stimulation with SF/HGF and formed similar numbers of invasive foci (data not shown). The depth of invasion after the 12-d culture period was, however, significantly more shallow in the MT3-MMP transfectants (192 ± 29 μm in control transfectants versus 48 ± 21 μm in MT3-MMP transfectants; mean depth of invasion ± 1 SD in ten randomly selected sections in a single representative experiment of four performed) and the cells formed cyst-like structures, rather than the tubular network normally formed by invading control MDCK cells (Fig. 5 D). Similar to the findings obtained with the MT1-MMP and the MT2-MMP transfectants, the alteration in morphogenic properties was limited to the type I collagen substratum as MT3-MMP overexpressing MDCK cells displayed a normal tubulogenic response on Matrigel (Fig. 5 E).

### *MT-MMP–dependent Regulation of MDCK Invasion through Interstitial Stroma*

Whereas increasing the expression of MT-MMPs in MDCK cells altered invasion and branching morphogenesis in type I collagen gels, this reconstituted matrix displays physical properties distinct from those of cross-linked collagen in vivo (Birkedal-Hansen, 1987; Rosenthal et al., 1998). To determine the ability of MT-MMPs to remodel a more physiologic matrix, control or MT-MMP transfectants were cultured atop an acellular sheet of peritoneum-derived interstitial tissue (Fig. 6). This tissue consists of a network of type I collagen and elastin fibers sandwiched between two discontinuous basement membranes (Fig. 6 A, inset; Parsons et al., 1983). Significantly, SF/HGF-stimulated control MDCK cells were unable to cross this more complex barrier (Fig. 6, A and D). Cells overexpressing MT1-MMP, however, vigorously invaded the peritoneal tissue after SF/HGF stimulation, forming large pits similar to those seen on type I collagen and leaving only small stretches of peritoneum intact (Fig. 6, B and D). As expected, this degradative process was inhibited completely by TIMP-2 (data not shown) or BB-94 (Fig. 6, C and D). An even more dramatic effect was seen with MT2-MMP transfectants, as these cells completely degraded large stretches of the tissue within three to four days of SF/HGF stimulation, eventually eliminating nearly all visible tissue via a BB-94–sensitive process (Fig. 6 E).

In marked contrast to MT1- and MT2-MMP–overexpressing cells, neither the MT3-MMP nor MMP-1, -2, -3, -7, -9, -11, or -13 stable transfectants displayed invasive behavior on the peritoneum, and were essentially indistinguishable from wild-type– or control-transfected MDCK cells (data not shown). As the dissolution of this interstitial matrix occurred in the presence of high concentrations of serum that contains a full complement of endogenous proteinase inhibitors, these data demonstrate that MT1-MMP, as well as MT2-MMP, confer MDCK cells with the ability to digest collagen-rich tissues in an in vivo-like setting.

### *MT1-MMP and MT2-MMP Function as Membrane-anchored Invasive Factors*

Whereas MT1-MMP and MT2-MMP modified the morphogenic program displayed by SF/HGF-stimulated MDCK cells, the ability of these enzymes to regulate collagen-invasive activity directly cannot be ascertained by this experimental approach, as the host cell coexpresses a complement of endogenously derived proteinases (Montesano et al., 1991a,b, 1999). Consequently, COS-1 cells, which do not express MT-MMPs (Sato et al., 1994), were transiently transfected with MT1-, MT2-, or MT3-MMP and collagen-invasive activity monitored. As shown in Fig. 7 A, cells transfected with FLAG epitope-tagged constructs expressed each of the respective proteinases. Furthermore, though the mature forms of MT2-MMP or MT3-MMP have not yet been identified in the literature, the enzymatic activity of the proteinases was monitored indirectly as the ability of the transfected cells to process exogenously supplied progelatinase A to its mature form. Using this assay, each of the MT-MMP transfectants displayed comparable activity (Fig. 7 B). Significantly, when the MT-MMP–transfected COS-1 cells were cultured atop the type I collagen substratum, both MT1- and MT2-MMP–overexpressing cells acquired an invasive activity (Fig. 7, D and E) that was inhibitable by both BB-94 and TIMP-2 (Fig. 7, G and H). Invasion was completely dependent on mem-

*Figure 5.* Characterization of MT2-MMP and MT3-MMP overexpressing MDCK cells. A, Western blot analysis of MT2-MMP protein (arrow) in Triton X-114 cell extracts of transfected cells. The expression of the MT3-MMP transcript in transfected cells was shown by Northern blotting. A 36B4 control was run to confirm RNA integrity and equal loading. MT2-MMP stable transfectants did not express detectable levels of MT1-MMP or MT3-MMP (Western and Northern blot analysis, respectively), whereas MT3-MMP stable transfectants did not express detectable levels of MT1-MMP or MT2-MMP, as assessed by Western blot. B, Cell morphology and scattering response to SF/HGF was unaffected by either MT2-MMP or MT3-MMP overexpression (compare to Fig. 3 B). C, Unstimulated MT2-MMP–overexpressing cells formed a confluent monolayer on collagen as assessed in cross-sections (top left-hand corner) or by TEM (bottom left-hand corner). Bar, 1 μm. When stimulated with SF/HGF for 12 d, MT2-MMP transfectants formed a pseudostratified-like epithelial layer with basilar extensions intruding into the underlying collagen (arrows in light section and TEM in the middle panel of the bottom row). The extension of these processes into the collagen was blocked by BB-94. D, Unstimulated MT3-MMP transfectants formed a phenotypically normal confluent monolayer on type I collagen as depicted in a 12-d-old culture. When stimulated with SF/HGF, MT3-MMP overexpressors invaded shallowly and formed cyst-like structures (shown en face, and in cross-section) distinctly different from control cell invasion (see Fig. 1, B and D). The invasive activity of MT3-MMP transfectants was blocked completely by BB-94. Bar, 100 μm. E, Invasion and tubulogenesis by control vector-, MT1-MMP–, MT2-MMP–, or MT3-MMP–transfected cells cultured atop Matrigel with SF/HGF for 12 d in complete media. Bar, 100 μm.

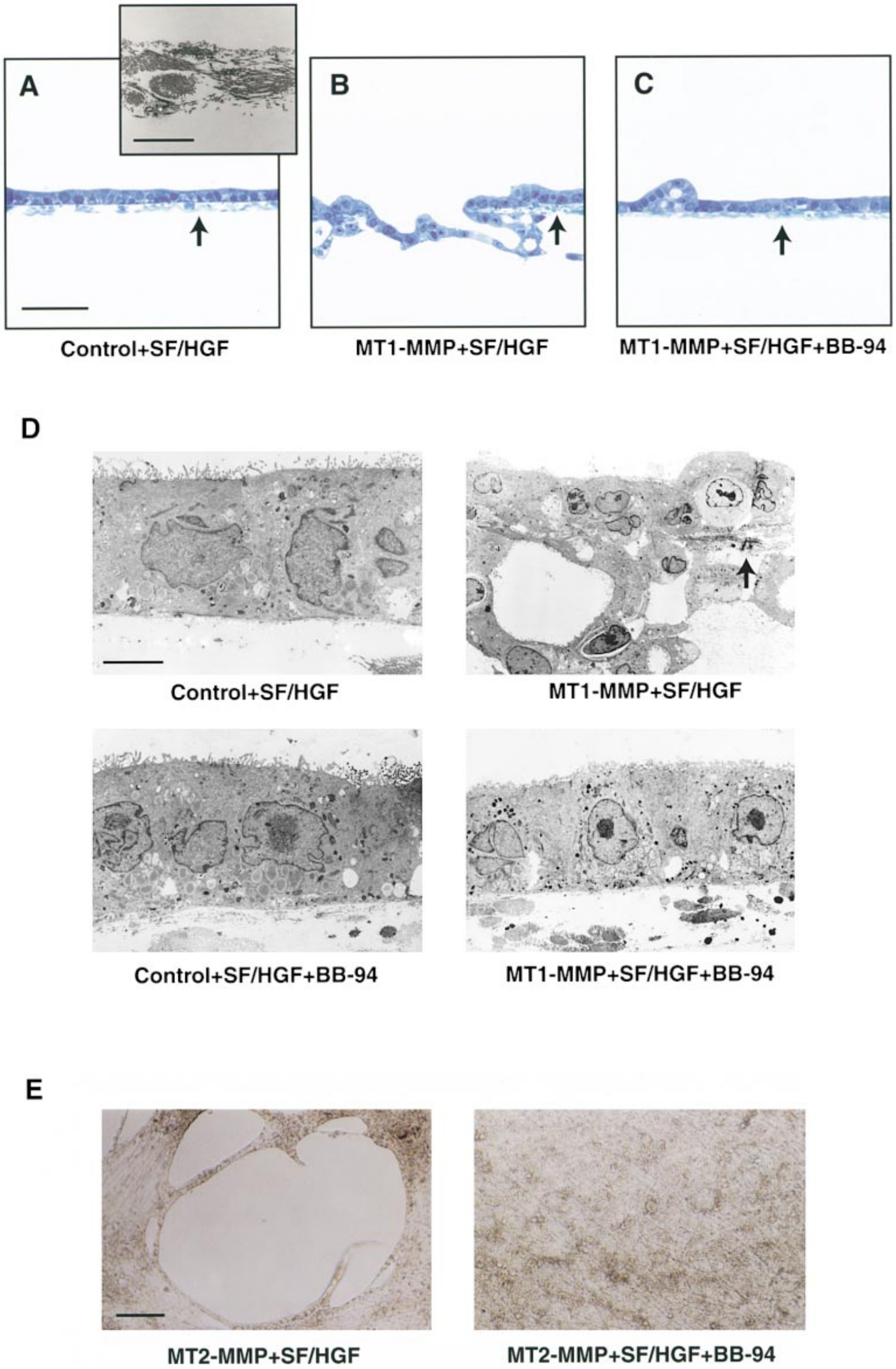


*Figure 6.* Invasion of peritoneal tissue by MDCK transfectants in vivo. A, Vector control-transfected MDCK cells cultured on the peritoneal matrix (arrow) did not express invasive activity after stimulation with SF/HGF for 12 d in complete media. Bar, 100 μm. The inset shows the peritoneal matrix by TEM after the mesothelial cell layers were removed. The tissue contains numerous collagen and elastin fibrils sandwiched between two discontinuous basement membranes. Bar, 1 μm. B, MT1-MMP–overexpressing cells degraded large blocks of tissue and invaded into the underlying collagen after stimulation with SF/HGF for 12 d. A small segment of remaining peritoneal tissue is indicated by the arrow. C, In the presence of BB-94, MT1-MMP transfectants failed to invade the peritoneum after 12 d in culture (arrow). D, TEM analysis of peritoneal tissue invasion by control or MT1-MMP transfectants cultured for 12 d with SF/HGF in the absence or presence of BB-94 as indicated. Only a portion of the peritoneum can be identified after culture with MT1-MMP–overexpressing cells (arrow). Bar, 1 μm. E, MT2-MMP–overexpressing cells completely degraded entire tracts of the peritoneum after stimulation with SF/HGF for 12 d as shown in an en face view of a live culture. The addition of BB-94 to these cultures blocked tissue dissolution and maintained an intact peritoneal matrix. Bar, 100 μm.

brane anchoring of the proteinases to the cell surface, as COS-1 cells transiently transfected with either soluble transmembrane-deleted MT1-MMP or MT2-MMP failed to express an invasive phenotype (1.0 ± 0.8 invasive foci/field and 2.0 ± 1.0, $n = 3$; invasive foci/field for ΔMT1-MMP and ΔMT2-MMP, respectively). MT3-MMP–transfected cells extended only a few short processes into the underlying collagen (Fig. 7 F) in a fashion similar to that observed in control-transfected cells (Fig. 7 C). Thus, both MT1-MMP and MT2-MMP, but not MT3-MMP, confer invasion-null cells with the ability to penetrate type I collagen matrices.

## *Discussion*

To traverse type I collagen-rich tissues, normal, as well

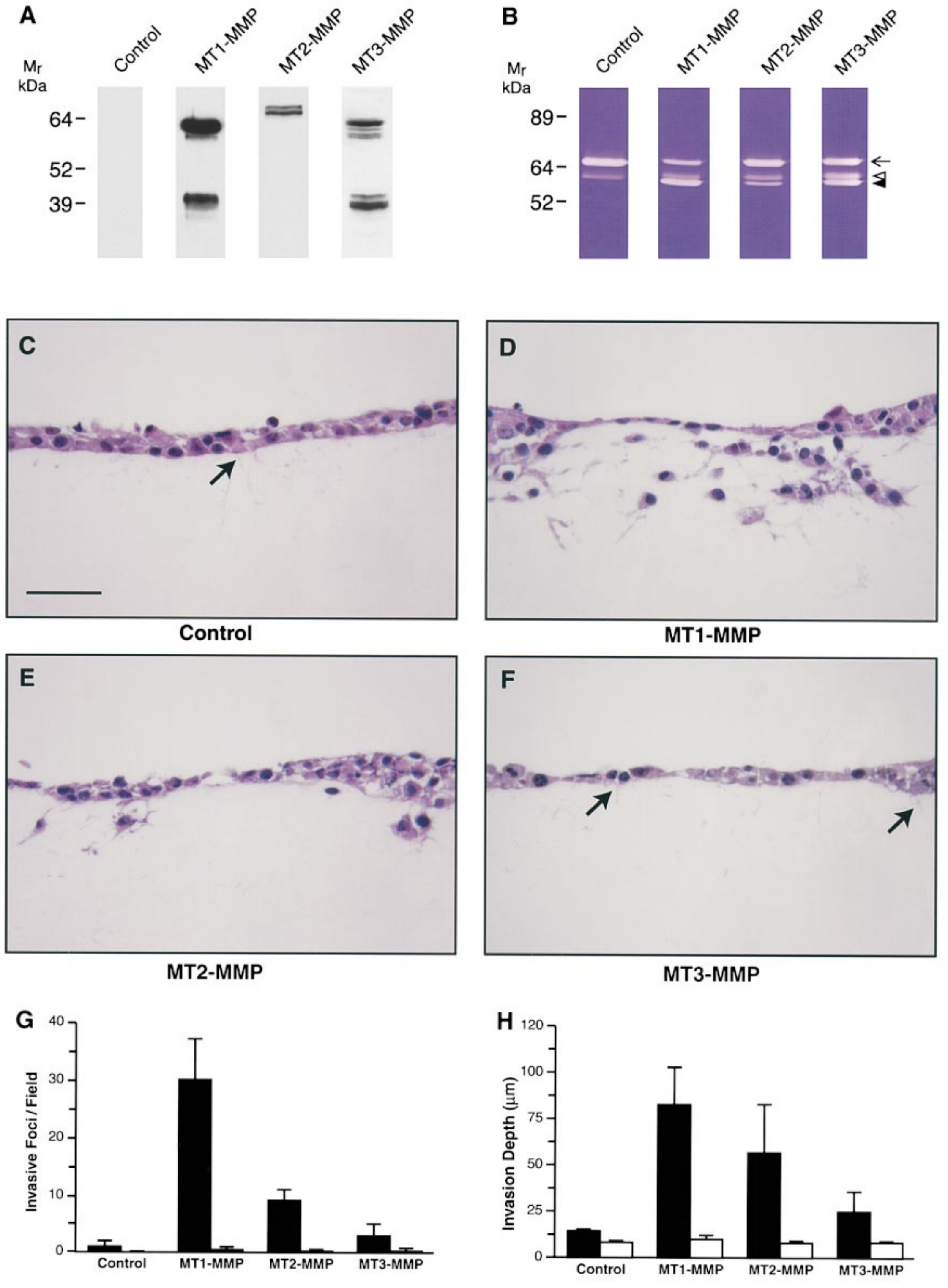


*Figure 7.* Collagen-invasive activity of MT-MMPs in transiently transfected COS-1 cells. A, Western blot analysis of COS-1 cells transiently transfected with FLAG epitope-tagged MT1-, MT2-, and MT3-MMP. Triton X-114 extracts were immunoblotted with anti-FLAG M2 mAb. B, MT-MMP activity in transiently transfected COS-1 cells was assessed by monitoring the activation of proMMP-2. The pro, intermediate, and active forms of MMP-2 are indicated by the arrow, clear arrowhead, and black arrowhead, respectively. C, Transverse light sections of control vector-transfected COS-1 cultured atop type I collagen gels for 5 d in the presence of SF/HGF were unable to express invasive activity, save for the extension of short processes (arrow) into the underlying substratum. COS-1 cells overexpressing MT1-MMP (D) or MT2-MMP (E) displayed invasive activity whereas MT3-MMP transfectants (F) were indistinguishable from control-transfected COS-1 cells. The number of invasive foci (G) and invasion depth (H) were assessed in transverse light sections of COS-1 cultures after a 5-d incubation period with SF/HGF in the absence or presence of BB-94 (shaded and open bars, respectively). Results are expressed as the mean ± 1 SD in ten randomly selected cross-sections in a single representative experiment of three performed. Bar, 50 μm.

as neoplastic, cells coordinately express motile and collagenolytic phenotypes (Werb, 1997; Murphy and Gavrilovic, 1999). Though multiple MMP family members can proteolyze fibrillar collagens (Pulcher et al., 1997; Benbow et al., 1999; Nagase and Woessner, 1999), the ability of these enzymes to promote or modify invasive activity through tissue barriers comprised of type I collagen has not been defined. MT1-MMP is the prototypical member of the subclass of membrane-anchored MMPs that display unique characteristics relative to the larger family of secreted MMPs (Nagase and Woessner, 1999). Like MT2- and MT3-MMPs (as well as the more recently described MT5- and MT6-MMPs; Llano et al., 1999; Pei, 1999a,b; Velasco et al., 2000), MT1-MMP has a short stretch of hydrophobic amino acids near its COOH terminus that is embedded in the plasma membrane (Nagase and Woessner, 1999). MT1-MMP also contains a basic motif (i.e., $^{108}$RRKR) at the COOH-terminal end of its prodomain that can act as a recognition site for proteolysis by one or more members of the proprotein convertase family (Sato et al., 1994; Pei and Weiss, 1996). Thus, whereas all secreted MMPs (with the exception of stromelysin-3, which contains a similar recognition motif; Pei and Weiss, 1995; Santavicca et al., 1996) are normally released as proenzymes, the MT1-MMP prodomain can be removed, and the active enzyme generated before, or coincident with, its delivery to the cell surface (Pei and Weiss, 1996; Sato et al., 1996; Yana and Weiss, 2000). Activation schemes for MT2-MMP or MT3-MMP have not been delineated to date, but given the presence of similar basic recognition motifs in homologous regions of their prodomains (Nagase and Woessner, 1999), these enzymes are likely processed to their mature forms via similar mechanisms.

MMP family members can potentially cleave integrins and cadherins, as well as other surface-associated molecules that can initiate a process leading to stable epithelial-to-mesenchymal conversion (Lochter et al., 1997b; Werb, 1997; Sternlicht et al., 1999). Consequently, we predicted that cells overexpressing MT-MMPs would display aberrant phenotypic characteristics similar to those described previously (Lochter et al., 1997b). However, neither MT1-, 2-, nor 3-MMP affected MDCK proliferation, cell–cell interactions, or scattering responses to SF/HGF. Furthermore, all of the MT-MMP transfectants retained the ability to generate patent tubular networks in Matrigel. MT1-MMP overexpressing clones did, however, display a markedly enhanced ability to invade type I collagen matrices resulting in the formation of cyst-like structures that eventually dissolved the underlying matrix. Interestingly, the transformation of a branching tubulogenesis-like program into one more reminiscent of cyst development has been observed in collagenase-treated embryonic submandibular glands induced to undergo branching in vitro (Fukuda et al., 1988). MT1-MMP similarly disrupted the tubulogenic activity of SF/HGF-treated MDCK cells, presumably by degrading the collagen matrix at a rate permissive for invasion, but not branching.

Recent studies have suggested that MT-MMP functional activity requires its regulated trafficking to the plasma membrane via a mechanism that is partially dependent on the COOH-terminal valine found at the end of the cytosolic tails of MT1-, MT2-, and MT3-MMP (Urena et al., 1999). Furthermore, on the cell surface, MT1-MMP has been reported to concentrate into discrete zones near the invading front via additional targeting sequences embedded in the metalloproteinase's transmembrane domain and/or cytosolic tail (Nakahara et al., 1997). In this manner, MT1-MMP localized to the cell–substratum interface could mediate collagenolytic effects directly or indirectly by processing other collagenolytic enzymes to their active forms (i.e., progelatinase A or procollagenase-3; Sato et al., 1994; Knauper et al., 1996). However, in our system, the cytosolic tail-deleted mutant was not only processed to its mature form in normal fashion, but it also induced an invasive program indistinguishable from that of the wild-type enzyme. Similarly, we have also determined that a chimeric construct of MT1-MMP, wherein the wild-type transmembrane domain and tail has been replaced, also retains full invasive activity (our unpublished observation). Whereas neither the cytosolic tail nor the transmembrane domain of MT1-MMP appear to encode critical signals, membrane anchoring to the cell surface proved essential to the invasive process. When MDCK or COS-1 cells were transfected with a soluble form of the enzyme that expresses type I collagenolytic activity (D'Ortho et al., 1997; Ohuchi et al., 1997), the invasive phenotype of these MDCK clones reverted to that observed in the control transfectants. Thus, MT1-MMP only confers invasive activity when confined to the pericellular compartment where it can be concentrated at the cell–substratum interface into sequestered zones that would presumably optimize enzyme–substrate interactions, as well as restrict the access of circulating proteinase inhibitors. Membrane anchoring of MT1-MMP can also accelerate the enzyme's ability to catalyze proMMP-2 activation (Nagase and Woessner, 1999), but we were unable to implicate MMP-2 in the invasion process. Whereas previous studies have suggested that MMP-2 can act as a type I collagenase (Aimes and Quigley, 1995), these conclusions have recently been questioned (Seltzer and Eisen, 1999).

Given the ability of MT1-MMP to modify the invasive activities of recipient cells, we predicted that similar effects might be obtained with MT2-MMP and MT3-MMP. However, despite the facts that the overall domain structures of MT2- and MT3-MMP are virtually identical to that of MT1-MMP, and the enzymes display similar abilities to activate progelatinase A (Nagase and Woessner, 1999), each of the proteinases affected invasion in a manner distinct from that observed with MT1-MMP. In the case of MT2-MMP, the morphogenic program of SF/HGF-stimulated MDCK cells was completely ablated, even though the cells displayed an epithelial phenotype and scattered in response to SF/HGF. Interestingly, whereas MT2-MMP transfectants were unable to express a tubulogenic phenotype in type I collagen gels, foci of degradative activity were identified along the basilar face of SF/HGF-stimulated cells. As MT2-MMP transfectants retained normal invasive activity when stimulated atop Matrigel, we posited that MT2-MMP disrupted type I collagen invasion by degrading the underlying substratum in a discoordinated fashion that prevented the cells from establishing the adhesive interactions necessary for migration. Indeed, when MT2-MMP transfectants were cultured atop the peritoneum in an effort to present the cells with a more protease-resistant matrix, the cells not only invaded, but actually dissolved the peritoneum despite the presence of serum antiproteinases. We cannot rule out the possibility that MT2-MMP penetrated the peritoneum by degrading extracellular matrix components other than type I collagen, or that MT2-MMP interacted with an as yet unidentified MDCK-derived proteinase. However, COS-1 cells transfected with full-length, but not soluble, MT2-MMP expressed a collagen-invasive activity similar to that observed with MT1-MMP–transfected cells. Most probably, COS-1 cells regulated MT2-MMP expression and motility in a more balanced fashion than the MDCK transfectants, thus allowing invasion to proceed. Recently, D'Ortho et al. (1997) reported that soluble MT2-MMP cannot degrade type I collagen, but the membrane-anchored form of the enzyme appears to express activities distinct from those displayed by the truncated proteinase. Nonetheless, we do note that MT2-MMP was not as efficient as MT1-MMP in driving collagen invasion. Whether these differences reflect differences in the enzymes' respective substrate repertoires or the efficiency with which the metalloproteinase zymogens are processed to their active forms remains to be determined.

The ability of MT1-MMP and MT2-MMP to regulate invasive activity is consistent with recent studies demonstrating that these two proteinases are widely expressed in normal tissues, as well as a variety of carcinomatous states (e.g., Ueno et al., 1997; Polette and Birembaut, 1998; Nakada et al., 1999). However, MT3-MMP expression appears to be much more restricted in both normal and neoplastic tissues (e.g., Ueno et al., 1997; Nakada et al., 1999). In contrast to MT1-MMP and MT2-MMP, MT3-MMP only subtly modified the SF/HGF-induced response by favoring a more cyst-like morphogenic program. Based on recent studies of recombinant soluble MT3-MMP (Shimada et al., 1999), this effect could be due to the ability of the proteinase to partially solubilize intact type I collagen by cleaving within the telopeptide regions that harbor interchain cross-links, degrading gelatin (i.e., denatured type I collagen) after proteolysis of type I collagen, or hydrolyzing MDCK-derived extracellular matrix molecules and generating bioactive fragments capable of altering cell function (Werb, 1997). The inability of MT3-MMP to promote collagen-invasive activity should not be misconstrued to suggest that the proteinase cannot affect invasive activity through other biologically relevant matrices. Soluble MT3-MMP has been reported to degrade fibrillar type III collagen (Shimada et al., 1999) and we have also determined that MT3-MMP overexpressing MDCK cells display marked fibrin-invasive activity (our unpublished observation). Thus, we favor a scenario in which distinct MT-MMPs promote invasive and/or morphogenic responses in a manner dictated by the enzymes' proteolytic profile and the characteristics of the connective tissue barrier confronting the cell (e.g., Belien et al., 1999; Koshikawa et al., 2000).

Given the ability of MT-MMPs to affect invasive activity, it was surprising that none of the secreted MMPs that we tested altered the MDCK or COS cell phenotypes in a discernable fashion. However, three issues bear consideration. First, few in vitro or in vivo studies have directly examined the role of specific MMPs in type I collagen invasion (Benbow et al., 1999). In vitro, MMP-9 and MMP-3 are the only metalloproteinases that have been indirectly linked to cell invasion programs through type I collagen gels (Lochter et al., 1997a; Mason et al., 1999). MMP-1 has also been reported to play a required role in keratinocyte migration across type I collagen-coated surfaces in vitro, but invasion was not examined (Pulcher et al., 1997). In our studies, no effects were observed on either cell migration, invasion, or morphogenesis when any of these MMPs were overexpressed. In this regard, it is worth noting that in vivo studies with MMP-$2^{-/-}$, MMP-$3^{-/-}$, MMP-$7^{-/-}$, and MMP-$11^{-/-}$ mice have demonstrated that these animals develop normally and display only subtle defects in their ability to remodel the extracellular matrix (Dunsmore et al., 1998; Itoh et al., 1998; Liu et al., 1998; Masson et al., 1998; Mudgett et al., 1998; Wang et al., 1999). MMP-$9^{-/-}$ mice exhibit abnormal patterns of skeletal growth plate vascularization and ossification, but interestingly, defects are temporary and primarily restricted to bone where MMP-9 acts as an angiogenic activator (Vu et al., 1998). MMP-9 has also been reported to participate in renal morphogenesis in vitro, but similar effects in knockout mice have not yet been described (Lelongt et al., 1997).

The somewhat limited effects of deleting soluble members of the MMP family stand in marked contrast to the more global and severe defects recently observed in MT1-$\text{MMP}^{-/-}$ mice (Holmbeck et al., 1999). In these animals, dwarfism, osteopenia, arthritis, and premature mortality have been primarily attributed to defects in type I collagen turnover. Interestingly, renal epithelial cells undergoing branching morphogenesis in vivo have been shown to transiently express MT1-MMP (Ota et al., 1998; Tanney et al., 1998). Furthermore, as MDCK cells can express low levels of MT1-MMP in vitro (Kadono et al., 1998; our unpublished observation), MT-MMPs may well participate in morphogenic programs in vivo. Specific defects in collagen-invasive or branching activity have not yet been examined in MT1-$\text{MMP}^{-/-}$ mice, but the multiple and severe defects that occur in these animals highlight the relative importance of membrane-anchored MMPs.

Second, despite the relative paucity of studies attempting to determine the means by which invading cells remodel type I collagen in vitro or in knockout animals, alterations in extracellular matrix structure/function have been observed in transgenic animals engineered to overexpress MMP-1, -3, or -7 (e.g., Sympson et al., 1994; Witty et al., 1995; D'Armiento et al., 1995; Rudolph-Owen et al., 1998; Sternlicht et al., 1999). However, these effects are complicated by the fact that MMPs induce epithelial-to-mesenchymal cell conversions in vivo with attendant changes in the expression profile of affected cell populations (Sternlicht et al., 1999). These types of analyses clearly underline the importance of MMPs in regulating tissue architecture and function, but the role of individual proteinases remains difficult to establish.

Finally, the above issues notwithstanding, increasing evidence suggests that the matrix remodeling events that occur coincident with invasive or morphogenic processes are most efficiently mediated by cell surface-associated proteinases. In this manner, a balance is struck between proteolysis and motility such that sufficient matrix is cleared to allow forward movement while leaving a substrate underfoot for propulsive movement (Werb, 1997; Hiraoka et al., 1998; Murphy and Gavrilovic, 1999). MT-MMPs may be specifically adapted for this purpose as the proteinases are activated and then tethered to the cell surface. Indeed, when either MT1- or MT2-MMP were truncated to generate secreted proteinases, all proinvasive effects were lost, despite the fact that the enzymes are efficiently processed to their active forms. Similarly, though MDCK clones overexpressing MMP-2, -3, or -11 were each able to activate a portion of the secreted metalloproteinase, no effect on invasion or tubulogenesis was observed. Thus, the inability of secreted MMPs to participate in invasive events most likely reflects their inefficient localization to the surface of MDCK or COS cells. Indeed, under appropriate conditions, even secreted proteinases may display important functional activities after membrane binding. For example, active MMP-2 can associate with the integrin $\alpha_v\beta_3$, whereas MMP-9 can bind to cell surfaces via interactions with the $\alpha_2$(IV) chain of collagen or CD44 (Brooks et al., 1996; Olson et al., 1998; Yu and Stamenkovic, 2000). In the latter case, a membrane-anchored, but not soluble, mutant form of MMP-9 was shown to regulate tumor invasion and angiogenesis (Yu and Stamenkovic, 2000). Taken together, these data suggest that either membrane-anchored or membrane-associated proteinases are ideally positioned for regulating movement through three-dimensional structures. Given the multiple normal and neoplastic cell types known to express MT-MMPs, we posit that this class of enzymes will prove to play key roles in allowing invading cells to negotiate and remodel interstitial matrix barriers.

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