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Membrane Type I Matrix Metalloproteinase Usurps Tumor Growth Control Imposed by the Three-Dimensional Extracellular Matrix

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Summary

Cancer cells are able to proliferate at accelerated rates within the confines of a three-dimensional (3D) extracellular matrix (ECM) that is rich in type I collagen. The mechanisms used by tumor cells to circumvent endogenous antigrowth signals have yet to be clearly defined. We find that the matrix metalloproteinase, MT1-MMP, confers tumor cells with a distinct 3D growth advantage in vitro and in vivo. The replicative advantage conferred by MT1-MMP requires pericellular proteolysis of the ECM, as proliferation is fully suppressed when tumor cells are suspended in 3D gels of protease-resistant collagen. In the absence of proteolysis, tumor cells embedded in physiologically relevant ECM matrices are trapped in a compact, spherical configuration and unable to undergo changes in cell shape or cytoskeletal reorganization required for 3D growth. These observations identify MT1-MMP as a tumor-derived growth factor that regulates proliferation by controlling cell geometry within the confines of the 3D ECM.

Introduction

During the initial phases of the neoplastic process, tumorigenic epithelial cells proliferate at accelerated rates in the two-dimensional (2D) environment that exists atop the underlying basement membrane (Hanahan and Weinberg, 2000; Liotta and Kohn, 2001; Chambers et al., 2002). As transformation progresses, cancer cells acquire the ability to penetrate the basement membrane and infiltrate the subjacent stromal matrix (Hanahan and Weinberg, 2000; Liotta and Kohn, 2001; Chambers et al., 2002). Coincident with the expression of the tissue-invasive phenotype, the tumor cell adapts itself for an abrupt transition in growth environments. No longer confined to the planar surface of the extracellular matrix (ECM), the invading cells are forced to proliferate within a dense three-dimensional (3D) matrix composed largely of type I collagen or crosslinked fibrin (Hanahan and Weinberg, 2000; Hiraoka et al., 1998; Liotta and Kohn, 2001; Chambers et al., 2002). However, despite the fact that the ECM is imbued with potent growth-suppressive properties (Nishiyama et al., 1989; Koyama et al., 1996; Henriet et al., 2000), cancer cells can proliferate at accelerated rates within this 3D environment by circumventing antigrowth signals (Hanahan and Weinberg, 2000; Chambers et al., 2002).

Strategies used by cancer cells to grow rapidly within a 3D ECM remain undefined. A potential role for proteinases in regulating tumor cell proliferation in vivo has been posited on the basis of the growth-suppressive activities displayed by broad spectrum inhibitors directed against enzymes belonging to the serine proteinases, cysteine proteinases, matrix metalloproteinase (MMP), or adamalysin gene families (Hidalgo and Eckhardt, 2001; Coussens et al., 2002). These proteinases cleave a wide variety of substrates that could affect cell growth including cell adhesion molecules, membrane-anchored as well as soluble growth factors, growth factor receptors, chemokines, and ECM macromolecules (Brinckerhoff and Matrisian, 2002; Coussens et al., 2002; Egeblad and Werb, 2002). However, antiproteinases almost uniformly exert more significant effects on tumor cell growth in vivo than in vitro (Hidalgo and Eckhardt, 2001; Coussens et al., 2002). As such, current opinion favors the possibility that proteinases indirectly control the proliferative activity of cancer cells by either cleaving targets peculiar to the in vivo environment (e.g., matrix-associated growth factors) or by affecting the angiogenic response necessary for supporting tumor expansion (Hidalgo and Eckhardt, 2001; Coussens et al., 2002; Egeblad and Werb, 2002).

To date, the consensus that proteinases do not regulate tumor cell growth directly has been based largely on in vitro model systems wherein cancer cell behavior is classically assessed in a 2D environment atop either a plastic substratum or a planar coat of ECM macromolecules. As cell behavior is critically affected by the physical presentation of the surrounding ECM (Cukierman et al., 2001, 2002; Walpita and Hay, 2002), we considered the possibility that proliferative responses of matrix-embedded cancer cells are controlled differentially under 3D conditions more relevant to the in vivo milieu encountered by tissue-invasive cells. Herein, we demonstrate that the membrane-anchored matrix metalloproteinase, MT1-MMP, confers tumor cells with a 3D growth advantage by directly driving proliferative responses within the confines of physiologically relevant ECM matrices in vitro or in vivo. MT1-MMP-enhanced growth is reversed completely by either suspending cancer cells in a 3D matrix constructed from type I collagen fibrils harboring knockin mutations surrounding the major MMP cleavage site in the triple helix or by blocking fibrinolysis. In the absence of ECM proteolysis, matrix-embedded tumor cells are locked in a compact configuration and are unable to alter cell geometry in a fashion consistent with a proliferative response. These findings identify MT1-MMP as collagenolytic/fibrinolytic growth

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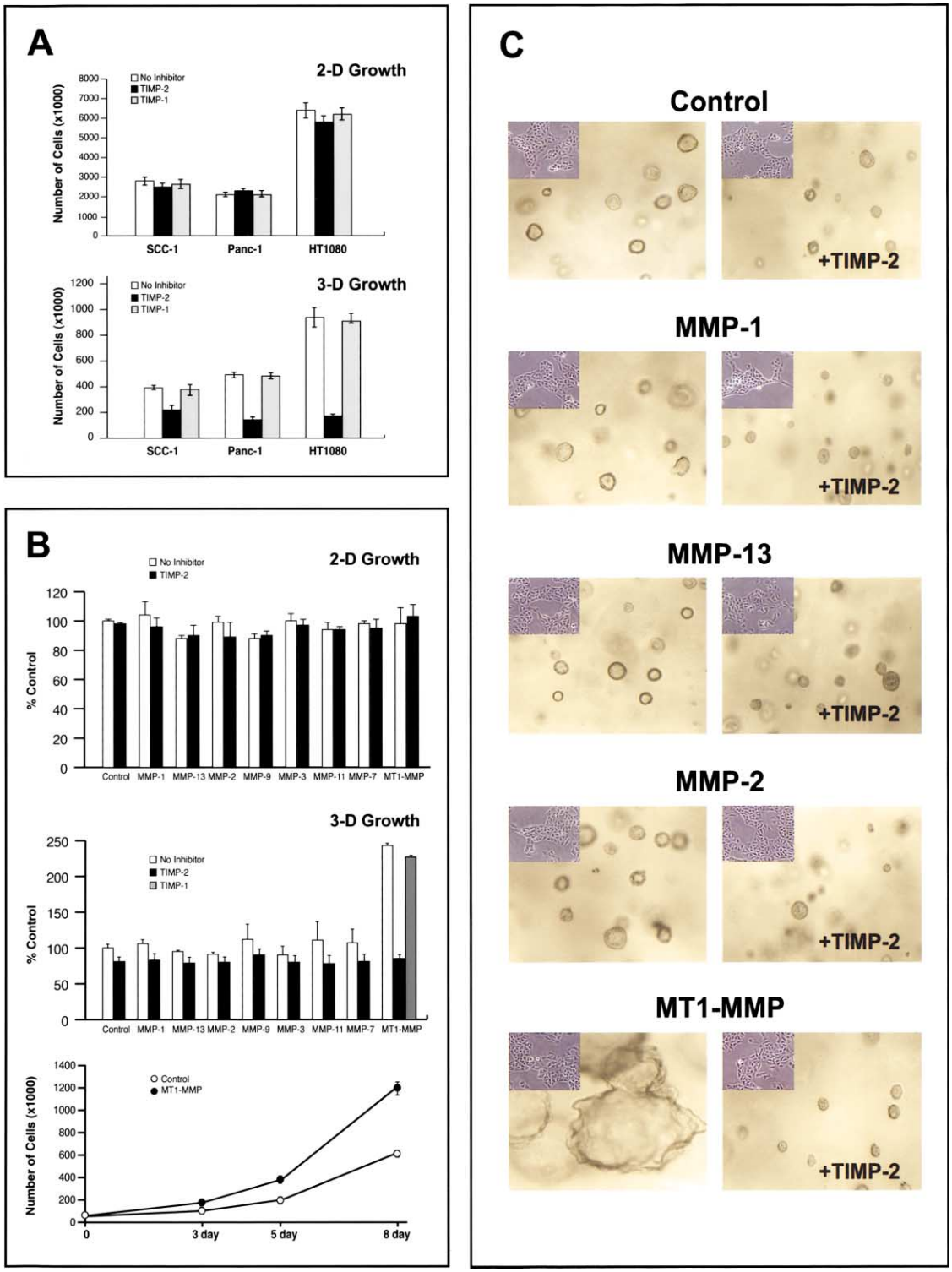


Figure 1. MMP Regulation of Cell Proliferation

(A) SCC-1, Panc-1, or HT-1080 cells were seeded at an initial density of 5×10^4 cells/well atop (2D) or within (3D) type I collagen gels in the absence or presence of TIMP-2 (5 μg/ml) or TIMP-1 (12.5 μg/ml) and cultured for 8 days, at which time cell number was determined.

(B) Control or MMP-transfected MDCK cells were cultured atop collagen gels for 8 days and cell number determined in the absence or presence of TIMP-2 (5 μg/ml; top). Under 3D growth conditions, MT1-MMP-transfected cells proliferated at a significantly faster rate than control cells (middle; $p < .001$) via a TIMP-2-, but not TIMP-1-, sensitive process. Results are expressed as the mean ± 1 SEM for triplicate cultures.

(C) Control, MMP-1-, MMP-13-, MMP-2-, or MT1-MMP-transfected MDCK cells were cultured within type I collagen gels in the absence or presence of TIMP-2 (5 μg/ml) for 8 days (100×). Insets show subconfluent cells on 2D collagen gels after 5 days.

factor that provides cancer cells with the ability to accelerate proliferative responses by defeating the growth-suppressive signals embedded within the 3D ECM.

Results

MMP-Dependent Regulation of 3D Growth

Under planar growth conditions atop the surface of type I collagen gels, squamous carcinoma cells (SCC-1), pancreatic carcinoma cells (Panc-1), or fibrosarcoma cells (HT-1080) proliferate rapidly relative to tumor cells embedded within the type I collagen matrix where growth decreases by ~70% (Figure 1A). Further, whereas neither serine- (aprotinin, soybean trypsin inhibitor), cysteine- (E-64), aspartyl- (pepstatin), nor matrix metallo-proteinase inhibitors affect tumor cell growth under 2D conditions (Figure 1B and data not shown), 3D proliferation of SCC-1, Panc-1, and HT-1080 cells is inhibited ~50%–80% by the MMP inhibitor, TIMP-2 (Figure 1B). Similar results were obtained with the synthetic MMP inhibitor, BB-94 (data not shown). Hence, within a growth-suppressive, 3D matrix of type I collagen, tumor cell proliferation is controlled by an MMP-dependent process.

To identify the TIMP-2-sensitive proteinase(s) that control 3D growth, a nontumorigenic cell line known to express low levels of a limited repertoire of MMPs, i.e., MDCK cells (Hotary et al., 2000), was engineered to stably express a series of MMPs previously associated with tumor growth in vivo (Hidalgo and Eckhardt, 2001; Coussens et al., 2002; Egeblad and Werb, 2002). As observed with the tumor cell lines, the proliferative activity of control MDCK clones is suppressed strongly when shifted from a 2D to 3D growth environment (Figure 1B). However, consistent with the limited potential of wild-type MDCK cells to express MMPs, TIMP-2 only modestly affects 3D growth of control clones (Figure 1B). Despite a paucity of endogenous MMPs, the replicative response of MDCK stable clones is not enhanced under 2D or 3D growth conditions by overexpressing the secreted MMPs, MMP-1 (collagenase-1), MMP-13 (collagenase-3), or MMP-2 (gelatinase A) (Figure 1B). Similarly, stable transfectants overexpressing MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-9 (gelatinase B), or MMP-11 (stromelysin-3) proliferate at normal rates under 2D conditions (100% $\pm$ 5%, 98% $\pm$ 2%, 88% $\pm$ 3%, and 94% $\pm$ 5% of control transfected cells, respectively) or 3D conditions (96% $\pm$ 12%, 107% $\pm$ 19%, 112% $\pm$ 21%, or 111% $\pm$ 25% of control transfected cells, respectively; $n = 3$). By contrast, in MDCK clones expressing the membrane-anchored MMP, MT1-MMP, cell number increases at an accelerated rate under 3D, but not 2D, growth conditions (Figure 1B). Changes in 3D cell growth rates are also accompanied by marked changes in the morphology of the embedded cysts (Figure 1C). TIMP-2, an efficient MT1-MMP inhibitor (Hotary et al., 2000, 2002), suppresses the growth rate of the MT1-MMP transfectants to control levels under 3D growth conditions and completely reverses the aberrant cyst morphology (Figures 1B and 1C). Likewise, MDCK clones expressing a catalytically inactive form of MT1-MMP (Yana and Weiss, 2000) did not stimulate proliferation relative to control transfected clones (i.e., 109% $\pm$ 3% of control transfected cells; $n = 3$). TIMP-1, a potent inhibitor of secreted MMPs that only inefficiently regulates MT1-MMP activity (Hotary et al., 2002), did not affect the accelerated growth rate of MT1-MMP-transfected MDCK clones or wild-type cancer cells (Figures 1A–1C).

MT1-MMP as a Direct-Acting Growth Promoter

MMPs are normally synthesized and secreted as inactive proenzymes, whereas the MT1-MMP prodomain is encrypted with a proprotein convertase recognition motif that allows the zymogen to undergo intracellular processing to its active form prior to its display at the cell surface (Yana and Weiss, 2000; Brinckerhoff and Matrisian, 2002). To rule out the possibility that MT1-MMP controlled cell proliferation only as a function of its efficient processing to an active proteinase, chimeric forms of MMP-1, MMP-2, and MMP-13 (MMP-1_{RXKR}, MMP-2_{RXKR}, MMP-13_{RXKR}) were expressed wherein an Arg-X-Arg-X-Lys-Arg (RXRXKR) motif recognized by proprotein convertases was inserted between the propeptide and catalytic domains of each of the respective MMPs (Pei and Weiss, 1995). Under these conditions, each of the RXKR constructs is secreted as the active proteinase in stably transfected clones (Figure 2A). However, none of the three active MMP chimeras accelerates growth under 3D conditions relative to control transfected clones (Figure 2B and data not shown).

Following intracellular processing, the mature, active form of MT1-MMP can catalyze the activation of either the MMP-2 or MMP-13 zymogens (Brinckerhoff and Matrisian, 2002), leaving open the possibility that MT1-MMP-dependent growth acceleration occurs because of its ability to activate MMP-2 or MMP-13. Though MDCK cells secrete virtually undetectable levels of MMP-2, the serum-supplemented media contains significant levels of the proenzyme (Hiraoka et al., 1998; Hotary et al., 2000). Nonetheless, the growth rate of the MT1-MMP-transfectants remains robust in MMP-2-depleted media (Figure 2C). Further, the ability of MMP-2 or MMP-13 to augment MT1-MMP-dependent growth in *cis* or *trans* was also examined by culturing MT1-MMP transfectants with MDCK clones overexpressing either MMP-2 or MMP-13. In these coculture experiments, neither the size nor the morphology of the 3D spheroids generated by the MT1-MMP transfectants is altered in the presence of the MMP-2 or MMP-13 overexpressing clones (Figure 2D). Taken together, these data imply that MT1-MMP regulates 3D growth directly.

MT1-MMP Confers Tumor Cells with a Growth Advantage In Vitro and In Vivo

While MT1-MMP conferred a growth advantage to a nontumorigenic cell line, neoplastic tumor cell lines may employ alternate strategies for maximizing proliferation within the 3D environment (Hanahan and Weinberg, 2000; Chambers et al., 2002). Consequently, the ability of MT1-MMP overexpression to promote tumor cell growth was examined in SCC-1 cells as well as an MT1-MMP-deficient, breast carcinoma cell line (MCF-7 cells; Sounni et al., 2002). As shown in Figure 3, control transfected SCC-1 cells proliferate as loose aggregates in 3D collagen gels (panels A–C). By contrast, the growth rate of MT1-MMP-transfected SCC-1 cells increases by

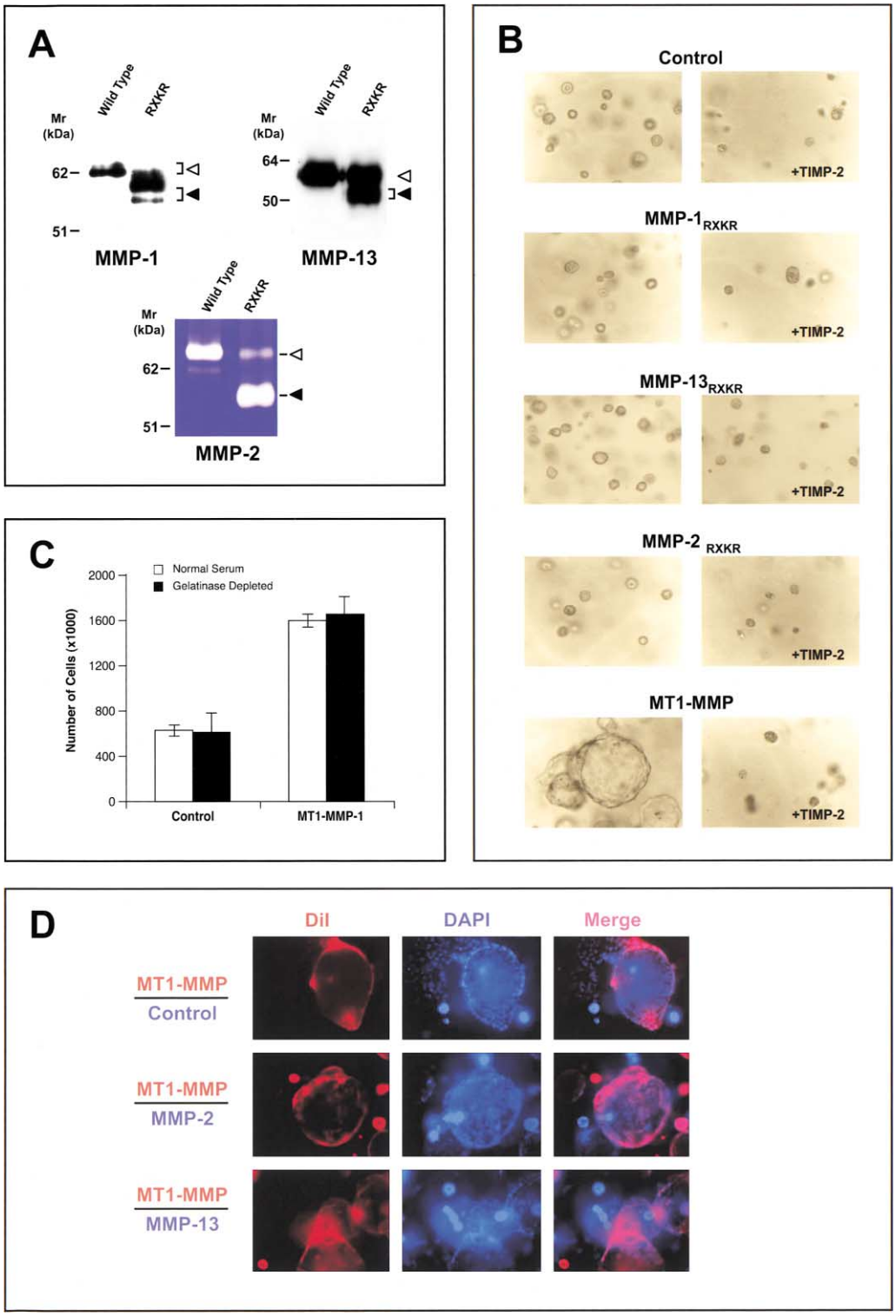


Figure 2. MT1-MMP Enhancement of Cell Growth

(A) Processing of RXKR-inserted MMPs as assessed by Western blotting (MMP-1 and MMP-13) or gelatin zymography (MMP-2). Open and closed arrows indicate the pro and active forms of the enzymes, respectively, while brackets enclose glycosylated and nonglycosylated forms.

(B) MDCK cells were stably transfected with the empty vector, RXKR-inserted MMP-1 ($MMP1_{RXKR}$), MMP-13 ($MMP13_{RXKR}$), MMP-2 ($MMP2_{RXKR}$), or wild-type MT1-MMP and cultured for 8 days within collagen gels in the absence or presence of TIMP-2 (5 μg/ml).

(C) Proliferative response of control or MT1-MMP expressing cells after 8 days in gelatinase-depleted serum. Expressed as the mean ± 1 SEM for triplicate cultures.

(D) DiI-labeled (red) MT1-MMP transfectants (2.5×10^4) were cocultured with 2.5×10^4 unlabeled control, MMP-2-, or MMP-13-transfected cells in 3D collagen for 8 days. Cultures were DAPI stained (blue), and red and blue digital images of the same field were collected and merged. In merged images, MT1-MMP-expressing cells appear pink/red while control, MMP-2-, or MMP-13-transfected cells appear blue.

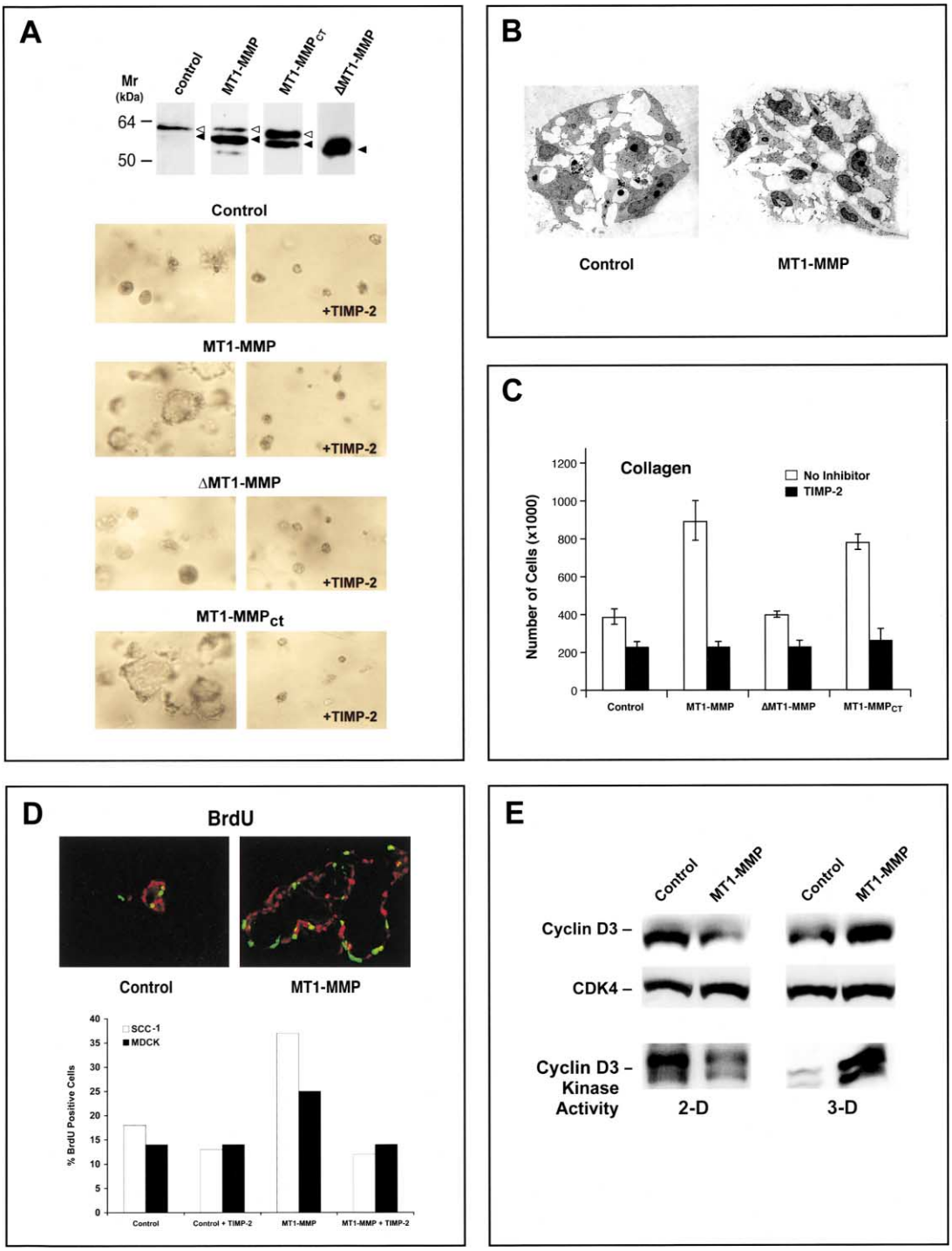


Figure 3. Regulation of SCC-1 Proliferation by MT1-MMP in 3D Culture

(A) Control SCC-1 or SCC-1 clones expressing MT1-MMP, soluble MT1-MMP (ΔMT1-MMP), or cytosolic tail-deleted MT1-MMP ($MT1\text{-}MMP_{ct}$) were cultured in 3D collagen for 8 days in the absence or presence of TIMP-2 (5 μg/ml). Immunoblots show representative expression levels of each construct. MT1-MMP levels in cells transfected with the wild-type enzyme is approximately 50% that detected in HT-1080 cells (data not shown). Open and closed arrows indicate pro and active forms of MT1-MMP, respectively.

(B) Transmission electron micrographs of control and MT1-MMP-transfected SCC-1 cells growing as loose aggregates for 8 days in 3D collagen (500×).

(C) Growth of control, MT1-MMP-, ΔMT1-MMP-, and $MT1\text{-}MMP_{ct}$-transfected SCC-1 cells in 3D culture in the absence or presence of TIMP-2 (5 μg/ml) expressed as the mean ± 1 SEM for triplicate cultures.

(D) BrdU uptake in control or MT1-MMP-transfected MDCK (photos and open bars) and SCC-1 (closed bars) cells was monitored in the absence or presence of TIMP-2 (5 μg/ml) in 3D cultures. The percentage of BrdU-positive cells (green) among all cells (red) per longitudinal section was determined after 8 days in culture. Bar graphs show representative counts for one complete section.

(E) Relative expression levels for cyclin D3 and CDK4 expression as well as cyclin D3 kinase activity was determined in control and MT1-MMP-transfected SCC-1 cells after 8 days in 2D or 3D collagen culture. Results are representative of three experiments performed.

approximately 2.5-fold under 3D conditions, coincident with a decrease in the density of cell packing within the tumor cell aggregates (Figures 3A–3C). Consistent with their MT1-MMP-deficient status, control clones of MCF-7 breast carcinoma cells only marginally increase in cell number over an 8 day culture period under 3D growth conditions (i.e., from 50×10^3 to $64 \pm 6 \times 10^3$; $n = 3$). However, stable expression of MT1-MMP in MCF-7 cells leads to a 4.2 ± 0.3-fold increase in cell growth (i.e., to $25.2 \pm 2.1 \times 10^4$ cells; $n = 3$). Significantly, increased growth rate reflects an increase in the proliferative index for MT1-MMP-transfected cells, since apoptotic rates are not affected by MT1-MMP expression (Figures 3D and data not shown). Likewise, the ability of TIMP-2 to reverse the MT1-MMP-dependent growth advantage represents a decrease in the proliferative index alone (Figures 3C and 3D) with no change in apoptosis as assessed by TUNEL (data not shown).

Given that MT1-MMP-dependent effects are limited to changes in proliferative indices, cell cycle changes associated with the transition of cancer cells from 2D to 3D growth environments were assessed. While neither cyclin D1, D3, A, or E nor CDK2, CDK4, or CDK6 expression are affected by MT1-MMP expression in cells cultured atop or within collagen gels, cyclin D3 kinase activity is markedly upregulated in MT1-MMP-transfected cells under 3D, but not 2D, growth conditions (Figure 3E). Changes in cyclin D3 kinase activities were confirmed in additional SCC-1 clones and could be reversed completely by TIMP-2 (data not shown). Under 2D growth conditions, neither proliferation nor apoptosis is altered by overexpressing MT1-MMP in SCC-1 or MCF-7 cells.

Recent studies suggest that the cytosolic tail and transmembrane domains of MT1-MMP play a major role in regulating its activity and function (Seiki, 2002). As the processing of MT1-MMP to its catalytically active form is unaffected by deleting the cytosolic tail or transmembrane domain (Hiraoka et al., 1998; Yana and Weiss, 2000; Hotary et al., 2000, 2002), we sought to determine whether the growth-promoting activity of MT1-MMP is dependent on either of these domains. Hence, SCC-1 cell lines were generated that expressed membrane-anchored MT1-MMP with either the cytosolic domain or the transmembrane domain deleted (MT1-MMP$_{ct}$ or ΔMT1-MMP, respectively). While tail-deleted MT1-MMP confers a similar growth advantage to transfected carcinoma cells, the soluble, transmembrane-deleted form of MT1-MMP does not display growth-enhancing activity (Figures 3A and 3C). Thus, only the membrane-anchored display of MT1-MMP at the cell surface is a necessary requirement for promoting tumor cell growth.

In vivo, type I collagen is the major extracellular matrix in the dermis where its concentration is estimated at 30 mg/ml (Hay, 1991). To determine whether MT1-MMP regulates tumor cell behavior in this more stringent environment, tumor mass, proliferation, and apoptosis were determined in a mouse model following the intradermal injection of wild-type SCC-1 cells, control transfectants, or MT1-MMP-transfected clones expressing high, intermediate, or low levels of the proteinase (clones MT1-MMP-1c, MT1-MMP-3c, and MT1-MMP-6, respectively). As shown in Figure 4, the tumor mass and proliferative index of MT1-MMP transfectants increases at accelerated rates in vivo. The growth-promoting phenotype correlates with the level of MT1-MMP expression (data not shown) and is independent of apoptotic indices (Figure 4). Contrary to recent reports (Sounni et al., 2002; Deryugina et al., 2002), VEGF expression by MT1-MMP transfectants was not increased in vivo or in vitro (data not shown).

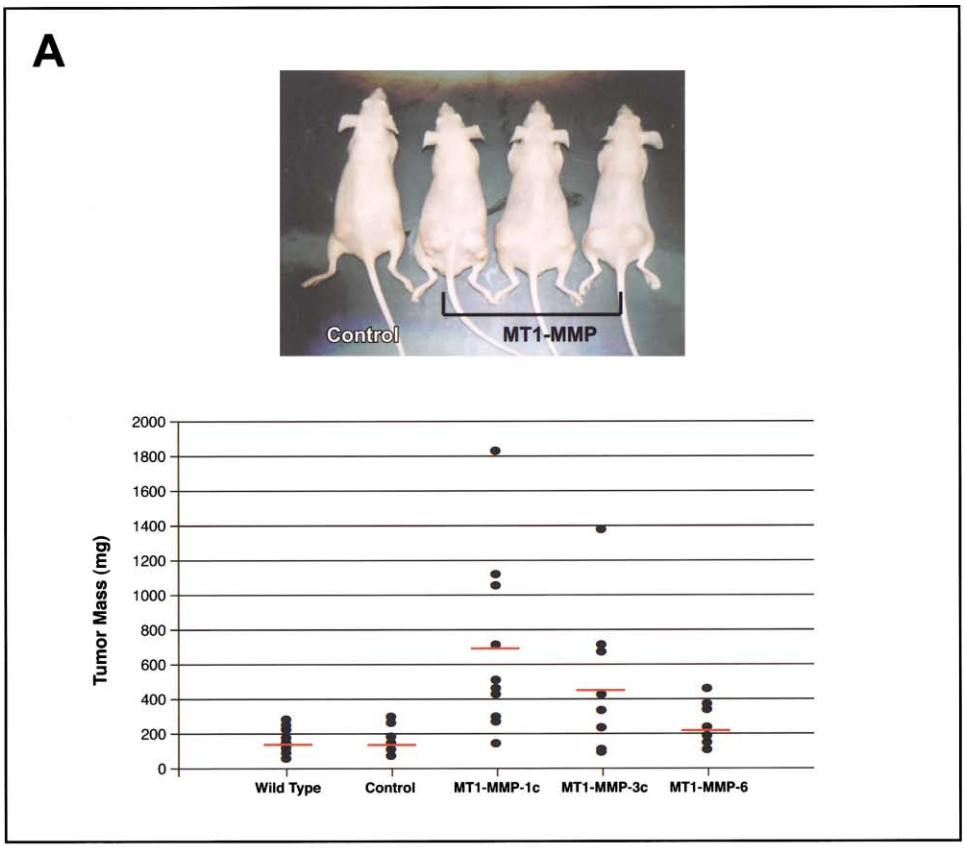
In preliminary studies designed to assess MT1-MMP structure/function relationships underlying growth enhancement in vivo, the cytosolic tail-deleted form of MT1-MMP increased tumor mass similarly to wild-type MT1-MMP (i.e., mean tumor mass in 5 mice injected at two sites with a control transfected clone was 227 ± 52 mg versus 997 ± 115 mg for MT1-MMP$_{ct}$). By contrast, 2 of 3 clones overexpressing soluble MT1-MMP grew at rates indistinguishable from the control clone (i.e., 237 ± 31 mg and 225 ± 50 mg). Taken together, these data suggest that membrane-anchored MT1-MMP confers a growth advantage in vitro as well as in vivo by stimulating cell proliferation.

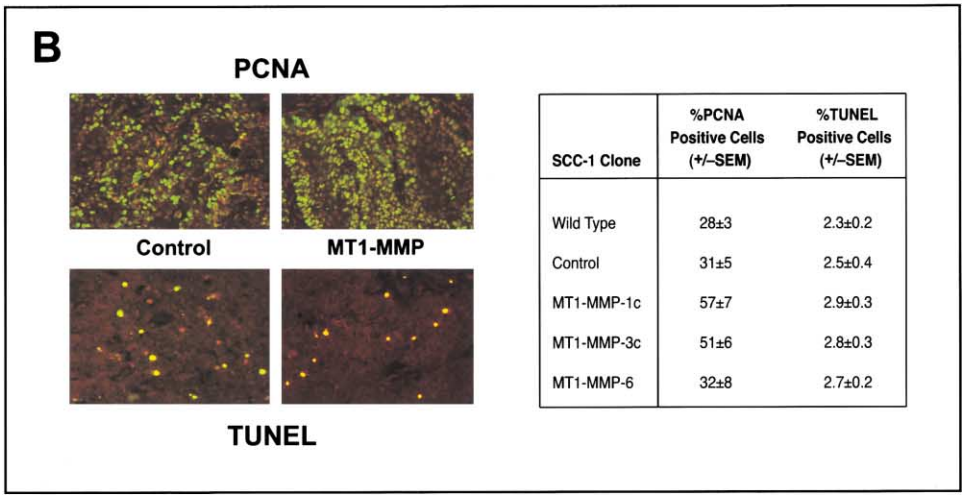
MT1-MMP-Dependent Type I Collagenolysis Is Required for 3D Growth

MMPs can alter cell behavior by proteolyzing a range of nonextracellular matrix targets (Coussens et al., 2002; Egeblad and Werb, 2002). Though MT1-MMP might regulate proliferative responses by targeting cell-derived factors independent of the surrounding matrix, MT1-MMP does not confer a growth advantage when cells are suspended in a 3D gel of either soft agar (Figure 5A) or in Matrigel, a matrix rich in laminin and type IV collagen (data not shown). As the MT1-MMP-dependent proliferative response is dependent on the 3D collagen matrix, these observations are consistent with the generation of a collagen-derived growth enhancer or proteolytic inactivation of a diffusible growth suppressor. However, the latter hypothesis is made less likely given that MT1-MMP-transfected cells cocultured in close proximity to control transfectants in 3D collagen gels do not affect the proliferation rate of the control clones (Figure 5B).

Alternatively, candidates for collagen-derived growth enhancers that may account for MT1-MMP-dependent phenotype include gelatin, a known $\alpha_v\beta_3$ ligand that can affect cell growth (Petitclerc et al., 1999; Xu et al., 2001). Gelatin is generated from triple-helical collagen following its proteolysis by MT1-MMP at a single site to generate two fragments that spontaneously undergo thermal denaturation at 37°C (Ohuchi et al., 1997). However, while pericellular gelatin staining is detected surrounding MT1-MMP-transfected cells in the absence, but not the presence, of TIMP-2 (Figure 5C), exogenous gelatin incorporated into collagen gels fails to stimulate the growth of control or MT1-MMP-transfected cells in the presence or absence of TIMP-2 (Figure 5D). Likewise, growth was unaffected by monoclonal antibodies (LM609 or HU177), which interfere specifically with gelatin binding to cell surface integrins (Figure 5E; Petitclerc et al., 1999; Xu et al., 2001).

While the formation of collagen degradation products could not be directly implicated in MT1-MMP-dependent proliferative responses, the growth-suppressive properties of 3D collagen itself might prove sensitive to proteolytic attack. Hence, tumor cell growth was com-





SCC-1 Clone	%PCNA Positive Cells (+/–SEM)	%TUNEL Positive Cells (+/–SEM)
Wild Type	28±3	2.3±0.2
Control	31±5	2.5±0.4
MT1-MMP-1c	57±7	2.9±0.3
MT1-MMP-3c	51±6	2.8±0.3
MT1-MMP-6	32±8	2.7±0.2

Figure 4. SCC-1 Tumor Growth in Nude Mice

(A) Tumors generated in the flanks of nude mice are shown 2 weeks after the subcutaneous injection of 5×10^6 wild-type, control, or MT1-MMP-transfected SCC-1 cells (clone MT1-MMP-1c). Chart shows individual tumor masses (10 for each clone) with the mean indicated by a red bar. The levels of MT1-MMP in clones 3c and 6 were approximately 60% and 25%, respectively, of that expressed in clone 1c.

(B) Proliferative and apoptotic indices were determined in tumor sections from nude mice inoculated with control or MT1-MMP-1c-transfected SCC-1 cells by PCNA staining and TUNEL, respectively. Propidium iodide counterstaining (red) was used to determine total cell numbers.

pared in collagen gels isolated from wild-type mice and transgenic mice harboring a knockin mutation that disrupts the site of collagenolytic attack (i.e., r/r collagen; Liu et al., 1995). Significantly, the growth of MT1-MMP-transfected cells in r/r collagen is suppressed to levels similar to those displayed by control transfected cells suspended in wild-type collagen supplemented with TIMP-2 (Figures 5F and 5G). Whereas TIMP-2 significantly inhibits the growth of MT1-MMP-transfected cells in wild-type collagen, their growth rate remains unperturbed when r/r gels are supplemented with TIMP-2 (Figure 5B). Further, when suspended in r/r collagen, Panc-1

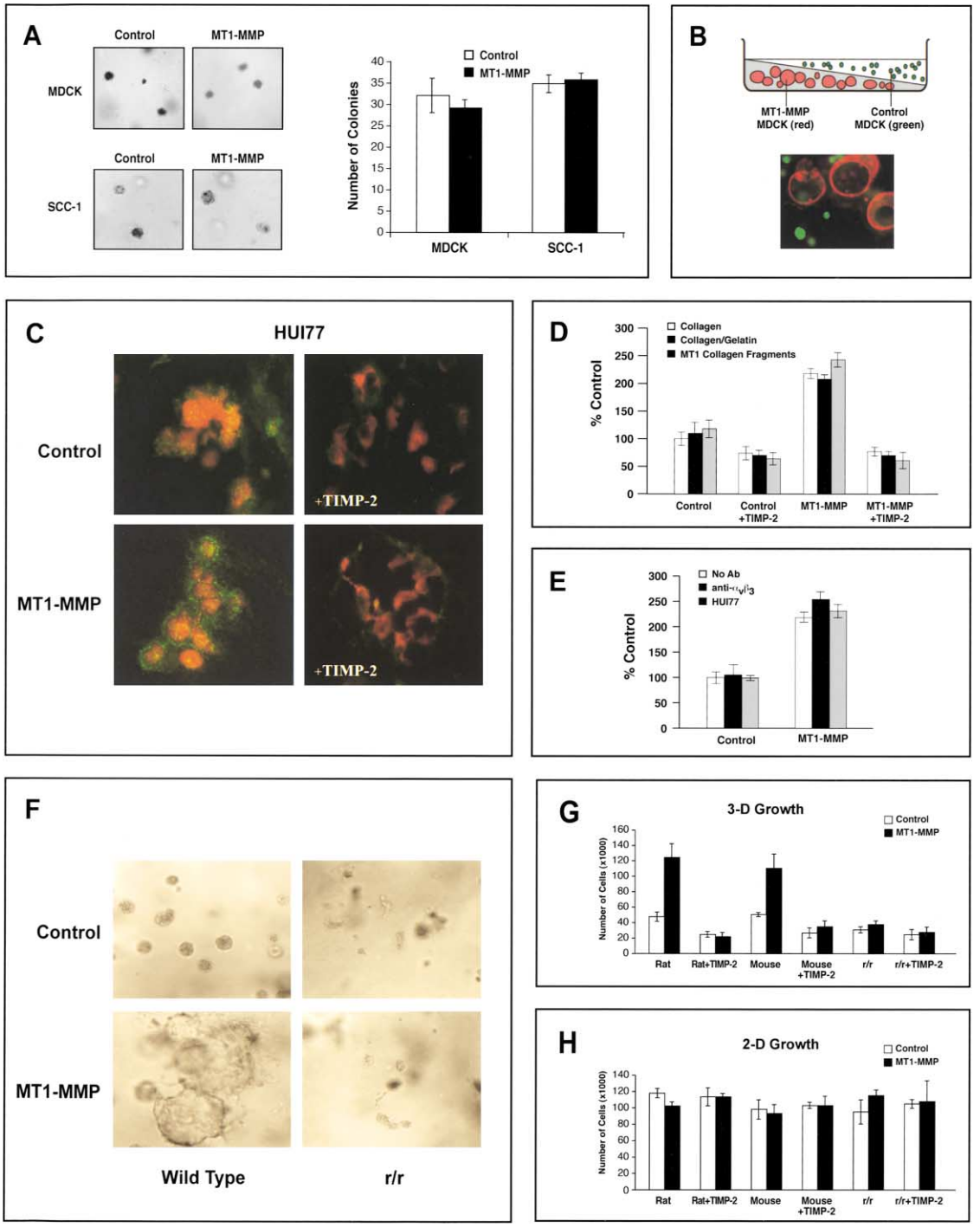


Figure 5. Characterization of the MT1-MMP-Mediated Growth Enhancement

(A) Control and MT1-MMP-transfected MDCK or SCC-1 cells (5×10^4) were cultured in 0.5% agar for 8 days. Results are expressed as the mean number of colonies ± 1 SEM in triplicate cultures.

(B) MT1-MMP-transfected MDCK cells (2.5×10^4) were DiI-labeled (red) and cocultured with DiO-labeled (green) control (2.5×10^4) MDCK cells as illustrated (top). After 8 days, red and green digital images of the same field were collected.

(C) MT1-MMP transfectants cultured in 3D collagen for 8 days displayed an enhanced ability to generate collagen degradation products in the pericellular milieu relative to control-transfected cells. Degraded collagen (detected by mAb HUI77) appears as punctuate green staining around propidium iodide-labeled cells (red). In the presence of TIMP-2 (5 μg/ml), staining for degraded collagen is reduced to background levels.

(D and E) Control and MT1-MMP-transfected SCC-1 cells were cultured for 8 days in collagen gels impregnated with 1% gelatin or 0.5% MT1-MMP-generated collagen fragments in the absence or presence of TIMP-2 (5 μg/ml; D) or with mAb LM609 (20 μg/ml) or HUI77 (100 μg/ml) (E). Results are expressed as the mean percent of control cell numbers ± 1 SEM of triplicate cultures.

(F) Control or MT1-MMP-transfected SCC-1 cells were buried in wild-type or r/r mouse collagen for 8 days and morphology assessed.

(G and H) Cell numbers were determined for control and MT1-MMP-transfected SCC-1 cells (7.5×10^3) cultured within (3D) (G) or atop (2D) (H) collagen gels isolated from wild-type or r/r mice for 8 or 5 days, respectively, in the absence or presence of TIMP-2 (5 μg/ml). Results are expressed as the mean cell number ± 1 SEM for triplicate cultures.

and HT-1080 as well as MT1-MMP-transfected MCF-7 cells all display a reduced proliferative activity similar to that observed in 3D gels of wild-type collagen supplemented with TIMP-2 (i.e., after 8 days in culture in wild-type versus r/r collagen, the proliferation of Panc-1 cells decreased from $44.4 \pm 2.0 \times 10^4$ to $13.2 \pm 8 \times 10^4$, HT-1080 cell number decreased from $67.8 \pm 4.4 \times 10^4$ to $6.7 \pm 4.0 \times 10^4$, and the proliferation of MT1-MMP-transfected MCF-7 cells decreased from 25.2×10^4 to $3.5 \pm 5.0 \times 10^4$; $n = 3$). In contrast to the inhibitory effects exerted by r/r collagen under 3D growth conditions, proliferation of control transfected or MT1-MMP-transfected carcinoma cells is not altered under 2D growth conditions atop r/r collagen gels (Figure 5H).

Geometric Control of 3D Tumor Cell Proliferation Is Regulated by MT1-MMP

The ability of 3D collagen to regulate cell proliferation could reflect integrin- or discoidin receptor-mediated signaling (e.g., Cukierman et al., 2002) or alternatively, growth suppression consequent to enmeshing the tumor cells in a physical cage that prevents the well-characterized changes in cell geometry and cytoskeletal rearrangements necessary to drive a proliferative response (e.g., Chen et al., 1997; Ingber, 2003; Tan et al., 2003). If collagen fibers act as a 3D growth regulator by constraining tumor cell shape, then a structurally distinct fibrillar network should exert similar, if not identical, effects on tumor cell geometry and growth. Fibrin, like type I collagen, enmeshes cancer cells in a dense, crosslinked fibrillar network in vivo that can be proteolyzed by MT1-MMP (Hiraoka et al., 1998; Hotary et al., 2002). Significantly, 3D fibrin gels suppressed cell growth comparably to collagen gels (e.g., the growth of wild-type MDCK cells or SCC-1 cells in 3D fibrin is depressed by 79% and 80%, respectively, relative to 2D growth conditions; $n = 2$). Further, as in the case of 3D collagen gels, only MT1-MMP confers MDCK transfectants with a growth advantage in 3D fibrin gels (a 2.2 ± 0.2-fold increase in cell number relative to control transfectants; $n = 4$) while likewise stimulating SCC-1 and MCF-7 cell proliferation in a TIMP-2-sensitive fashion (Figures 6A and 6B).

In the presence of serum-derived mitogens, anchorage-dependent growth atop planar substrates will not proceed through the cell cycle restriction point into S phase unless adherent cells can spread, engage contractile machinery, and rearrange their actin cytoskeleton (Chen et al., 1997; Ingber, 2003; Tan et al., 2003). Given the density of the meshwork of crosslinked collagen and fibrin that surrounds embedded cells (Figure 6C), cell spreading in response to serum-derived growth factors might be predicted to require matrix proteolysis. Hence, changes in cell geometry were monitored in 3D matrices in the absence or presence of MT1-MMP proteolytic activity. As shown in Figure 6D, while MT1-MMP-expressing SCC-1 cells readily stretch within 3D collagen or fibrin gels as well as intact skin, inhibiting MT1-MMP activity with TIMP-2 traps cells in a compact, spherical configuration. Confocal images of phalloidin-stained SCC-1 cells embedded in 3D collagen or fibrin matrices reveals the organization of a centrally located network of stress fibers in stretched cells as compared to TIMP-2-treated cells where the actin network is confined to a thin, cortical shell (Figure 6E). By contrast, MT1-MMP-deficient MCF-7 cells suspended in 3D gels are locked into spheres that can only undergo shape change and rearrange their actin cytoskeleton following expression of MT1-MMP in the absence, but not the presence, of TIMP-2 (Figure 6E). While cell spreading and cytoskeletal restructuring is unaffected by MT1-MMP under 2D growth conditions (data not shown), the percentage of MT1-MMP-transfected SCC-1 cells undergoing shape change in 3D collagen, fibrin, or skin (79%, 91%, and 57%, respectively) is decreased to 11%, 4%, and 14% in the presence of TIMP-2. Likewise, the number of MCF-7 cells able to undergo a 3D shape change increases from 6% in collagen gels and 5% in fibrin gels to 61% and 52%, respectively, following MT1-MMP expression ($n = 2$). As expected, 3D growth of cancer cells is inhibited dramatically when the actin network is disrupted with 1 μg/ml cytochalasin D (e.g., the growth of control and MT1-MMP-expressing SCC-1 cells decreases from $228 \pm 16 \times 10^3$ and $642 \pm 48 \times 10^3$, respectively, to $20 \pm 4 \times 10^3$ and $24 \pm 2 \times 10^3$ cells during 8 days in culture) (Ingber, 2003). Thus, within the confines of the 3D ECM, MT1-MMP-dependent pericellular proteolysis regulates the critical changes in cell geometry that drive the proliferative response.

Discussion

As epithelial-derived cancer cells engage gene programs necessary to promote invasion from primary sites into the underlying stroma, they must adapt to the new growth conditions dictated by life within a dense, 3D meshwork of ECM macromolecules (Liotta and Kohn, 2001; Chambers et al., 2002). Under normal circumstances, the type I collagen-rich interstitial matrix has been thought to effectively regulate cell growth by either ligating collagen-specific receptors (Cukierman et al., 2002) or sequestering ECM bound growth factors (Egeblad and Werb, 2002). Yet, within the same 3D ECM, tissue-invasive cancer cells almost immediately begin to proliferate at accelerated rates (Chambers et al., 2002). Thus, in the earliest stages of postinvasive growth, cancer cells are able to replicate in an autonomous fashion until the metabolic requirements of the tumor mass can no longer be met by simple diffusion and an angiogenic switch is activated in order to recruit new blood vessels (Hanahan and Weinberg, 2000).

Recent studies have demonstrated that the in vitro culture of normal cells within a 3D matrix exerts profound effects on intracellular signaling cascades, cytoskeletal organization, and gene expression (Cukierman et al., 2001, 2002; Walpita and Hay, 2002). Less is known about the behavior of cancer cells under 3D growth conditions, but we reasoned that the factors regulating the preangiogenic proliferative activity of tumor cells in situ would be more accurately recapitulated if cells were suspended within a dense, structurally ordered matrix of crosslinked, type I collagen fibrils. Indeed, under 3D growth conditions, MMP inhibitors were able to suppress tumor cell proliferation in vitro in a fashion similar to that described in vivo (Hidalgo and Eckhardt, 2001; Coussens et al., 2002). Thus, while MMPs were not re-

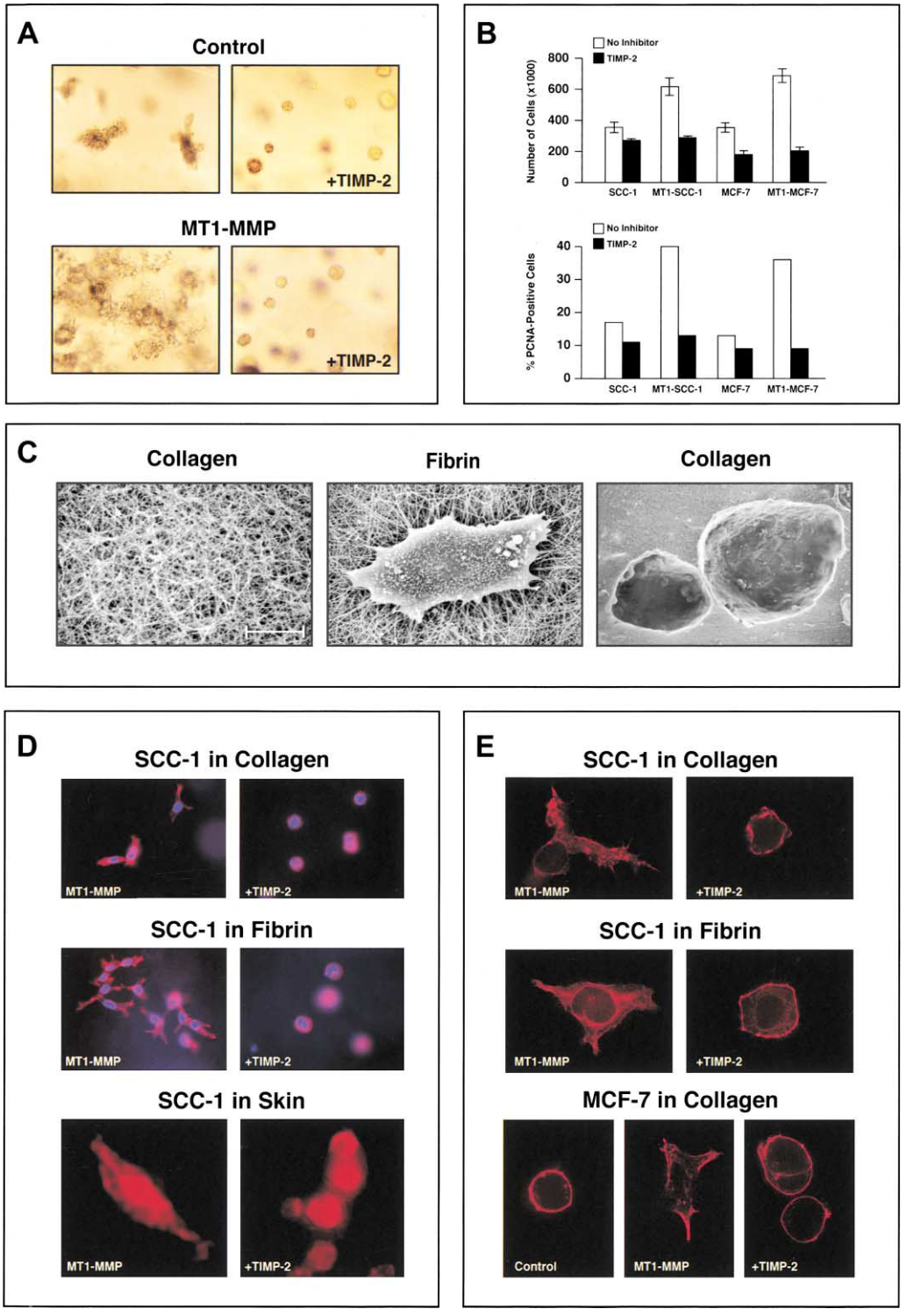


Figure 6. MT1-MMP Promotes Tumor Cell Shape Change and Proliferation in 3D Matrices

(A) Control or MT1-MMP-transfected SCC-1 cells were cultured in 3D fibrin gels for 8 days in the absence or presence of TIMP-2 (5 μg/ml).

(B) Proliferative responses of control and MT1-MMP-transfected SCC-1 or MCF-7 cells were determined in 3D fibrin after 8 days in culture in the absence or presence of TIMP-2 (5 μg/ml). Top chart shows total cell numbers expressed as the mean ± 1 SEM, while the bottom chart shows the percentage of PCNA-positive cells.

(C) Scanning electron micrographs illustrate (from left to right) the density of the collagen and fibrin matrices (scale bar equals 10 μm) relative to a single cell (on fibrin) or a freeze-fractured multicellular cyst from a MT1-MMP-transfected MDCK culture in collagen (scale bar equals 100 μm).

(D) Fluorescent images were obtained of MT1-MMP-expressing SCC-1 cells embedded in 3D collagen gels, fibrin gels, or live skin cultured for 2 days in the absence or presence of TIMP-2.

(E) Confocal sections of phalloidin-stained MT1-MMP-transfected SCC-1 cells in 3D collagen and fibrin, or control and MT1-MMP-transfected MCF-7 cells in 3D collagen. Images were collected after 2 days in culture in the absence or presence of TIMP-2 (5 μg/ml).

quired to support the proliferative response of tumor cells seeded *atop* a 3D collagen gel, a role for MMPs was unmasked when proliferation proceeded *within* the confines of an organized collagen matrix.

Tumor tissues generate a complex mix of MMPs in vitro and in vivo (Liotta and Kohn, 2001; Brinckerhoff and Matrisian, 2002; Egeblad and Werb, 2002). As such, the broad spectrum effects of metalloproteinase inhibitors preclude attempts to identify unequivocally individual target proteinases. Consequently, we initially screened a series of MMPs known to be upregulated in cancer tissue for their ability to confer recipient cells with a 3D-specific growth advantage. However, among the MMPs tested, only MT1-MMP conferred both nontumorigenic and tumorigenic cell lines with a 3D ECM-specific growth advantage. Furthermore, similar to cancer cells growing in vivo, the accelerated growth of MT1-MMP-transfected cells was driven almost entirely by effects on proliferation rather than changes in apoptotic rates (Chambers et al., 2002).

With the identification of MT1-MMP as a growth promoter, we next focused on the identification of potential target substrates. Recently, the number of MMP substrates has expanded to include non-ECM targets such as growth factors, proteinase inhibitors, growth factor receptors, and chemokines (Coussens et al., 2002; Egeblad and Werb, 2002). Nonetheless, MT1-MMP-dependent growth was strictly dependent on the hydrolysis of the surrounding type I collagen matrix. Native collagen is a ligand for β_1 integrins, which could have exerted transdominant effects on the cell cycle (Eckes et al., 2000), but we reasoned that the 3D collagen gel might alternatively prevent the changes in cell geometry necessary to drive proliferative responses (Chen et al., 1997; Ingber, 2003; Tan et al., 2003). Recent studies have demonstrated that cell growth under 2D conditions not only requires cell attachment, but also spreading, traction, and the consequent rearrangement of the actin cytoskeleton (Ingber, 2003). By decorating nonadhesive surfaces with small islands of integrin ligands, cell spreading is restricted and adherent cells are unable to exert contractile forces, generate stress fibers, or mount a proliferative response (Chen et al., 1997; Tan et al., 2003). While these artificial constructs likely recapitulate the early growth of tumor cells confined to the surface of the basement membrane, the dense network of fibrillar collagen presents the embedded tumor cells with a more relevant shape change-restricted environment encountered by invading cancer cells. As such, other physiologically relevant fibrillar matrices that are sensitive to MT1-MMP-dependent proteolysis were predicted to similarly affect cell geometry and growth.

Fibrin, the insoluble product of fibrinogen, is deposited around growing tumor cells in vivo, where it is crosslinked by transglutaminases to form a dense matrix (Hiraoka et al., 1998; Hotary et al., 2002). Though fibrin binds to tumor cells via integrins distinct from those used to bind to type I collagen (Feng et al., 1999), the crosslinked fibrils are likewise sensitive to MT1-MMP-dependent pericellular proteolysis (Hiraoka et al., 1998). Indeed, both crosslinked fibrin and collagen potently suppressed 3D shape changes, actin cytoskeleton rearrangements, and cell growth when MT1-MMP-dependent proteolysis was blocked. Thus, in either 3D matrix, MT1-MMP-dependent proteolysis allowed tumor cells to escape from growth regulatory structural constraints and to engage the necessary cell cycle machinery to drive an accelerated proliferative response. Tumor cell growth was not completely inhibited when pericellular proteolysis was blocked, but the 3D gels used in our studies are less dense and less heavily crosslinked than the ECM barriers encountered in vivo, thus presumably allowing for shape changes mediated by the mechanical distortion of the surrounding gels. Recent studies have stressed the role of the CDK complex inhibitor, $p27^{kip1}$, in regulating the proliferative responses of cells cultured atop type I collagen-coated surfaces (Koyama et al., 1996; Henriet et al., 2000). Similar to these reports, $p27^{kip1}$ levels were elevated when tumor cells were cultured under 2D growth conditions in our system. However, upon shifting the cells to the 3D growth environment, $p27^{kip1}$ levels decreased in both control and MT1-MMP-transfected SCC-1 cells and equivalent amounts of $p27^{kip1}$ were detected within the CDK4/cyclin D3 complexes (unpublished observation). Thus, under the more physiological conditions of 3D growth, $p27^{kip1}$ disassociation from CDK complexes may not represent a general means of cell cycle control in MT-MMP-expressing cells.

Of the major collagenolytic MMPs expressed by tumor cells (i.e., MMP-1, MMP-13, MMP-2, and MT1-MMP; Brinckerhoff and Matrisian, 2002; Egeblad and Werb, 2002), only MT1-MMP conferred recipient cells with a 3D growth advantage. As such, it is tempting to speculate that this MMP alone plays a preeminent role in regulating cancer cell proliferation. However, we have recently reported that another membrane-anchored MMP, MT2-MMP, can also arm recipient cells with collagen-degradative activity (Hotary et al., 2000). Similarly, MT1-MMP, MT2-MMP, and MT3-MMP can each mediate fibrinolysis (Hotary et al., 2002). Further studies indicate that MT2-MMP can similarly enhance the proliferative activity of cells embedded in type I collagen or fibrin gels while the growth-enhancing effects of MT3-MMP are confined to fibrin gels alone (unpublished observation). As MT1-MMP, MT2-MMP, and/or MT3-MMP are expressed in a wide variety of tumor tissues in vivo, either in the cancer cell itself or the surrounding stroma (Liotta and Kohn, 2001; Coussens et al., 2002; Egeblad and Werb, 2002), we posit that the 3D growth of neoplastic and accessory cell populations are regulated by MT-MMP family members. This conclusion would appear to contradict recent studies documenting the inability of synthetic MMP inhibitors to effectively suppress some, but not all, types of tumor growth in vivo (Hidalgo and Eckhardt, 2001; Coussens et al., 2002). However, in contrast to TIMP-2, synthetic inhibitors can affect members of the adamlysin family, whose role in tumor growth and progression remains the subject of speculation (Hidalgo and Eckhardt, 2001; Coussens et al., 2002). Given the almost uniform ability of TIMP-2 to block tumor cell growth in vivo (e.g., Montgomery et al., 1994; Brand et al., 2000; Coussens et al., 2002), the primary actions of the endogenous antiproteinase appear most consistent with its ability to target growth-promoting MT-MMPs.

Recently, a new model of tumor progression has been forwarded wherein a subset of mutant alleles acquired early in the neoplastic process are predicted to not only confer cells with a replicative advantage at the primary

site, but also drive the later-onset metastatic process (Bernards and Weinberg, 2002). Interestingly, the ability of MT1-MMP to confer recipient cells with a 3D growth advantage complements precisely its ability to arm cells with the ability to breach connective tissue barriers (Hiraoka et al., 1998; Hotary et al., 2000, 2002). Hence, we propose that mutant alleles that regulate MT1-MMP expression (e.g., the β-catenin/Tcf4 complex; Takahashi et al., 2002) could allow tumor cells to move from planar growth environments into the underlying 3D matrix where the invading cancer cell would use the proteinase to defeat growth regulatory checkpoints. As MT1-MMP also imbues cancer cells with the ability to intravasate, extravasate (unpublished observation), and regulate a neovascular response (Deryugina et al., 2002; Sounni et al., 2002), membrane-anchored metalloproteinases highlight the ability of early onset gene products to exert unexpectedly diverse effects on tumor cell phenotype.

Experimental Procedures

Cell Lines

MDCK, HT-1080, Panc-1, MCF-7 (all from ATCC), and the squamous cell carcinoma cell line, UM-SCC-1 (T. Carey, University of Michigan), were maintained in DMEM with 10% fetal bovine serum (FBS; Hyclone), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Stably transfected cell lines were selected and maintained in geneticin (Life Technologies).

Expression Vectors and Transfections

Expression vectors, isolation of stable cell clones, and characterization of cell lines overexpressing MMPs-1, 2, 3, 7, 9, 11, 13, MT1-MMP, transmembrane-deleted MT1-MMP (ΔMT1-MMP), catalytically inactive MT1-MMP (MT1-MMP $Glu^{240} \rightarrow Ala$), and cytosolic tail-truncated MT1-MMP ($MT1\text{-}MMP_{ct}$) have been described (Hotary et al., 2000; Yana and Weiss, 2000). $MMP\text{-}1_{RXKR}$, $MMP\text{-}2_{RXKR}$, and $MMP\text{-}13_{RXKR}$ constructs were generated using a sequential PCR method (Pei and Weiss, 1995) to insert in-frame a 10 amino acid sequence from MMP-11 (Asp87-Phe98) into the coding sequence of MMP-1 (between Gln99 and Phe100), MMP-2 (between Asn109 and Tyr110), and MMP-13 (between Glu103 and Tyr104). Control cell lines were generated by transfecting cells with the empty vector (pCR3.1 Uni; Invitrogen). Results shown are representative of at least three stable clones.

2D/3D Culture Conditions and Cell Growth Assays

Type I collagen was prepared from the tails or skin of rats or mice [wild-type or homozygous (r/r) $Col1a1^{tmlJae}$] at a final concentration of 2.7 mg/ml in 0.2% acetic acid as described (Hotary et al., 2000). Fibrin gels were prepared by mixing plasminogen-free fibrinogen (6 mg/ml; Calbiochem) with human thrombin (4 U/ml; Sigma-Aldrich) containing 200 μg/ml aprotinin (Hiraoka et al., 1998). For 2D substrata, collagen was mixed on ice with 10× MEM (Life Technologies) and 0.34 N NaOH in an 8:1:1 ratio. For 3D cultures, 5×10^4 cells were added to the collagen or fibrin mix prior to gelling. Experiments were performed in 10% serum before or after MMP-2/MMP-9 depletion by gelatin-sepharose affinity chromatography (Amersham Pharmacia Biotech; Hiraoka et al., 1998). In selected experiments, the recombinant MMP inhibitors, TIMP-1 and TIMP-2 (endotoxin-free; Fuji Industries), were included at a final concentration of 5 μg/ml for TIMP-2 and 12.5 μg/ml of TIMP-1 (equimolar as determined by active site titration; Hotary et al., 2002). For collagen/gelatin mixtures, gelatin prepared either by thermal denaturation of type I collagen at 60°C or by degrading collagen with recombinant MT1-MMP (Hiraoka et al., 1998) was added to type I collagen and the mixture gelled as described above. Monoclonal antibodies HU177 (Petitclerc et al., 1999) or LM609 (Wayner et al., 1991) were used at a final concentration of 100 μg/ml or 20 μg/ml, respectively.

In selected experiments, tumor cell growth was assessed in a soft agar assay. Agar (2.5%; Sigma) was diluted to 0.5% in tissue culture medium and 5×10^4 cells added to individual wells of 12-well tissue culture dishes.

Cell number in 3D cultures was determined by hemacytometry after dissolving gels in 2 mg/ml bacterial collagenase (Worthington). In agar, multicellular (>10 cells) colonies were counted in 10 randomly selected microscopic fields. Cell number and colony counts are expressed as the mean ± 1 SEM for triplicate cultures.

Cell Labeling, Immunofluorescence, and Proliferation/Apoptosis Assays

For fluorescent cell labeling experiments, cells were labeled with DiI or DiO (Molecular Probes). To create bicultures, 2.5×10^4 labeled cells were mixed with collagen as above and the slurry gelled with the culture dish angled at approximately 30°. Cells (2.5×10^4) from a second labeled (or unlabeled) population were embedded in collagen, and prior to gelling, layered over the first matrix (see Figure 5). Cultures were fixed in 4% paraformaldehyde, DAPI-stained, and cells at the interface of the two collagen layers examined.

Immunofluorescence was performed on frozen sections that were fixed in 1% paraformaldehyde, incubated overnight at 4°C with monoclonal antibody HUI77 (100 μg/ml; gift of Cell-Matrix, Inc., a subsidiary of CancerVax Corp.) to detect denatured collagen, and incubated with FITC-conjugated secondary antibody (1:400). BrdU incorporation was assayed following a 60 min pulse with 10 μM BrdU in 3D cultures on day 8 (Henriet et al., 2000). Proliferative indices in tumor tissue excised from nude mice or fibrin gel cultures were determined by staining sections for proliferating cell nuclear antigen (PCNA). Apoptosis was assessed in 3D cultures and in tumor sections using a TUNEL assay kit (Fluorescein Direct Apoptag; Intergen).

To assess cell shape changes in 3D collagen or fibrin, cells were fixed in 4% paraformaldehyde after 2 days in cultures permeabilized with 0.5% Triton X-100, labeled overnight with Texas red-conjugated phalloidin (1:100 in PBS; Molecular Probes), and postfixed in 4% paraformaldehyde. Confocal images were collected with an Ultraview LCI Confocal Imaging system (Perkin Elmer). For skin cultures, patches of live skin (10–15 mm^2) were removed from nude mice and approximately 300 DiI-labeled SCC-1 cells (control or MT1-MMP-transfected) injected into the dermis through a 30-gauge needle. The skin was immersed in culture medium with or without TIMP-2 (10 μg/ml) and digital images of labeled cells captured after 2 days in culture.

Transmission and Scanning Electron Microscopy

Three-dimensional collagen and fibrin gel cultures were prepared for transmission (TEM) and scanning (SEM) electron microscopy as described previously (Hotary et al., 2002). For freeze-fracture SEM, gels were immersed in liquid N_2 and fractured.

Western Blots and Zymography

MMP protein expression was assessed by Western blotting or zymography (Yana and Weiss, 2000). Following transfer, membranes were probed with anti-MMP-1, anti-MMP-13, or anti MT1-MMP rabbit polyclonal antisera (Hotary et al., 2002) and incubated with HRP-conjugated anti-rabbit IgG secondary antibody. Protein bands were visualized using ECL (Pierce Chemical Co.).

Cyclin-CDK Expression and Kinase Assays

To assay cyclin and CDK protein expression, cells were lysed and protein (50 μg) was separated by SDS-PAGE and transferred to nitrocellulose membranes (Datta et al., 1996). Blots were probed with antibodies to cyclins A, B, D_1, D_3, CDK2, CDK4, or CDK6 (Pharmingen or Santa Cruz Biotechnology), incubated with isotype-specific HRP-conjugated secondary antibody and visualized by ECL. For kinase activity determinations, cell lysates were immunoprecipitated with anti-cyclin antibodies and incubated with protein A/G sepharose beads (Datta et al., 1996). Immune complexes were assayed with purified GST-Rb protein (Santa Cruz) or H_1-histone (Roche Diagnostic Corporation) as substrate (Datta et al., 1996).

Tumor Growth in Nude Mice

5×10^6 SCC-1 cells (control or stable transfectants) were suspended in DMEM (0.2 ml) and injected subcutaneously into each flank of 5-

to 6-week-old female nude mice (NU/NU; Charles River). Tumors were allowed to develop for two weeks, at which time the mice were sacrificed and photographed. Tumors were excised for mass and volume measurements.

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