

Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP

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As cancer cells traverse collagen-rich extracellular matrix (ECM) barriers and intravasate, they adopt a fibroblast-like phenotype and engage undefined proteolytic cascades that mediate invasive activity. Herein, we find that fibroblasts and cancer cells express an indistinguishable pericellular collagenolytic activity that allows them to traverse the ECM. Using fibroblasts isolated from gene-targeted mice, a matrix metalloproteinase (MMP)-dependent activity is identified that drives invasion

independently of plasminogen, the gelatinase A/TIMP-2 axis, gelatinase B, collagenase-3, collagenase-2, or stromelysin-1. In contrast, deleting or suppressing expression of the membrane-tethered MMP, MT1-MMP, in fibroblasts or tumor cells results in a loss of collagenolytic and invasive activity *in vitro* or *in vivo*. Thus, MT1-MMP serves as the major cell-associated proteinase necessary to confer normal or neoplastic cells with invasive activity.

Introduction

The metastatic spread of tumor cells to distant organs via hematogeneous routes represents the most important cause of morbidity and mortality in cancer (Hanahan and Weinberg, 2000; Chambers et al., 2002). In order for malignant cells to intravasate into the host vasculature, neoplastic cells express a tissue-invasive phenotype that allows them to infiltrate an ECM largely composed of a dense, cross-linked meshwork of type I collagen fibrils (Hay, 1991).

To date, multiple proteinases have been identified that impact on tumor cell behavior *in vivo* as a consequence of their ability to cleave growth factors, growth factor receptors, cell-associated molecules, or chemokines/cytokines (Egeblad and Werb, 2002; Turk et al., 2004). However, with specific regard to tissue-invasive activity the required proteolytic machinery remains the subject of speculation. Current evidence suggests that cancer cells negotiate collagenous barriers by mobilizing redundant proteolytic systems that each serve to dissolve the interceding matrix, thus precluding a necessary role for any

single proteinase (Brinckerhoff and Matrisian, 2002; Matrisian et al., 2003). Further, it has been proposed that even if ECM hydrolysis is inhibited, tumor cells can adopt an amoeboid phenotype that accommodates tissue trafficking by mechanical means alone (Friedl and Wolf, 2003).

Although the genomic instability that characterizes the neoplastic process may allow each distinct cancer cell to adopt its own unique strategy for negotiating the ECM, recent gene-profiling studies support a generic program for initiating the metastatic process (Ramaswamy et al., 2003). Indeed, tissue-infiltrating cancer cells of multiple origins have been noted to assume a mesenchymal cell-like phenotype similar to that displayed by fibroblasts (Thiery, 2002; Friedl and Wolf, 2003). Like tumor cells, fibroblasts are also endowed with the ability to remodel and infiltrate collagen-rich tissues as a function of their roles in growth, development, wound healing, and inflammation (Grinnell, 2003; Dong et al., 2004). Interestingly, the proteolytic or nonproteolytic systems engaged by this “professional” collagen-remodeling cell type also remain undefined, as does the possibility that normal and neoplastic cells share a conserved stratagem for invading the ECM.

Herein, we report that fibroblasts and tumor cells are able to tunnel through dense barriers of cross-linked type I

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Abbreviations used in this paper: CAM, chicken chorioallantoic membrane; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase.

collagen *in vitro* or *in vivo* via a virtually indistinguishable proteolytic process that requires matrix metalloproteinase (MMP) activity. Further, we demonstrate that fibroblasts deficient for the membrane-anchored metalloproteinase MT1-MMP are unable to display pericellular collagenolytic activity and are incapable of expressing a collagen-invasive phenotype *in vitro* or *in vivo*. Likewise, gene silencing of MT1-MMP in human carcinoma or fibrosarcoma cells similarly results in a complete loss of membrane-associated collagenolytic activity and renders tumor cells unable to infiltrate host tissues. These data identify MT1-MMP as a required pericellular collagenolysin that confers both normal and neoplastic cells with tissue-invasive activity.

Results

MMP-dependent invasion by fibroblasts and tumor cells

After *in vitro* fibrillogenesis, type I collagen forms a gel-like network of interlocking fibrils stabilized by intermolecular, aldimine cross-links similar to those generated *in vivo* (Hay, 1991). When cultured atop this three-dimensional substratum and exposed to a chemotactic gradient of PDGF-BB in the presence of serum, fibroblasts invade the collagen gel (Fig. 1 A). Tumor cells of mesenchymal or epithelial origin (e.g., HT-1080 and SCC-1 cells, respectively) also infiltrate the three-dimensional collagen barriers over the course of the 4-d culture period (Fig. 1 D). Coincident with the invasive process, both fibroblasts and tumor cells remodel the collagen substratum, leaving a network of tunnels that crisscross the traversed matrix (Fig. 1, B and E). Significantly, the channels formed are not the products of mechanical remodeling alone (Sawhney and Howard, 2002), as tunnel walls are lined by immunodetectable collagen denaturation products (Fig. 1 C). Likewise, expression of the collagen-invasive phenotype by fibroblasts or tumor cells correlates with the accumulation of type I collagen degradation products (Fig. 1, F and G).

To identify the class of proteolytic enzymes responsible for driving this invasive/degradative process, fibroblasts or tumor cells were stimulated in the absence or presence of cysteine (E-64d), aspartyl (pepstatin A), serine (SBTI), or MMP (BB-94) inhibitors (Hiraoka et al., 1998; Hotary et al., 2000). Under these conditions, the synthetic MMP inhibitor BB-94 alone is able to block invasion (Fig. 1, A and D), tunnel formation (Fig. 1, B and E), and collagenolysis (Fig. 1, C, F, and G). Similar results are obtained with breast, pancreatic, or prostate carcinoma cell lines where BB-94 inhibits invasion and collagen degradation by more than 90% (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>). However, requirement for proteolytic activity during invasion is confined to physiologically relevant, cross-linked collagen gels. When matrices are prepared from pepsin-extracted collagen, which, unlike collagen networks assembled *in vivo*, cannot form intermolecular cross-links and are stabilized by non-covalent forces alone (Snowden and Swann, 1979; Capaldi and Chapman, 1982), tunnels are no longer observed in the wake of the migrating cells (Fig. 1 E) and BB-94 does not block fibro-

blast or tumor cell invasion (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>).

Fibroblast-mediated pericellular collagenolysis

As fibroblasts and tumor cells both express collagenolytic activity during invasion, efforts were initiated to identify the proteolytic systems that mediate pericellular degradation. During matrix remodeling events *in vivo*, fibroblasts express MMP zymogens that can undergo processing to active forms (Brinckerhoff and Matrisian, 2002). Similar to the repertoire of MMPs expressed by fibroblasts activated *in vivo* (Okada et al., 1997; Bullard et al., 1999; Wu et al., 2002), fibroblasts stimulated with PDGF atop a three-dimensional collagen substratum *in vitro* express MMP-13, MMP-8, MMP-2, MT1-MMP, MT3-MMP, MMP-3, and MMP-9 as well as the MMP inhibitor TIMP-2 (Fig. 2 A). Further, wild-type cells process proMMP-2 to an active form as observed *in vivo* (Wang et al., 2000; Fig. 2 A).

When fibroblasts are stimulated atop a bed of rhodamine-labeled collagen fibrils in the presence of serum, the field of collagenolytic activity is confined to the cell-matrix interface where circulating antiproteases may have limited access (Fig. 1 B). As observed during invasion (Fig. 1), pericellular collagen degradation is likewise sensitive to inhibition by BB-94 or TIMP-2 (Fig. 2, B and C) and unaffected by serine, cysteine, or aspartyl proteinase inhibitors (not depicted).

Murine fibroblasts potentially degrade subjacent collagen by mobilizing the direct-acting collagenolysins, MMP-13, MMP-8, MMP-2, or MT1-MMP (Brinckerhoff and Matrisian, 2002). In addition, MT1-MMP may also promote collagenolysis by catalyzing the processing of either proMMP-2, proMMP-8, or proMMP-13 to active enzymes (Holopainen et al., 2003; Seiki et al., 2003). To identify individual MMPs or MMP cascades that drive the collagenolytic phenotype, fibroblasts were isolated from MMP-13-, MMP-8-, MMP-2-, or MT1-MMP-deleted mice and the ability of resting or PDGF-stimulated cells to degrade type I collagen assessed. As shown in Fig. 2 A, each of the knockout cells express the full repertoire of MMPs detected in wild-type littermate cells, save for the targeted gene product. Though MMP-13 or MMP-8 are potent collagenolysins (Brinckerhoff and Matrisian, 2002) and can be detected in fibroblast releasates (not depicted), fibroblasts null for either of these MMPs degrade collagen comparably to control cells in either the absence or presence of PDGF (Fig. 2, B and C). Similarly, although recent reports posit a preeminent role for MMP-2 in degradative events after its TIMP-2-dependent activation by MT1-MMP (Brinckerhoff and Matrisian, 2002; Seiki et al., 2003), MMP-2^{-/-} as well as TIMP-2^{-/-} fibroblasts also mount normal collagenolytic responses, effectively eliminating a required role for the MT1-MMP/TIMP-2/MMP-2 axis in this proteolytic process (Fig. 2, B and C). MMP-3 has been implicated in the activation of both procollagenases and proMMP-9 (Benbow et al., 1999; Ramos-DeSimone et al., 1999), but neither MMP-3^{-/-} nor MMP-9^{-/-} fibroblasts display defects in the collagenolytic phenotype (Fig. 2, B and C).

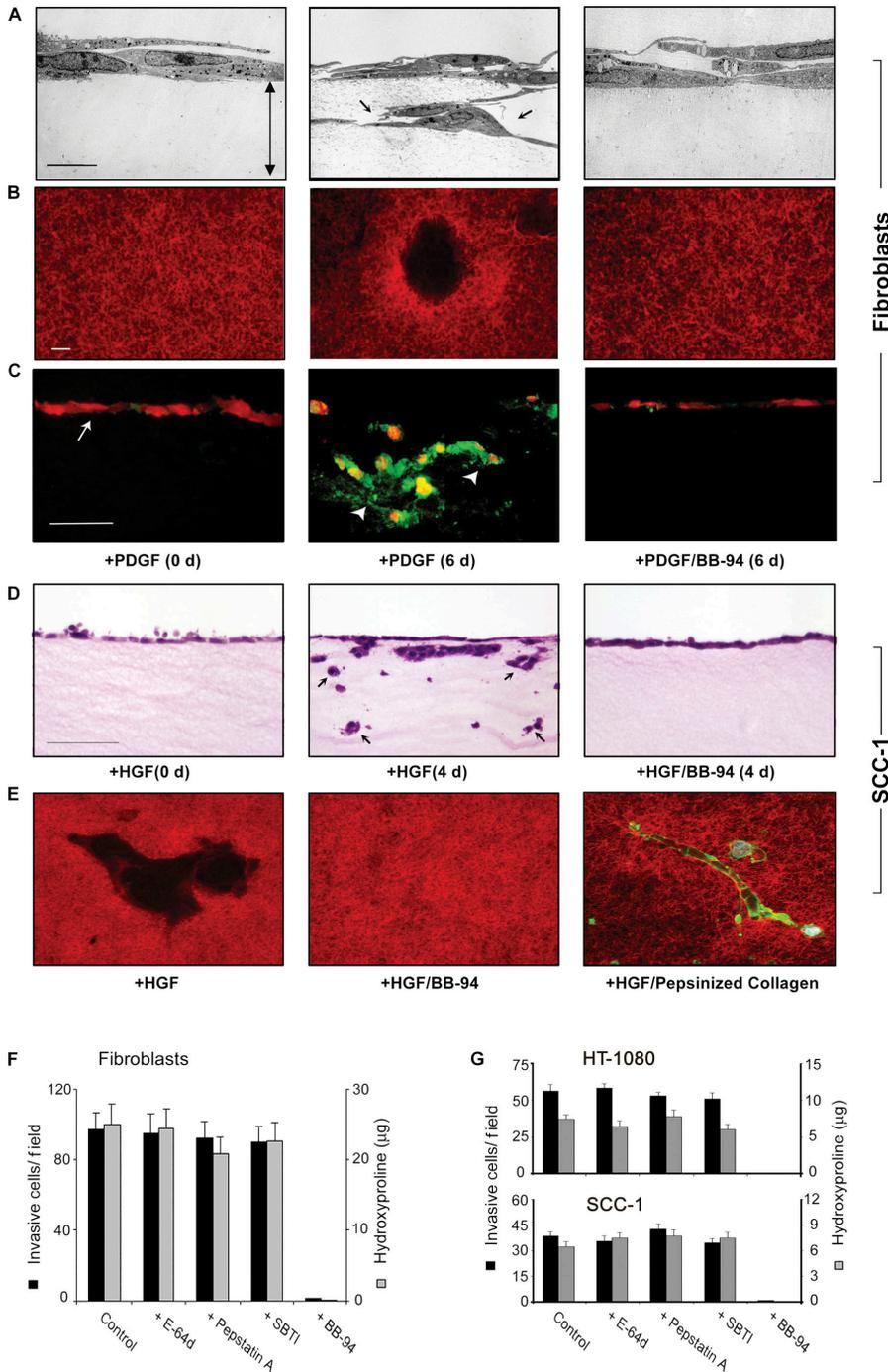


Figure 1. Collagen-invasive and -degradative activities of fibroblasts and tumor cells. (A) Transmission electron micrographs of fibroblast monolayers cultured atop three-dimensional collagen gels (2.2 mg/ml) with PDGF-BB (10 ng/ml) in the presence of 10% serum at the start of the culture period (0 d) or after 6 d in either the absence or presence of 5 µM BB-94. Zones of collagen clearing are observed in areas surrounding invading cells (middle, arrows). Double-headed arrow indicates the position of the underlying gel. Bar, 10 µm. (B) Laser confocal micrographs of rhodamine-labeled collagen gels (2.2 mg/ml) incubated with fibroblasts and PDGF in 10% serum for 0 or 6 d in the absence or presence of BB-94. Bar, 10 µm. (C) Immunofluorescent staining of collagen cross sections reveals zones of denatured collagen (stained green, arrowheads) surrounding invading fibroblasts (stained red, arrow) in the absence, but not the presence, of BB-94. Bar, 100 µm. (D) Light micrographs of SCC-1 monolayers cultured atop collagen gels (2.2 mg/ml) and stimulated with hepatocyte growth factor (HGF; 50 ng/ml) in 10% serum for 0 or 4 d in the absence or presence of BB-94. Arrows indicate islands of invasive cells. Bar, 100 µm. (E) HGF-stimulated SCC-1 cells were cultured atop three-dimensional gels of rhodamine-labeled collagen gels for 4 d in the presence of 10% serum, and tunneling behavior was assessed by laser confocal microscopy. Tunnels were not observed in the presence of BB-94. SCC-1 cells (stained with DAPI and phalloidin) invade rhodamine-labeled, pepsin-extracted collagen gels without generating tunnels. (F and G) Fibroblast (F) or tumor cell (HT1080 and SCC-1; G) invasion and collagenolytic activities were monitored in three-dimensional collagen gels (2.2 mg/ml) supplemented with 10% serum in the absence or presence of inhibitors for 6 or 4 d, respectively. Results are expressed as the mean ± 1 SEM of three or more experiments.

As fibroblasts null for each of the major secreted collagenases degrade collagen in an area restricted to the pericellular space, we next considered a direct attack by the membrane-anchored metalloprotease MT1-MMP. Indeed, MT1-MMP-deleted fibroblasts display an almost complete defect in collagenolytic activity relative to wild-type cells though the cells express MMP-13, MMP-8, MMP-2, and TIMP-2 comparably to control fibroblasts and retain a residual ability to process proMMP-2 to its active form (Fig. 2, A–C), presumably via MT3-MMP (Hotary et al., 2002). Consistent with a direct role for MT1-MMP in the collagenolytic phenotype, collagen degradation by wild-type fibroblasts was blocked completely by either

BB-94 or exogenous TIMP-2, MMP inhibitors whose substrate profile includes the membrane-anchored MMPs (Fig. 2, B and C), but not by high concentrations of TIMP-1, an MMP inhibitor that preferentially targets secreted MMPs (not depicted; Hotary et al., 2002, 2003). The collagenolytic-null phenotype of MT1-MMP^{-/-} fibroblasts is reversed by reexpressing full-length MT1-MMP, but not by an MT1-MMP construct harboring an inactivating point mutation in the catalytic domain (i.e., MT1-MMP E²⁴⁰→A; Fig. 2 B). Consistent with the restricted ability of secreted proteinases to operate effectively in the presence of serum antiproteinases, the collagenolysis-null phenotype of MT1-MMP^{-/-} fibroblasts cannot be rescued when cells are transfected

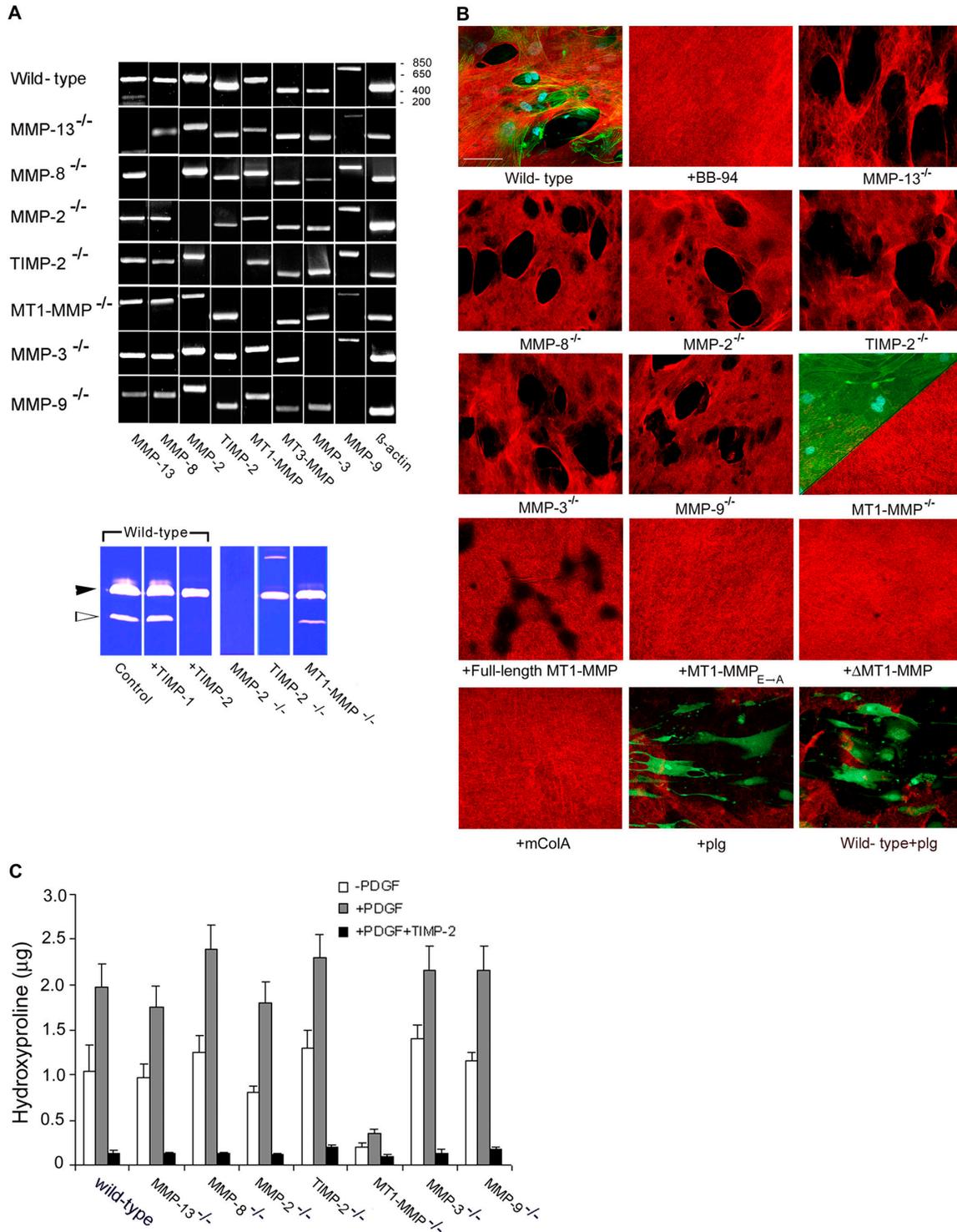


Figure 2. **Fibroblast MMP expression profile and collagenolytic activity.** (A, top) RT-PCR analysis of MMP expression of wild-type and null fibroblasts cultured atop collagen gels in 10% serum for 3 d with PDGF. (bottom) Gelatin zymography of serum-free supernatants recovered from the wild-type, MMP-2^{-/-}, or MT1-MMP^{-/-} fibroblasts cultured with PDGF alone (Control) or with either TIMP-1 or TIMP-2. Wild-type fibroblasts express pro-MMP-2 (black arrowhead) and generate mature MMP-2 (white arrowhead) via a TIMP-2-sensitive process. No MMP-2 was detected in MMP-2^{-/-} cultures, whereas mature MMP-2 was not generated in the TIMP-2^{-/-} cultures. The identity of the high M_r gelatinolytic species in the supernatant of TIMP-2^{-/-} fibroblasts is unknown. (B and C) Collagenolytic activity of fibroblasts seeded on a type I collagen film as assessed by confocal laser microscopy (B) or hydroxyproline release (C). Fibroblasts were seeded atop a 100 μg/2.2 cm² film of rhodamine-labeled collagen and stimulated with PDGF without or with BB-94 in 10% autologous mouse serum or cultured under serum-free conditions with PDGF in the presence of 20 μg/ml of plasminogen for 5 d. (B) Wild-type or MT1-MMP^{-/-} fibroblasts were labeled with calcein-AM (green) and DAPI (blue) in the merged images (first, ninth, and last two images in the series). Bar, 100 μm. (C) Hydroxyproline release was monitored in 10% serum without or with PDGF in the absence or presence of TIMP-2. Results are expressed as the mean ± 1 SEM of three or more experiments.

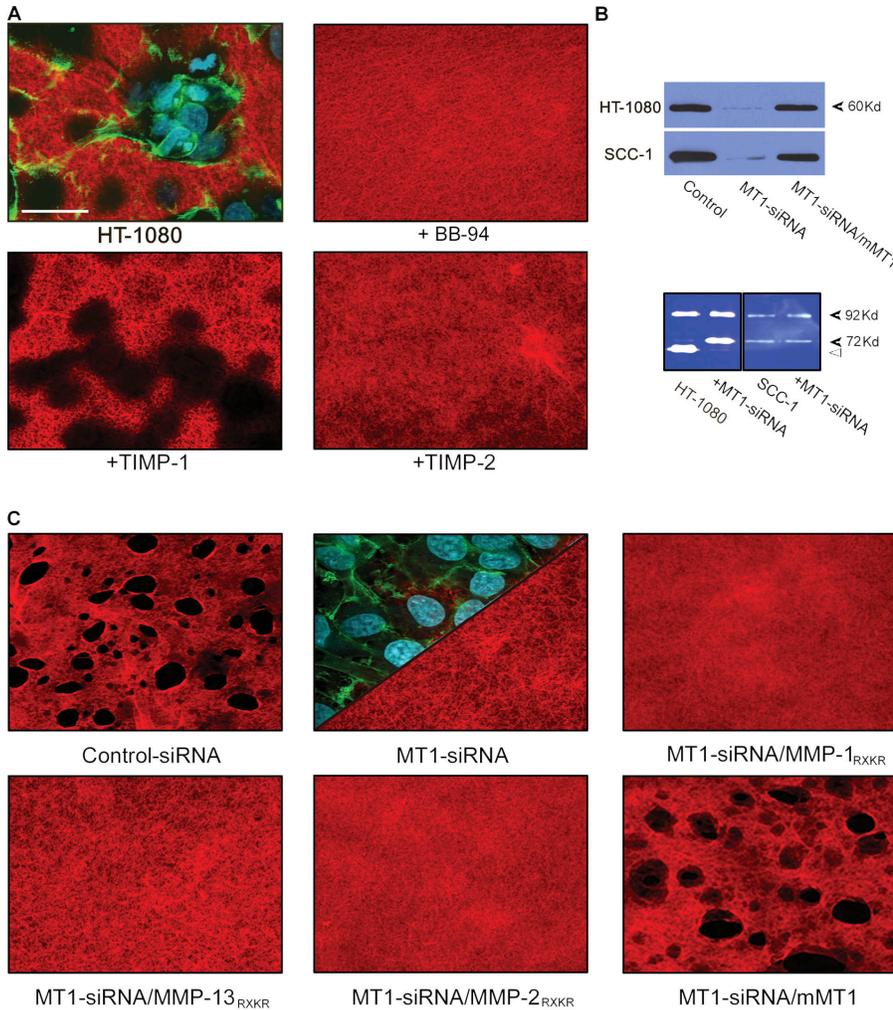


Figure 3. MT1-MMP regulates collagen-degradative activity in tumor cells. (A) Degradative activity of HT-1080 cells (5×10^4) cultured atop a film of rhodamine-labeled type I collagen ($100 \mu\text{g}/2.2 \text{ cm}^2$) in the presence of 10% serum alone (HT-1080; stained with phalloidin/DAPI), or with BB-94, TIMP-1, or TIMP-2. Zones of collagen degradation were monitored by confocal laser microscopy after 3 d. (B) Western blot analysis of MT1-MMP (top) expression in HT-1080 or SCC-1 cells before or after electroporation with a 21-bp MT1-MMP siRNA (MT1-siRNA) or after coelectroporation of MT1-siRNA with a mouse MT1-MMP plasmid (mMT1). Gelatin zymography (bottom) of serum-free supernatants recovered from control or MT1-siRNA-treated HT-1080 or SCC-1 cells after 2 d in culture. MMP-2 (72 kD) and MMP-9 (92 kD) expression are not affected by MT1-siRNA as assessed by zymography. Control HT-1080 cells generate pro-MMP-2 (black arrowhead) and mature MMP-2 (white arrowhead). (C) HT-1080 cells were either treated with a control-siRNA, MT1-siRNA, or coelectroporated with MT1-siRNA and either MMP-1_{RXKR}, MMP-13_{RXKR}, MMP-2_{RXKR}, or mMT1-MMP expression vectors, cultured for 3 d atop a film of rhodamine-labeled type I collagen in 10% serum, and zones of collagen degradation monitored by confocal laser microscopy. In the top section of the split image of MT1-siRNA-treated HT-1080 cells, the phalloidin/DAPI-stained tumor cells are shown atop the collagen film, whereas the lower section displays the underlying collagen layer alone. Results are representative of four performed. Bar, 100 μm .

with a fully active, but soluble, form of MT1-MMP (i.e., Δ MT1-MMP; Fig. 2 B). Though mouse adult fibroblasts can express low levels of a species-specific orthologue of mammalian MMP-1, termed mCoLA (Balbin et al., 2001), subjacent proteolysis is not affected by transfecting wild-type or MT1-MMP^{-/-} cells with a mCoLA expression vector (Fig. 2 B).

Importantly, the inability of MT1-MMP^{-/-} fibroblasts to degrade collagen in a serum-containing environment is not due to a generalized defect in proliferation or the mobilization or activation of secreted collagenases. Although MT1-MMP can affect cell proliferation (Hotary et al., 2003), this effect is limited to three-dimensional growth and, as such, wild-type and null fibroblasts replicate at indistinguishable rates atop the collagen substratum (at day 5, $2.1 \pm 0.3 \times 10^5$ and $1.9 \pm 0.2 \times 10^5$ cells for wild-type and MT1-MMP^{-/-} fibroblasts, respectively). Further, when MT1-MMP^{+/+} or MT1-MMP^{-/-} cells are triggered under serum-free conditions and the medium supplemented with exogenous plasminogen, both wild-type and null cells extend collagenolytic activity beyond their cell borders in a manner consistent with the activation of secreted collagenases (Fig. 2 B; Netzel-Arnett et al., 2002) and degrade collagen comparably (i.e., wild-type and MT1-MMP^{-/-} fibroblasts solubilize 8.7 ± 1.1 and $7.9 \pm 1.0 \mu\text{g}$ of hydroxyproline, respectively). Nonetheless, in an antiproteinase-rich milieu that

recapitulates that encountered in vivo, membrane-anchored MT1-MMP serves as the dominant collagenase.

Tumor cell-dependent collagenolysis

Tumor cells, like fibroblasts, also express secreted and membrane-anchored MMPs (Seiki et al., 2003) and efficiently degrade subjacent collagen via a BB-94-sensitive process (Fig. 3 A). Consistent with a role for MT1-MMP in pericellular collagenolysis, high dose TIMP-1 is unable to block degradation, whereas proteolysis is inhibited completely by TIMP-2 (Fig. 3 A). To determine directly the role of MT1-MMP in the tumor cell collagenolytic phenotype, the expression of the membrane-anchored enzyme was targeted with a siRNA construct in HT-1080 or SCC-1 cells. As shown in Fig. 3 B, MT1-MMP is silenced effectively without affecting MMP-2 or MMP-9 expression, though, as expected, the MT1-MMP-dependent processing of proMMP-2 to its active form is suppressed. Further, like MT1-MMP^{-/-} fibroblasts, MT1-MMP siRNA-treated cancer cells display no defects in two-dimensional proliferation or migration across collagen-coated surfaces (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>). However, in the absence of endogenous MT1-MMP, the pericellular collagenolytic activity of HT-1080 cells or SCC-1 cells was lost completely (Fig. 3 C). Further, the inability of MT1-MMP

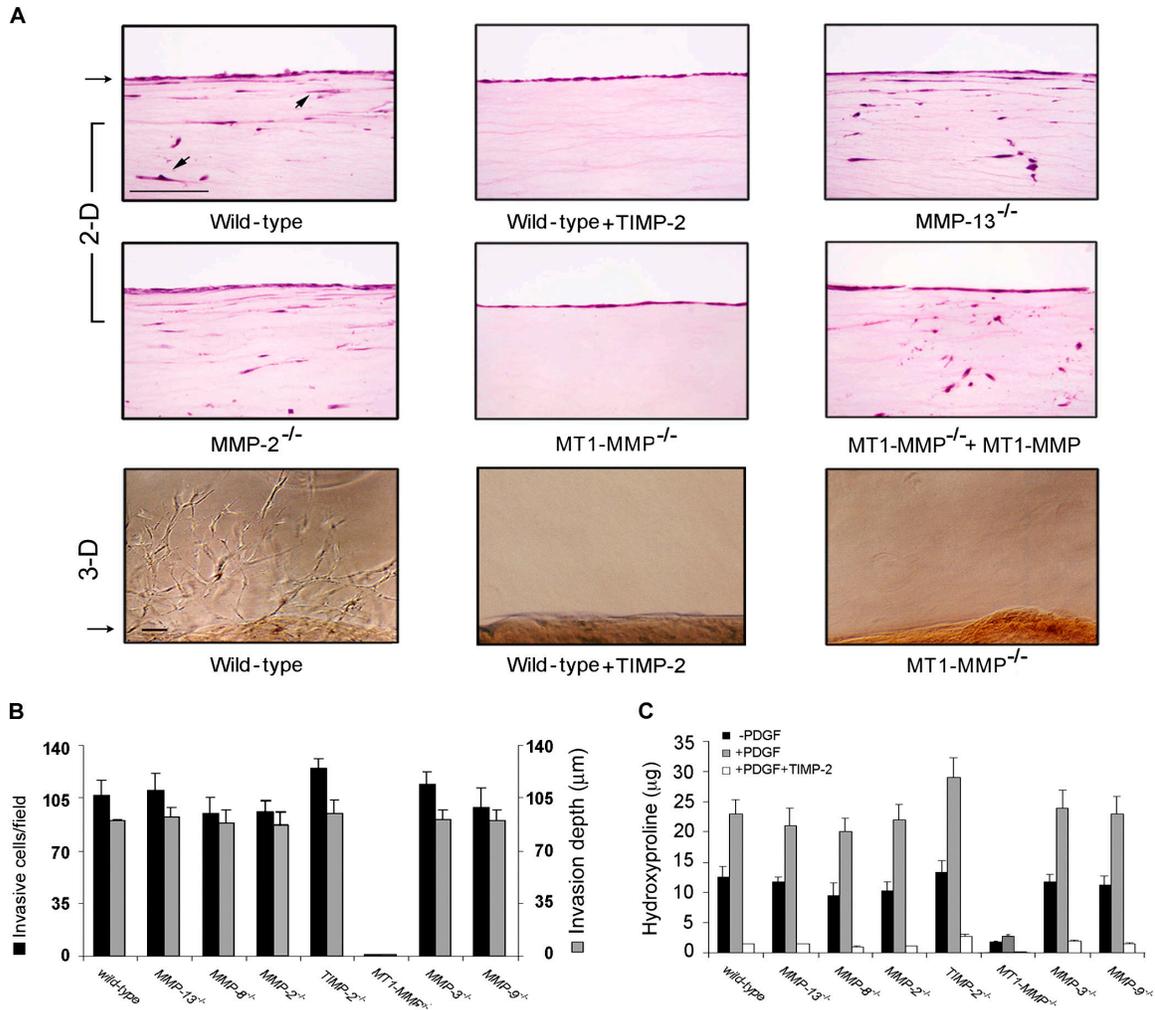


Figure 4. **Fibroblast MT1-MMP mediates collagen-invasive activity.** (A–C) Wild-type and null fibroblasts were cultured atop type I collagen gels (2.2 mg/ml; top two rows, 2-D) or within three-dimensional collagen gels (2.2 mg/ml; bottom row) in 10% serum and stimulated with a PDGF gradient (10 ng/ml) for 6 d in the absence or presence of TIMP-2. The arrow to the left of the panels marks the surface of the collagen monolayer or the edge of the embedded island of fibroblasts. Arrows within the gel indicate positions of invading fibroblasts. Bars, 100 µm. Invasion (B) and collagen degradation (C) were quantified in three-dimensional collagen gels in the presence of 10% wild-type serum save for experiments with MMP-2^{-/-}, MMP-9^{-/-}, or TIMP-2^{-/-} fibroblasts, where null serum was isolated from each of the respective mouse strains. Results are expressed as the mean ± 1 SEM of three or more experiments.

siRNA-treated tumor cells to mediate subjacent collagenolysis is not reversed when the cells are transfected with chimeric constructs of human MMP-1, MMP-13, or MMP-2 that undergo constitutive processing to active forms (i.e., MMP-1_{RXKR}, MMP-13_{RXKR}, or MMP-2_{RXKR} expression vectors; Hotary et al., 2003; Fig. 3 C). Although siRNAs can potentially induce nonspecific effects by mediating off-target gene regulation (Scacheri et al., 2004), collagenolytic activity is restored completely when the MT1-MMP siRNA-treated tumor cells are transfected with a mouse MT1-MMP construct that displays only limited homology through the human MT1-MMP siRNA-targeted sequence (Fig. 3 C). Thus, cancer cells, like fibroblasts, rely on MT1-MMP to express subjacent collagenolytic activity.

MT1-MMP regulates the collagen-invasive phenotype of fibroblasts in vitro and in vivo

Although fibroblasts and tumor cells both use MT1-MMP as a pericellular collagenolysin, the invasive phenotype displayed

by cells negotiating three-dimensional ECM barriers is a more complex form of cellular behavior and conceivably requires complementation by other proteolytic systems (Murphy and Gavrilovic, 1999). Hence, monolayers of wild-type and null fibroblasts were established atop three-dimensional collagen gels and the cells exposed to a chemotactic gradient of PDGF. Under these conditions, MMP-13^{-/-}, MMP-8^{-/-}, MMP-2^{-/-}, TIMP-2^{-/-}, MMP-3^{-/-}, and MMP-9^{-/-} fibroblasts each invade the collagen gels comparably to wild-type cells while degrading similar amounts of collagen (Fig. 4, A–C). Likewise, though plasmin can activate secreted MMPs, fibroblasts isolated from plasminogen-null mice and suspended in plasminogen-null sera display an invasive phenotype indistinguishable from controls (i.e., 98 ± 10% of control; n = 3). Recent papers have promoted a potential role for the transmembrane serine prolyl peptidase, fibroblast activation protein/seprase, in collagen remodeling events (Gherzi et al., 2002), but the null fibroblasts also retained full invasive activity (i.e., and 95 ± 6% of control; n = 3). In contrast, MT1-MMP^{-/-} fibroblasts are

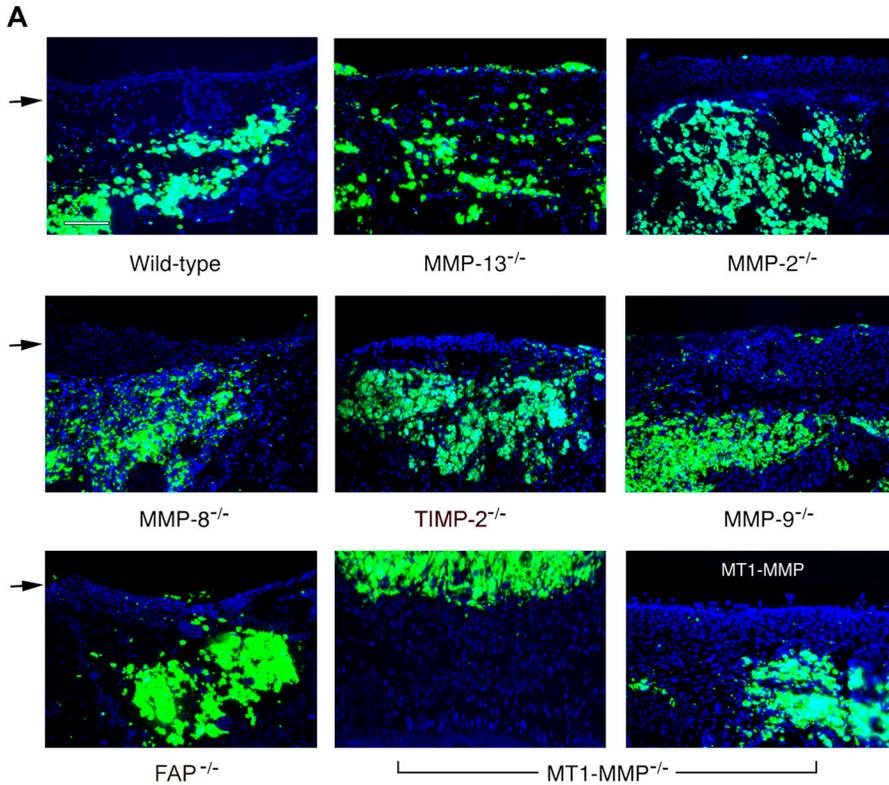
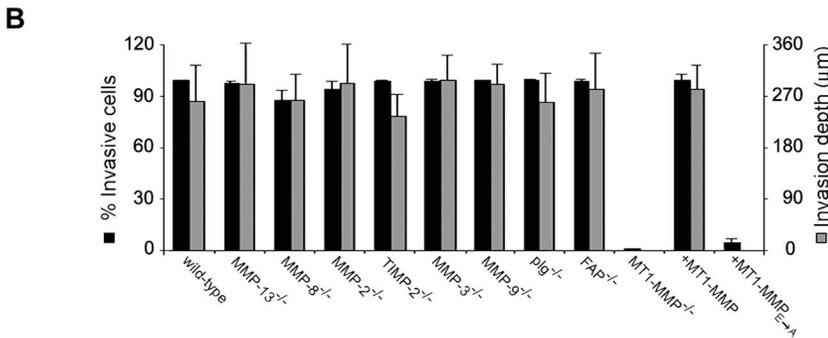


Figure 5. CAM invasion by wild-type and MMP-null fibroblasts. (A) Fibroblasts from wild-type, MMP-deficient, or FAP-null mice were labeled with fluorescent microbeads (green) and seeded atop the CAM of 11-d-old chicks and cultured for 3 d. Fluorescent micrographs of CAM cross sections demonstrate the ability of mouse fibroblasts to penetrate the CAM surface (DAPI-stained, blue nuclei of chick cells at the CAM surface are highlighted by arrows). The MT1-MMP^{-/-} defect was reversed by transient transfection of MT1-MMP^{-/-} cells with MT1-MMP. Results are representative of four performed. Bar, 100 μ m. (B) Invasion was quantified as the percentage of wild-type or null fibroblasts that traversed the CAM surface and the mean depth of invasion of the leading front of three or more fibroblasts. Results are expressed as the mean \pm 1 SD of at least three experiments.



unable to express an invasive phenotype over the 6-d culture period (Fig. 4, A–C). Though cell behavior can be altered when cells are embedded within, as opposed to cultured atop, a three-dimensional matrix (Cukierman et al., 2002, Hotary et al., 2003), MT1-MMP^{-/-} cells sustain a similar, and complete, deficiency in three-dimensional invasive behavior relative to wild-type fibroblasts (Fig. 4 A). However, under either two- or three-dimensional conditions, the MT1-MMP^{-/-} invasion-null phenotype is rescued when cells are transfected with an expression vector encoding wild-type (Fig. 4 A), but not either soluble Δ MT1-MMP, inactive MT1-MMP E²⁴⁰ \rightarrow A, mColA, human MMP-1_{RXKR}, human MMP-13_{RXKR}, or human MMP-2_{RXKR} (not depicted). However, defects in MT1-MMP^{-/-} fibroblasts are confined to the type I collagen substrate as the null cells migrate across collagen-coated surfaces comparably to controls, exert a normal collagen-contractile response (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>), and, as reported previously, invade three-dimensional matrices of cross-linked fibrin via an MT3-MMP-dependent process (Hotary et al., 2002).

Although fibroblasts use MT1-MMP to cross barriers of type I collagen assembled in vitro, the extracellular matrix in vivo is a more complex mix of collagens, glycosaminoglycans, glycoproteins, and peptidoglycans (Hay, 1991). To determine whether or not MT1-MMP likewise regulates tissue-invasive activity in vivo, wild-type or null fibroblasts labeled with fluorescent nanobeads were applied to the surface of the chicken chorioallantoic membrane (CAM), a type I collagen-rich ECM barrier commonly used to study invasive processes (Kim et al., 1998). As reported previously (Armstrong et al., 1982), wild-type fibroblasts readily invade the CAM in the course of a 3-d culture period (Fig. 5 A). Though the pattern of distribution of the invading fibroblasts is variable between tissue sections and individual chick CAMs (unpublished data), both the percentage of fibroblasts that cross the CAM surface and the depth of the leading front of invasion is highly reproducible. By either of these criteria, neither MMP-13^{-/-}, MMP-8^{-/-}, MMP-2^{-/-}, MMP-3^{-/-}, MMP-9^{-/-}, plasminogen^{-/-}, nor fibroblast activation protein^{-/-} fibroblasts display discernible defects in invasive activity (Fig. 5, A and B). In contrast, MT1-MMP-null fi-

broblasts are completely unable to penetrate the CAM surface unless the cells are transfected with wild-type, but not a MT1-MMP E²⁴⁰→A, expression vector (Fig. 5, B and C).

MT1-MMP-dependent tumor cell invasion and intravasation

To determine if a required role for MT1-MMP in fibroblast invasion extends to tumor cells, collagen-invasive activity of the MT1-MMP siRNA-treated cancer cells was assessed in vitro and in vivo. As shown in Fig. 6, type I collagen invasion and the associated degradation of the surrounding matrix by HT-1080 or SCC-1, although unaffected by the control siRNA, are suppressed almost completely by the MT1-MMP siRNA (Fig. 6, A and B). Validated siRNA-mediated targeting of either tumor cell MMP-1, MMP-2, or MMP-9 did not affect collagen-invasive activity (unpublished data). A role for MT1-MMP in tumor cell invasion was not limited to HT-1080 or SCC-1 cells, as similar results are obtained when MT1-MMP expression is silenced in MDA-MB-231, PANC-1, or DU-145 cells where the number of invasive foci is reduced by more than 75% (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>). MT1-MMP siRNA-treated tumor cells retain full ability to invade either pepsin-extracted collagen gels devoid of intermolecular cross-links or fibrin matrices (Fig. S3). Similarly, (though in contrast with other recent reports; Ueda et al., 2003; Iida et al., 2004) invasion through Matrigel, an extract of noncross-linked ECM macromolecules (Kleinman et al., 1982), is unaffected in MT1-MMP-targeted tumor cells (Fig. S3). However, invasion through cross-linked collagen matrices and the associated collagenolytic activity are restored fully by transfecting the MT1-MMP siRNA-treated tumor cells with the mouse MT1-MMP construct (Fig. 6, A and B).

Unlike fibroblasts, tumor cells transplanted atop the CAM not only invade the surrounding tissues but gain access to the host vascular and/or lymphatic beds where human cell DNA can be detected as Alu sequences via PCR-based assay on extracts prepared from the lower CAM (Kim et al., 1998). To determine if MT1-MMP plays a necessary role in driving cancer cell invasion and intravasation, tumor cells were treated with either siRNA controls or MT1-MMP siRNA, and the in vivo phenotype was monitored. After a 3-d incubation period, HT-1080 and SCC-1 cells treated with siRNA controls readily invade the CAM surface (Fig. 6 C). The invasive phenotype was not limited to the chick extracellular matrix, as tumor cells cultured atop acellular human dermis and explanted onto the CAM likewise penetrated this type I/III collagen-rich barrier (Fig. 6 D). After penetrating the CAM interstitium, the tumor cells further breach the vascular bed and gain entry to the lower CAM as detected by Alu sequence (Fig. 6 E). In contrast, MT1-MMP siRNA-treated cells are unable to express invasive activity and fail to access the lower CAM unless transfected with mouse MT1-MMP (Fig. 6, C and E). Neither siRNAs directed against MMP-2 nor MMP-1 inhibit significantly tumor cell invasion (i.e., percent invasion for control siRNA-, MMP-2 siRNA-, and MMP-1 siRNA-treated HT-1080 cells is $23.9 \pm 6.7\%$, $22.2 \pm 6.3\%$, and $21.2 \pm 2.6\%$, respectively). These data support a necessary, if not critical, role for MT1-MMP

in the metastatic process by arming cancer cells with the ability to invade interstitial tissues and gain access to the host vasculature.

Discussion

In vivo, cancer cells initiate metastatic behavior by penetrating the type I collagen-rich ECM, infiltrating vascular or lymphatic compartments, and disseminating to distant sites (Hanahan and Weinberg, 2000; Chambers et al., 2002). Multiple proteolytic systems have been shown to impact on tumor cell behavior in vivo (Matrisian et al., 2003; Turk et al., 2004), but the identity of the enzymes that confer tissue-invasive activity remain controversial. As tumor cells engage the proteolytic machinery necessary to invade surrounding tissues, they adopt a fibroblast-like morphology—a phenotypic switch that suggests that tumor cells access purposefully the same gene programs engaged by normal cells that traffic through the ECM (Thiery, 2002; Friedl and Wolf, 2003). Herein, we demonstrate that the membrane-anchored metalloproteinase MT1-MMP serves as the dominant purveyor of the tissue-invasive activities necessary to support the trafficking of normal and neoplastic cells through the ECM. Although fibroblasts as well as tumor cells also mobilize secreted collagenases, which may associate with the cell surface (Dumin et al., 2001; Netzel-Arnett et al., 2002), such binding interactions were unable to confer these cells with invasive activity. Presumably, only MT1-MMP is directed specifically to sites targeted for degradation by a combination of motifs embedded not only in the transmembrane domain and intracellular cytosolic tail but also the collagen-binding hemopexin domain on the extracellular face of the enzyme (Tam et al., 2002; Seiki et al., 2003). This conclusion should not be construed to suggest that secreted collagenases are inoperative in vivo. Rather, the ability of secreted collagenases to resorb bulk tissue would be restricted to those sites where the antiproteinase shield is overwhelmed by a relative proteinase excess or inactivated by either reactive oxidants or proteolytic attack (Weiss, 1989).

The ability of fibroblasts and tumor cells to mobilize a pericellular collagenase would appear to provide a mechanistic basis for the tunnel tracks we detected in cell-trafficked collagen matrices. Indeed, the unique ability of MT1-MMP, rather than secreted MMPs, to drive invasion correlates precisely with the singular role that the membrane-anchored proteinase plays in pericellular collagenolysis. Although invasion may require MT1-MMP to cleave collagen as well as one or more cell surface targets in tandem (Seiki et al., 2003), it should be noted that MT1-MMP-null cells display no defects in collagen adhesion, collagen-contractile activity, or migration, and invade three-dimensional gels constructed of cross-linked fibrin or noncross-linked collagen comparably to wild-type cells. Furthermore, invasion is inhibited when tumor cells confront barriers of a collagenase-resistant mutant form of type I collagen (Ellerbroek et al., 2001; Hotary et al., 2003). Finally, we have found that invasive activity can be conferred by a chimeric MT1-MMP construct wherein the catalytic domain of the wild-type enzyme has been replaced with that of the “classic” collagenase MMP-1 (unpublished

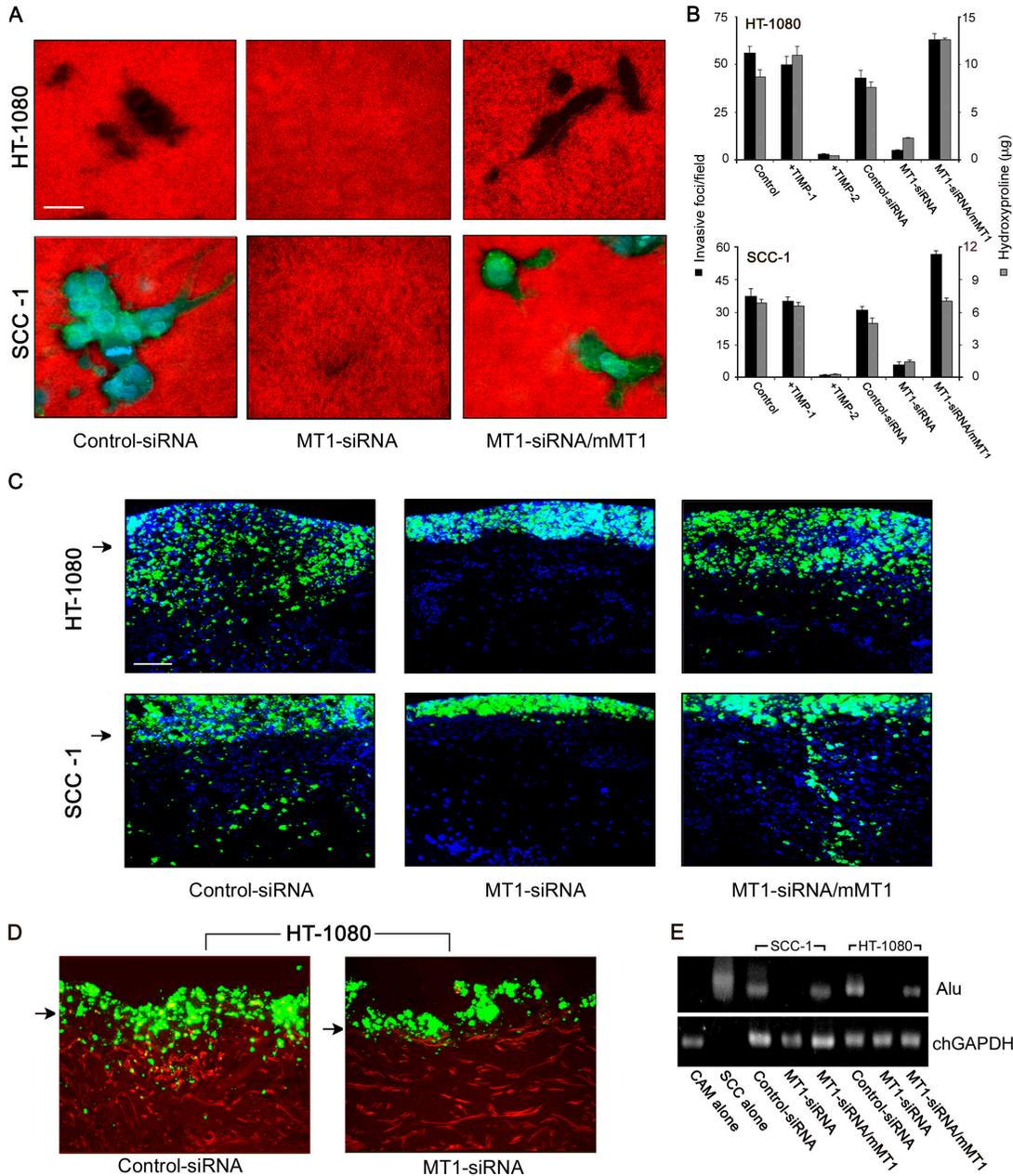


Figure 6. MT1-MMP drives cancer cell invasion and intravasation. (A and B) HT-1080 or SCC-1 cells were incubated alone with TIMP-1, TIMP-2, a 21-bp MT1-MMP siRNA, or a 21-bp control siRNA and cultured atop a rhodamine-labeled type I collagen gel (2.2 mg/ml) in 10% serum with 50 ng/ml HGF for 3 d. Tunneling behavior of HT-1080 and SCC-1 cells (A) was monitored by confocal laser microscopy (bottom, merged images of phalloidin/DAPI-labeled SCC-1 cells and the surrounding rhodamine-labeled collagen are shown). Invasive foci and hydroxyproline release for HT-1080 and SCC-1 cells were quantified as described in B. Where indicated, MT1-MMP siRNA-treated cells were transfected with a mouse MT1-MMP (mMT1-MMP) expression vector (MT1-siRNA/mMT1). (C) CAM invasion by fluorescent nanobead-labeled HT-1080 or SCC-1 cells incubated alone, electroporated with MT1-siRNA alone, or coelectroporated with MT1-siRNA and a mMT1-MMP expression vector. Tumor cell invasion was visualized by fluorescent microscopy of CAM cross sections after a 3-d incubation period. The CAM surface is marked by arrows. Percent invasion for control-siRNA-, MT1-siRNA-, and mMT1-MMP-transfected MT1-siRNA-treated HT-1080 cells was $24 \pm 7\%$, $1 \pm 1\%$, and $25 \pm 3\%$, respectively, and for SCC-1 cells was $15.8 \pm 1\%$, $1 \pm 1\%$, and $16 \pm 4\%$, respectively (mean \pm 1 SD; $n = 3$). (D) Invasion of human dermal explants by HT-1080 cells after electroporation with a control siRNA or MT1-siRNA and cultured atop the CAM for 3 d. The percentage of invading cells and invasion depth for control siRNA- and MT1 siRNA-treated HT-1080 cells, respectively, were $16.9 \pm 5.9\%$ and $144.1 \pm 47.6 \mu\text{m}$ and $2.9 \pm 0.2\%$ and $22.9 \pm 10.8 \mu\text{m}$. (E) Tumor cell intravasation/extravasation was detected as Alu-sequences by PCR on DNA extracted from the lower CAM for either HT-1080 or SCC-1 cells after a 3-d incubation period. Chick GAPDH (chGAPDH) serves as the loading control. Results are representative of four experiments performed. Bars, 100 μm .

data). Together, these results support a model where MT1-MMP confers fibroblasts and tumor cells with invasive activity by acting as a membrane-tethered collagenase.

In vivo, tissue-invasive fibroblasts and cancer cells not only negotiate collagenous barriers, but the more complex

mix of ECM-associated macromolecules that constitute the interstitial matrix (Hay, 1991). When applied to the CAM surface, wild-type fibroblasts and cancer cells both cross the CAM surface and invade the underlying mesenchyme via a process dependent completely on MT1-MMP—a finding con-

sistent with the ability of MT1-MMP to cleave multiple interstitial collagen types, glycoproteins and proteoglycans (Seiki et al., 2003). However, carcinoma as well as fibrosarcoma cells not only invade locally but also intravasate into the chick vasculature and/or lymphatic bed (Kim et al., 1998). Despite the more aggressive phenotype of the transformed cells, the invasive activity of cancer cells is likewise paralyzed by silencing MT1-MMP expression. Although the specificity of siRNA-mediated silencing must be interpreted cautiously (Scacheri et al., 2004), we note that the inhibitory effects exerted on tumor cells are reversed by reexpressing an MT1-MMP construct designed to escape siRNA targeting and that the observed tumor cell phenotype recapitulates that of MT1-MMP^{-/-} fibroblasts. Hence, contrary to expectations that multiple proteolytic systems can confer invasive activity (Egeblad and Werb, 2002; Matrisian et al., 2003; Turk et al., 2004), in the absence of MT1-MMP, none of the five cancer cell lines studied herein are able to mobilize any of their remaining repertoire of serine-, cysteine-, aspartyl-, or metalloproteinases alone, or in combination with chick proteinases, to drive invasion or intravasation.

Although our findings highlight the importance of proteolytic remodeling of the ECM during invasion, recent attention has focused on the ability of tumor cells to potentially negotiate collagenous barriers by switching to an amoeboid-like form of movement that is permissive for the proteinase-independent patterns of migration more commonly associated with myeloid cells (Huber and Weiss, 1989; Friedl and Wolf, 2003; Wolf et al., 2003). However, we note that tumor cell amoeboid movement was observed *in vitro* within the confines of three-dimensional gels constructed from pepsin-extracted collagen that no longer contains the telopeptide region critical for the structural integrity of type I collagen gels (Snowden and Swann, 1979; Capaldi and Chapman, 1982). Similarly, non-proteolytic systems have been reported to confer tumor cells with the ability to traverse Matrigel-coated surfaces (Hotary et al., 2000; Sahai and Marshall, 2003), a noncross-linked composite of basement membrane-associated macromolecules (Kleinman et al., 1982). As collagens found in the interstitial matrix are normally cross-linked *in vivo* (Hay, 1991), we posit that MT1-MMP plays a dominant role in most tissue-invasive processes as MT1-MMP-suppressed tumor cells were unable to negotiate type I collagen gels, the CAM interstitial matrix, or human dermis. This conclusion remains at odds with reports that MMP inhibition fails to block tumor cell migration *in vivo* (Wolf et al., 2003) and that MT1-MMP^{-/-} mice develop normally (Holmbeck et al., 1999). First, with respect to the amoeboid behavior of tumor cells observed *in vivo*, HT-1080 cells were treated with reversible MMP inhibitors *in vitro* and antiproteinases were washed away before *in vivo* analysis (Wolf et al., 2003). Hence, HT-1080 cells would be free to mobilize MT1-MMP and initiate invasion. Second, we find that the concentrations of MMP inhibitors required to block tumor cell migration in our long term *in vitro* studies are far higher than those reached in either animal studies or clinical trials (Matrisian et al., 2003). Third, although MT1-MMP^{-/-} mice display no discernible defects at birth, the syn-

thesis and maturation of type I collagen fibrils is confined largely to the postnatal period (Caulfield and Borg, 1979; Mays et al., 1988). Indeed, we have found that MT1-MMP^{-/-} fibroblasts can navigate through the type I collagen-poor neonatal dermis, but remain entrapped in the type I collagen-rich dermis of 4-wk-old mice (unpublished data). Although collagen content and structure can differ dramatically from tissue to tissue even during postnatal states (Hay, 1991), gene-expression profiles demonstrate that type I collagen is up-regulated consistently at both primary and metastatic sites in a wide variety of human carcinomas (Ramaswamy et al., 2003). Hence, MT1-MMP may play a required role for invasion even at sites where the collagen content of the normal, pre-affected tissue is low. Further, while our work emphasizes the importance of MT1-MMP, preliminary studies indicate that MT2-MMP may also bestow recipient cells with tissue-invasive activity (Hotary et al., 2000; unpublished data). However, the more restricted pattern of MT2-MMP expression *in vivo* suggests that this alternate collagenolysin likely plays a more limited role than MT1-MMP in regulating the invasive phenotype.

For more than 30 yr, various proteinases have been linked to tumor invasion, intravasation, or metastasis (Brinckerhoff and Matrisian, 2002; Matrisian et al., 2003; Turk et al., 2004). However, with increasing frequency, proteolytic enzymes appear to regulate tumor cell behavior in an indirect fashion, e.g., by solubilizing matrix-bound growth factors, or by processing cadherins, integrins, and chemokines (Egeblad and Werb, 2002; Seiki et al., 2003; Turk et al., 2004). In contrast, our data, coupled with more recent findings (Hotary et al., 2003), support a model wherein MT1-MMP functions as a direct-acting, pro-invasive factor that confers tumor cells with the ability to penetrate connective tissue barriers, proliferate within the confines of the three-dimensional ECM, and initiate the metastatic process. Given the widespread distribution of MT1-MMP both at the invading front of cancer cells and in the surrounding stroma (Seiki et al., 2003; Hlubek et al., 2004) as well as the role of tissue-infiltrating fibroblasts in supporting tumor growth (Dong et al., 2004), we posit that MT1-MMP serves as the key protease mobilized by normal and neoplastic cells as they traverse and remodel ECM barriers in the *in vivo* setting.

Materials and methods

Isolation and culture of mouse skin fibroblasts and human cancer cells

Fibroblasts were isolated from dorsal dermal explants of 2–4-wk-old mice with targeted deletions in MT1-MMP (Holmbeck et al., 1999), MMP-2 (Itoh et al., 1998; provided by S. Itohara, RIKEN Brain Science Institute, Saitama, Japan), MMP-9 (Liu et al., 1998; provided by R.M. Senior and J.M. Shipley, Washington University, St. Louis, MO), MMP-3 (Mudgett et al., 1998), MMP-8 (Balbin et al., 2003), seprase/fibroblast activation protein (Niedermeyer et al., 2000; provided by E. Ostermann and E. Patzelt, Boehringer Ingelheim, Vienna, Austria), plasminogen (Ploplis et al., 1995; provided by T. Bugge, National Institutes of Health, Bethesda, MD), or TIMP-2 (Wang et al., 2000) as well as their wild-type littermates as described previously (Havemose-Poulsen et al., 1998). MMP-13^{-/-} fibroblasts were recovered from knockout mice whose characterization will be described elsewhere. Fibroblasts (passage 2–6) were cultured in DME supplemented with either 10% heat-inactivated FCS (Atlanta Biologicals) or heat-inactivated mouse sera, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and 2 mM l-glutamine. Human HT-1080 fibrosarcoma cells (American Type Culture Col-

lection), SCC-1 squamous carcinoma cells (provided by T. Carey, University of Michigan, Ann Arbor, MI), MDA-MB-231 breast carcinoma cells (American Type Culture Collection), PANC-1 pancreatic carcinoma cells (American Type Culture Collection), and DU-145 carcinoma cells (American Type Culture Collection) were cultured as described previously (Hotary et al., 2003).

RT-PCR analysis

Fibroblasts were cultured atop type I collagen in the presence and absence of 10 ng/ml PDGF in media supplemented with 10% serum, and total RNA was isolated using TRIzol reagent (GIBCO BRL). RT-PCR was performed with 1 μ g of total RNA and 10 μ M of specific primers (Kim et al., 1998; Hotary et al., 2002) using One-Step RT-PCR System reagent (Life Technologies). The identity of each PCR product was confirmed by sequence analysis.

Expression vector construction and transfection

Fibroblasts or tumor cells were transiently transfected with control vector (PCR3.1 Uni; Invitrogen), mColA, mouse MT1-MMP (gift of M. Seiki, University of Tokyo, Tokyo, Japan), human MT1-MMP, catalytically inactive human MT1-MMP (EA mutant, Glu 240→Ala), transmembrane-deleted human MT1-MMP (Δ MT1-MMP), or expression vectors encoding proprotein convertase-activatable forms of human MMP-1, MMP-2, or MMP-13 (MMP-1_{RXKR}, MMP-2_{RXKR}, and MMP-13_{RXKR}) using Fugene6 (Roche) as described previously (Hotary et al., 2003).

Collagen degradation assays

Collagen gel films (~100 μ g distributed uniformly over an area of 2 cm²) were incubated with 0.05 M of sodium borate buffer, pH 9.0, for 1 h and labeled with tetramethylrhodamine isocyanate (Molecular Probes) for 45 min. Fluorescence images of rhodamine-labeled collagen were captured by confocal microscopy. Fibroblasts or tumor cells (5×10^4) were added to the collagen monolayer and incubated for either 5 or 3 d, respectively. Where indicated, cells were labeled with either phalloidin, calcein-AM, or DAPI (Molecular Probes). Collagen degradation products were quantified by hydroxyproline release after an ethanol precipitation step (70% vol/vol; Creemers et al., 1997). Degradation assays were performed in the presence of 10% FCS, in autologous mouse sera (i.e., from wild-type, MMP-2^{-/-}, MMP-9^{-/-}, or TIMP-2^{-/-} mice) or where indicated, under serum-free conditions in the presence of 20 μ g/ml of plasminogen (Enzyme Research). Results are presented as the mean \pm 1 SEM of at least three experiments.

Invasion assays

Type I collagen was acid or pepsin extracted from rat or mice tail tendons (Birkedal-Hansen, 1987; Hotary et al., 2000). Alternatively, pepsin-extracted bovine dermal collagen (Vitrogen; Nutacon) was commercially obtained. Collagen gels were prepared in 24-mm Transwell dishes (3- μ m pore size; Corning, Inc.) with 1 ml of type I collagen used at a final concentration of 2.2 mg/ml (Hotary et al., 2000). After gelling (45 min at 37°C), $1.5\text{--}2 \times 10^5$ cells in media supplemented with either 10% heat-inactivated FCS or 10% mouse serum (from either wild-type, TIMP-2^{-/-}, plasminogen^{-/-}, MMP-2^{-/-}, or MMP-9^{-/-} mice) were added to the upper chamber. PDGF-BB (Upstate Biotechnology) was added to the lower compartment of the Transwell chambers to initiate fibroblast invasion or 50 ng/ml hepatocyte growth factor (HGF; Genentech, Inc.) to trigger tumor cell invasion. Where indicated, gels and media were supplemented with recombinant tissue inhibitor of metalloproteinases (7.5 μ g/ml TIMP-1 or 2.5 μ g/ml TIMP-2; Fuji Chemical Industries), the synthetic MMP inhibitor BB-94 (5 μ M final concentration in 0.1% DMSO; British Biotechnology), the cysteine proteinase inhibitor, E-64d (100 μ M; Sigma-Aldrich), the aspartate proteinase inhibitor pepstatin A (50 μ M; Sigma-Aldrich), or the serine proteinase inhibitors, soybean trypsin inhibitor, SBTI (100 μ g/ml) or aprotinin (200 μ g/ml; Roche). Invasive activity was visualized by either phase-contrast microscopy or confocal laser microscopy of rhodamine-labeled type I collagen gels. The number of invading cells per high powered field and leading front depth of invasion (three or more cells) were quantified as the mean \pm 1 SEM of at least three experiments as described previously (Hotary et al., 2002). Collagen degradation was monitored by hydroxyproline release as described in the section Collagen degradation assays.

To analyze the three-dimensional invasive activity of fibroblasts or tumor cells, 10^5 cells were embedded in 100 μ l of collagen in a 96-well plate and cultured for 24 h. The collagen-cell plugs were then transferred to 24-well plates and further embedded in 1 ml of collagen gels and cultured for 7 d. Cell migration from the central plug into the surrounding collagen was monitored by phase-contrast microscopy.

Gelatin zymography and Western blotting

Gelatin zymography for secreted MMP-2 and MMP-9 and Western blot analysis were performed as described previously (Hotary et al., 2003).

siRNA electroporation

The antisense strand of siRNA was targeted against a 21-nt MT1-MMP sequence [5'-AACAGGCAAAGCTGATGCAGA-3'; nt 228–248]. The nucleotide sequence was scrambled to generate a siRNA control sequence [5'-AAGTGATCAAGCACCGAAGAG-3']. hMMP-1, hMMP-2, and hMMP-9 were targeted using 21-nt sequences (5'-AAGATGTGGACTAGTCCAGA-3', nt 86–106 for hMMP-1; 5'-AATACCATCGAGACCATGCGG-3', nt 274–294 for hMMP-2; and 5'-AAGGAGTACTCGACCTGTACC-3', nt 1066–1086 for hMMP-9). siRNA oligonucleotides (QIAGEN) were introduced into tumor cells (50–100 nM) using a nucleofector kit and electroporation (Amaxa Biosystems). Using fluorescent oligonucleotides, ~99% of the tumor cells were transfected. 20 h after electroporation, siRNA knockdown was complete for up to 72 h.

Chick chorioallantoic membrane assays

In vivo fibroblast or cancer cell invasion and intravasation assays were conducted using 11-d-old chick embryos wherein wild-type or null fibroblasts, HT-1080 cells, or SCC-1 cells (10^5 cells labeled with fluoresbrite 45-nm-diam carboxylate microspheres; Polysciences) were seeded atop the CAM for 3 d as described previously (Kim et al., 1998; Chambers et al., 2002). The percentage of invading cells was quantified as a function of the percentage of the total cell-associated fluorescence localized beneath the CAM surface in five or more randomly selected fields (ImageQuant [version 5.2]; Molecular Dynamics Inc.). Depth of invasion from the CAM surface was defined as the leading front of three or more invading cells in 10 randomly selected fields.

In selected experiments, HT-1080 cells were cultured atop an acellular fragment of human dermis (Alloderm; Life Cell), and then explanted atop the CAM for 3 d to initiate invasion.

Histology and microscopy

Frozen sections of fibroblast-collagen gel cocultures were incubated with a polyclonal antibody against the denatured fragment of type-I collagen (gift of R. Poole, McGill University, Montreal, Canada) as described previously (Hollander et al., 1995). Collagen gels were prepared for transmission EM as described previously (Hotary et al., 2002).

Confocal imaging was performed on a laser scanning fluorescent microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) equipped with acquisition software (Version 3.2 Service Pack 2) using either 40 \times or 63 \times C-Apochromat (1.2 NA) water immersion objectives at 25°C. Samples were mounted in fluorescent mounting media (DakoCytomation). Triple-color imaging of DAPI, Alexa 488-phalloidin or calcein-AM, and rhodamine-red was obtained at 364 nm, 488 nm, and 543 nm, respectively, for selective excitation.

All other bright-field, fluorescent, or phase-contrast images were captured using either an upright fluorescent microscope (Leica; 40 \times objective/0.7 NA) or inverted microscopy (Leica; 10 \times objective/0.3 NA), and digitally acquired using a Spot digital camera (Software v.3.3; Diagnostic Instruments) at RT. Image-processing software (Photoshop 7; Adobe) was used to overlay images and to enhance equally image color and clarity.

Online supplemental material

Figs. S1–S5 are available in the online supplemental material. Fig. S1 shows the effects of BB-94 on collagen invasion and degradation by carcinoma cell lines. Fig. S2 shows the effect of BB-94 on invasion through three-dimensional gels of pepsinized collagen. Fig. S3 shows the effects of MT1-MMP siRNA on tumor cell migration, growth, morphology, and invasion. Fig. S4 shows migration and contractile activity of MMP-null fibroblasts. Fig. S5 shows type I collagen invasion by MT1-MMP siRNA-treated carcinoma cells. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200408028/DC1>.

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Motility and contraction assays

Fibroblast or tumor cell motility was monitored by culturing 5×10^4 cells atop collagen gels within a cloning cylinder. After 24 h, the cylinder was removed and complete medium with 10% autologous serum was added with or without PDGF. The distance migrated by the advancing front of cells (i.e., three or more cells) from the confluent area initially delimited by the cylinder was measured in five randomly selected fields at a magnification of 10.

Contraction of collagen gels by mice skin fibroblasts was monitored by culturing 1×10^5 cells atop collagen gels in the presence of PDGF in 10% heat-inactivated in 24-well plates. After 2 d, the collagen gels were detached from the wells and allowed to contract at 37°C for 24 h. The diameters of the contracted gels were measured, and the contraction was calculated as the change in surface area.