Tracking the Cartoon mouse phenotype: Hemopexin domain-dependent regulation of MT1-MMP pericellular collagenolytic activity

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Running Title: Hemopexin domain control of MT1-MMP

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Keywords: MT1-MMP, mesenchymal stem cell, type I collagen, hemopexin domain

ABSTRACT
Following ENU mutagenesis, a phenodeviant line was generated, termed the ‘Cartoon mouse’, that exhibits profound defects in growth and development. Cartoon mice harbor a single S466P point mutation in the MT1-MMP hemopexin domain, a 200 amino acid segment that is thought to play a critical role in regulating MT1-MMP collagenolytic activity. Herein, we demonstrate that the MT1-MMPS466P mutation replicates the phenotypic status of Mt1-mmp-null animals as well as the functional characteristics of MT1-MMP-/- cells. However, rather than a loss-of-function mutation acquired as a consequence of defects in MT1-MMP proteolytic activity, the S466P substitution generates a misfolded, temperature-sensitive mutant that is abnormally retained in the endoplasmic reticulum (ER). By contrast, the wild-type hemopexin domain does not play a required role in regulating MT1-MMP trafficking as a hemopexin domain-deletion mutant is successfully mobilized to the cell surface and displays near-normal collagenolytic activity. Alternatively, when MT1-MMPS466P-expresssing cells cultured at a permissive temperature of 25°C that depresses misfolding, the mutant successfully traffics from the ER to the trans-Golgi network (ER→TGN) where it undergoes processing to its mature form, mobilizes to the cell surface and expresses type I collagenolytic activity. Together, these analyses define the Cartoon mouse as an unexpected gain-of- abnormal function mutation wherein the temperature-sensitive mutant phenocopies MT1-MMP-/- mice as a consequence of eliciting a specific ER→TGN trafficking defect.

Type I collagen, the dominant extracellular protein found in mammals, undergoes extensive proteolytic remodeling in the course of growth and development as well as multiple disease states, ranging from inflammation to cancer (1-3). While the mammalian proteome includes more than 500 distinct enzymes, only a small subset of proteinases display type I collagenolytic activity (1-3). In mice, the ability to cleave native type I collagen within its triple helical domain is restricted largely to the secreted matrix metalloproteinases, MMP-8, MMP-13 and possibly MMP-2, the membrane-anchored matrix metalloproteinases, MT1-MMP and MT2-MMP, and the cysteine proteinase, cathepsin K (1-3). Nevertheless, while the expression of each of these proteinases has been targeted in mouse models (4-8), only MT1-MMP-null animals exhibit profound defects in type I collagen remodeling in vivo that are associated with early morbidity and mortality (9,10).
Not unexpectedly, the unique proteolytic functions assigned to MT1-MMP have catalyzed comprehensive efforts to delineate the critical structural determinants that define its ability to operate as the dominant pericellular type I collagenase operative in mammalian systems (3,11). Currently, the membrane-anchored proteinase is divided structurally into at least 6 discrete regions; an N-terminal prodomain, a catalytic domain, a short linker sequence followed by the hemopexin domain, a single-pass transmembrane region and a short cytosolic tail (3,11). Independent of the obvious functional importance of its catalytic domain, increasing interest has focused on the ability of the MT1-MMP hemopexin domain to modulate proteolytic activity (3,11). Using a variety of structure/function-designed approaches, the MT1-MMP hemopexin domain has been reported to control i) the trafficking of the enzyme from the trans-Golgi network to the cell surface, ii) MT1-MMP association with cell surface transmembrane protein binding partners, iii) MT1-MMP homodimerization with consequent effects on proteolytic activity and iv) MT1-MMP-type I collagen binding interactions (12-30). Alternatively, more subtle roles for the hemopexin domain in regulating MT1-MMP activity have also been identified (31-33), thereby complicating efforts to assign a definitive role to its functional activity.

In an ENU mutagenesis screen designed to identify immunological phenodeviants, Beutler and colleagues recently identified a mutation giving rise to mice with craniofacial defects, stunted growth, infertility and a markedly shortened lifespan; a phenotype similar to that described for MT1-MMP-null mice (http://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=219; (9,10)). Indeed, Cartoon mice, like their MT1-MMP-null counterparts, also exhibit an osteopenic phenotype, increased cartilage formation and the complete absence of secondary ossification centers (Fig. 1B). Similarly, a profound loss of dermal adipose tissue, a characteristic finding in Mt1-mmp-/-- mice (34), is likewise observed in the Cartoon mouse mutants (Fig. 1C). Hence, the Cartoon mouse displays phenotypic changes similar, if not identical, to those observed in MT1-MMP-null mice.

In normal fibroblasts, MT1-MMP is mobilized to the cell surface when it proteolytically remodels pericellular collagen and promotes tissue-invasive activity (35-37). By contrast, MT1-
MMP-null fibroblasts display a complete inability to either degrade or invade type I collagen-rich extracellular matrix barriers (35-37). As such, primary dermal fibroblasts were isolated from wild-type, Cartoon and Mm1-mpnull mice in order to characterize their respective functional activities. Dermal fibroblasts recovered from Cartoon mice display normal morphology and cytoskeletal organization that are indistinguishable from MT1-MMP+/+ or MT1-MMP-/- fibroblasts (Fig. 2A). Further, relative to the complete deficiency of the proteinase in MT1-MMP-/- cells, MT1-MMP protein levels in cell lysates are comparable between wild-type and Cartoon mouse fibroblasts (Fig. 2B). However, when cultured atop a bed of fluorescently-labeled type I collagen fibrils, only wild-type, but not Cartoon or MT1-MMP-/- fibroblasts, display a collagenolytic phenotype (Fig. 2B,C). Likewise, as collagenolytic activity is a prerequisite for supporting invasive activity through native type I collagen gels (35-37), Cartoon fibroblasts — like MT1-MMP-/- fibroblasts — are unable to mount an invasive response (Fig. 2D,E). Hence, Cartoon mouse fibroblasts display a loss of pericellular collagenolytic activity that phenocopies the functional properties of MT1-MMP-null cells.

**Characterization of MT1-MMPs466P Activity**

To define the role of the hemopexin point mutation in regulating MT1-MMP activity, mouse wild-type or S466P mutant expression vectors were constructed and transfected into COS-1 cells that do not express detectable levels of the endogenous protein (38). Following transfection, and as observed in Cartoon mouse fibroblasts, both the wild-type and mutant protein (i.e., MT1-MMPs466P) are expressed at comparable levels in cell lysates (Fig. 3A). However, in apparent agreement with earlier studies reporting a required role for the MT1-MMP hemopexin domain in activating its downstream proteolytic target, pro-MMP-2 (12,21,23), only wild-type MT1-MMP-transfected cells effectively processed the MMP-2 zymogen to its active form in the extracellular compartment as assessed by gelatin zymography (Fig. 3A). As the highly specific anti-MT1-MMP monoclonal antibody used here is directed towards an epitope localized near the catalytic domain, a 46kD autotryptic degradation product that remains anchored to the cell surface, but no longer retains the catalytic domain, cannot be detected (32,39). As such, an HA-epitope tag was inserted into the juxtamembrane region to allow tracking of MT1-MMP turnover (32,39). Interestingly, whereas the wild-type proteinase generated the autotryptic MMP turnover (32,39), these results are not confined to mouse MT1-MMP as similar findings are found when COS-1 cells are transfected with a human MT1-MMPs466P mutant carrying an HA-epitope tag (Fig. 3C). As expected, the generation of the 46 kDa degradation product derived from wild-type MT1-MMP is blocked in the presence of the pan-specific MMP inhibitor, BB-94 (35), while mutant MT1-MMP does not undergo further processing in the absence or presence of BB-94 (Fig. 3D). A faint ~40 kDa band is often detected in MT1-MMPs466P transfected COS-1 cells in either the absence or presence of BB-94 (but not in Cartoon fibroblasts) and appears to reflect a degradation product of the overexpressed mutant protein. In any case, the inability of MT1-MMPs466P to activate the MMP-2 zymogen or undergo autotryptic degradation, are both consistent with a presumed loss of hemopexin-dependent MT1-MMP activity. Indeed, whereas COS-1 cells transfected with wild-type MT1-MMP readily degrade subjacent collagen, MT1-MMPs466P mutant-transfected cells are predictably devoid of detectable collagenolytic activity (Fig. 3E,F).

**Cell Surface Trafficking of Cartoon Mouse MT1-MMP**

Whereas MT1-MMP-dependent defects in proMMP-2 processing and collagenolytic activity are consistent with activities assigned previously to the hemopexin domain, MT1-MMP must undergo trafficking to the cell surface in order to function as a pericellular proteinase (3,11). To directly assess MT1-MMP routing to the cell surface, COS cells were transfected with either wild-type or the MT1-MMPs466P mutant, surface proteins biotinylated, and then captured by streptavidin-affinity chromatography prior to Western blotting. As expected, in cells expressing wild-type MT1-MMP, both active and degraded forms of the proteinase are detected on
the cell surface with the generation of the 46 kDa product blocked completely in the presence of BB-94 (Fig. 4A) (32). In marked contrast, the MT1-MMP_{S466P} mutant cannot be detected on the cell surface in either the absence or presence of BB-94 by surface biotinylation or immunostaining (Fig. 4A,B). Confirming this result, Cartoon mouse fibroblasts are likewise unable to traffic mutant MT1-MMP to the cell surface (Fig. 4C). Given that the absence of MT1-MMP_{S466P} on the cell surface might be alternatively explained by an accelerated rate of internalization, lysosomal routing and degradation, we stabilized membrane expression levels by deleting the MT1-MMP cytosolic tail that contains key internalization signals (13,32,40). As expected, when wild-type MT1-MMP is expressed as a tail-deleted mutant, i.e., MT1-MMP_{ΔCT}, cell surface expression is marginally increased in tandem with increased MMP-2 activation (Fig. 4D,E). By contrast, deleting the tail of the MT1-MMP_{S466P} mutant yields only barely detectable levels of the enzyme at the cell surface (Fig. 4D,E). Consistent with these findings, tail-deleted MT1-MMP displays a trend towards increased collagenolytic activity relative to the wild-type proteinase, while MT1ΔCT_{S466P}–transfected COS cells remain unable to degrade subjacent collagen fibrils to a detectable degree (Fig. 4F,G).

**Dysregulated Trafficking of Cartoon Mouse MT1-MMP**

Recent studies suggest that MT1-MMP hemopexin domain plays a regulatory role in sorting the proenzyme to the trans-Golgi network where the enzyme undergoes proprotein convertase-dependent processing to its proteolytically active form prior to its final routing to the cell surface (22). However, when COS cells are transfected with an MT1-MMP hemopexin deletion-mutant domain (i.e., MT1ΔPEX), the proteinase maintains its ability to traffic to the cell surface where it remains catalytically active as reflected in its ability to i) undergo autocatalytic degradation, ii) activate pro-MMP-2 and iii) degrade subjacent collagen fibrils (Fig. 5A-C). Although the level of collagenolytic activity displayed by MT1ΔPEX is modestly depressed relative to that of the wild-type enzyme (32), the hemopexin-deleted mutant maintains significant activity relative to the Cartoon mutant (Fig. 5C).

Independent of its ability to support tissue-invasive activity, MT1-MMP has also been shown to regulate bone marrow-derived mesenchymal stem cell (MSC) differentiation programs (41). When suspended in 3-D type I collagen hydrogels, MT1-MMP promotes osteogenic commitment and differentiation while in the absence of MT1-MMP, bone marrow-derived MSCs engage a default adipogenic program (41). As such, when cultured in a cocktail of adipogenic and osteogenic factors, MT1-MMP−/− MSCs preferentially commit to an adipogenic pathway as assessed the formation of Oil Red-O positive adipocytes or gene expression (i.e., AP2, adiponectin or PPARγ) (Fig. 5D,E). Similarly, following transduction of MT1-MMP−/− MSCs with an MT1-MMP_{S466P} expression vector, 3-D collagen-embedded MSCs remain locked in an adipogenic lineage commitment program (Fig. 5D,E). By contrast, when MT1-MMP+MSCs are transduced with the wild-type proteinase or MT1ΔPEX, adipogenesis is inhibited while lineage commitment is redirected towards osteoblastogenesis (Fig. 5D,F). Though MT1ΔPEX is less active than wild-type MT1-MMP in terms of either collagenolytic or MSC differentiation-inducing activity (Fig. 5C-F), these results demonstrate that the MT1-MMP hemopexin domain does not play a required role in regulating the enzyme’s proteolytic or functional activity.

In the absence of direct evidence supporting a required role for the hemopexin domain in regulating MT1-MMP processing, trafficking or activity, we next considered the potential impact of the S466P substitution on MT1-MMP structure by first interrogating the recently solved crystal structure of the MT1-MMP hemopexin domain (23). In wild-type MT1-MMP, Ser466 is characterized as a buried moiety within the mid-region of a beta-strand of a 4-fold propeller structure (Fig. 6). This serine residue is positioned in a closely packed environment with other buried residues, and the insertion of a bulky proline residue would be predicted to disrupt protein secondary structure by inhibiting the ability of its backbone to adopt a β-strand...
conformation while creating a potential steric clash with Ala417 in a neighboring strand (Fig. 6) (42). As β-strand structural changes can impact trafficking of secreted proteins (43-46), we compared the intracellular localization of MT1-MMP and MT1-MMP_{S466P}. As expected, wild-type MT1-MMP co-localized with markers for the endoplasmic reticulum (ER) and cis-Golgi compartments, i.e., calnexin and GM130, respectively (Fig. 7A,B). By contrast, MT1-MMP_{S466P} is confined almost entirely to the ER (Fig. 7A,B). Further, while wild-type MT1-MMP internalized from the cell surface co-localizes with the early endosomal marker, EEA1, MT1-MMP_{S466P} cannot be detected in this compartment (Fig. 7A,B).

As the processing of the MT1-MMP zymogen to its active form normally occurs in the trans-Golgi network (39,47), the localization of MT1-MMP_{S466P} to the ER raises the possibility that the proteinase remains locked in its zymogen form as a proenzyme. To monitor MT1-MMP processing in situ, a FLAG sequence was inserted into both wild-type MT1-MMP and MT1-MMP_{S466P} downstream of the RXKR_{111} proprotein convertase-recognition sequence (39). Using this approach, as wild-type MT1-MMP undergoes processing, the FLAG sequence is positioned at the newly exposed N-terminus where it can be recognized specifically by the FLAG M1 monoclonal antibody. Indeed, whereas the M1-positive product of processed MT1-MMP is readily detected in wild-type-transfected cells, the MT1-MMP_{S466P}-expressing cells fail to expose the FLAG N-terminus, confirming a failure to undergo proprotein convertase-dependent processing (Fig. 7C). Although confinement of pro-MT1-MMP_{S466P} to the ER might be predicted to trigger an unfolded protein stress response (48,49), no significant changes in pERK, pERF2α or pJNK are detected in wild-type versus mutant-transfected COS cells (Fig. 7D).

**MT1-MMP_{S466P} Is a Temperature-Sensitive Mutant that Retains Pericellular Collagenolytic Activity**

The inability of MT1-MMP_{S466P} to traffic to the cell surface precludes efforts to assess its proteolytic activity as a membrane-anchored proteinase. Regardless of whether the intracellular confinement of the mutant proteinase occurs as a consequence of the generation or exposure of a cryptic ER retention signal, the trafficking of ER-retained, misfolded proteins can sometimes be rescued at permissive temperatures, thereby allowing mutant proteins to traffic to the cell surface (43,45,50). As such, MT1-MMP_{S466P}-transfected COS cells were either incubated under standard conditions at 37°C or alternatively, cultured at 25°C for 12 h before returning the cells to 37°C. As expected, at 37°C, MT1-MMP_{S466P}-transfected COS cells fail to traffic the mutant to the GM130^+ cis-Golgi network (Fig. 8A). In marked contrast, the 25°C→37°C switch allows the mutant proteinase to bypass the ER block, traffic to the cis-Golgi network and then move to the cell surface (Fig. 8A-C). Similarly, despite lower levels of expression, Cartoon fibroblasts likewise traffic the endogenous MT1-MMP mutant to the cell surface (Fig. 8B). As such, we next sought to assess the ability of the membrane-anchored MT1-MMP_{S466P} mutant to express pericellular proteolytic activity. To this end, transfected COS cells were again cultured under non-permissive conditions at 37°C or alternatively, allowed to undergo the 25°C→37°C switch, before plating the cells atop fluorescently-labeled gelatin- or type I collagen-coated surfaces at 37°C. Whereas MT1-MMP_{S466P}-transfected COS cells pre-cultured under standard 37°C conditions predictably fail to display gelatinolytic or collagenolytic activity, the 25°C→37°C switch allows mutant expressing cells to display both proteolytic activities (Fig. 8D). Hence, MT1-MMP_{S466P} elicits a loss-of-function mutation by transforming MT1-MMP into an ER-retained, temperature-sensitive mutant that retains collagenolytic activity when conformation-specific defects in cell surface trafficking are circumvented.

**Discussion**

MT1-MMP^−/− mice display an almost bewildering array of phenotypic abnormalities, including major defects in bone and cartilage formation, angiogenesis and lymphangiogenesis, muscle and organ development as well as accelerated aging and exaggerated inflammatory responses (34,41,51-66). In vitro studies have further defined roles for MT1-MMP in cell motility,
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invasion and proliferation as well as the regulation of signal transduction cascades and metabolic homeostasis (59,67,68). Attempts to define the key MT1-MMP domains responsible for this range of activities have identified both proteolytic and non-proteolytic functions for the proteinase (59,67,68). Not unexpectedly, the reported ability of the MT1-MMP hemopexin domain to form homodimers, direct cell surface trafficking, generate hetero-oligomeric complexes with cell surface molecules, including β1 and β3 integrins, CD44 and tetraspanins, regulate proteolytic activity and control binding interactions with type I collagen, have led many investigators to conclude that this domain plays a required role in controlling MT1-MMP function (12-31). Indeed, support for this interpretation had apparently been strengthened following the serendipitous generation and preliminary characterization of the MT1-MMP S466P Cartoon mouse mutant (http://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=219). While a formal description of these mice has not yet been reported in the literature by this group, they ascribed the Cartoon mouse phenotype to the previously assigned importance of the hemopexin domain in regulating MT1-MMP activity (http://mutagenetix.utsouthwestern.edu/phenotypic/phenotypic_rec.cfm?pk=219).

As we now describe, Cartoon mice phenocopy many of characteristics assigned to MT1-MMP-null mice, including major defects in bone formation, an inability to form secondary ossification zones and the disrupted development of peripheral white fat depots (9,10,34,41). Furthermore, in apparent agreement with earlier studies stressing a required functional role for the MT1-MMP hemopexin domain (11-23), the S466P mutant proved incapable of activating proMMP-2 or expressing type I collagenolytic activity following expression in COS cells. However, rather than defining a defect in MT1-MMP proteolytic activity per se, further studies demonstrate that the S466P mutation interferes with MT1-MMP trafficking to the cell surface. While this outcome is consistent with reports that the hemopexin domain can control MT1-MMP exocytosis (22), the former experiments were performed by introducing domain swaps wherein the MT1-MMP hemopexin domain was replaced with the hemopexin domain of MT4-MMP, a GPI-anchored MMP whose structure is distinct from that of type I transmembrane MT-MMPs (11). Here, we show that deleting the entire hemopexin domain of MT1-MMP does not interfere with MT1-MMP trafficking or function at the cell surface, a finding consistent with earlier work from our laboratory as well as others where the MT1-MMP hemopexin domain was replaced with that of either the MT3-MMP or MMP-2 hemopexin domain without affecting cell surface trafficking (19,39). As such, the earlier results reported for the chimeric construct while the MT4-MMP hemopexin domain most likely arise as a consequence of unanticipated domain clashes. Indeed, as opposed to the Cartoon mouse mutation, the MT1-MMPΔPEX deletion mutant not only retains its ability to activate proMMP-2, but also to support collagenolytic and invasive activity as well as more complex functions, including MSC differentiation. Nevertheless, these results should not be misconstrued to suggest that the hemopexin domain is without function, at least in terms of tuning proteolytic activity. Using transmembrane-deleted mutants, Zhao and colleagues reported that the ability of secreted, wild-type MT1-MMP to hydrolyze triple-helical substrates (as defined by kcat/Km values) is decreased when the hemopexin domain is deleted, but only by 3-fold (31). Nevertheless, it should be stressed that while this study is consistent with our findings, these authors did not examine the ability of the mutant to degrade native collagen as a membrane-anchored proteinase in an intact cell system (31). Taken together, these studies highlight the fact that the hemopexin domain more likely serves a modulatory, as opposed to necessary, role in defining MT1-MMP functional activity.

Though the presence of the MT1-MMP hemopexin domain is not required for its export to the cell surface, we found that the single S466P point mutation precluded the export of Cartoon MT1-MMP from the ER to the trans-Golgi apparatus where the proenzyme normally undergoes proprotein convertase-dependent processing to its active form (39,47). The C-terminal hemopexin domain of MT1-MMP is comprised of a sheet of four anti-parallel β-stands
that form a four-bladed propeller-like structure (23). As the insertion of proline residues into β-sheet strands precludes normal folding (42,46), the associated conformational changes are not permissive for ER→Golgi trafficking. Interestingly, a number of human genetic disorders that are distinguished by defects in intracellular sorting and trafficking are also characterized by proline substitutions in β-sheet structures (43,45,69). While the mutant MT1-MMP protein does not appear to trigger an unfolded protein response, we note that ER retention is not necessarily associated with increased rates of degradation (70). In this regard, a recent report has concluded that bacterially-expressed recombinant MT1-MMP 466P does not display major changes in structural conformation (33), but it is apparent from our studies that significant alterations in protein folding do occur under physiologic conditions. The further characterization of MT1-MMP 466P as a temperature-sensitive mutant also allows us to conclude that the hemopexin domain point mutation does not, in and of itself, interfere with proprotein convertase-dependent processing, trafficking to the cell surface or preclude the expression of type I collagenolytic activity. Direct kinetic analyses of the collagenolytic activity of wild-type versus mutant MT1-MMP cannot be readily determined, but we note that a recombinant MT1-MMP 466P transmembrane-deletion mutant has been reported to retain full enzymatic activity against synthetic triple-helical substrates (33).

Finally, by establishing Cartoon fibroblast cultures, we confirmed that these cells share each of the functional defects observed in our model COS cell system. Indeed, the inability of Cartoon fibroblasts to degrade or invade type I collagen hydrogels is identical to that observed in MT1-MMP-null fibroblasts. Nevertheless, it is interesting to note that Cartoon mice live longer than MT1-MMP+/+ mice in identical C57BL/6J backgrounds (i.e., whereas MT1-MMP+/+ mice rarely live beyond 3 weeks, Cartoon mice display only a modest increase in longevity with partial morbidity observed by 3.5 weeks with no mice surviving beyond 6 weeks). While it remains possible that small amounts of MT1-MMP 466P are folded correctly and can traffic to the cell surface under select conditions in vivo, it is also noteworthy that MT1-MMP can exert protease-independent functions that potentially affect signal transduction cascades as well as transcriptional programs (59,67,68). Further studies will be needed to resolve these issues, but the findings described herein characterize the Cartoon mouse as an unexpected “gain-of-abnormal-function” mutation that elicits a specific, but reversible, defect in MT1-MMP trafficking.

Experimental procedures

Cell culture and mouse lines

COS-1 cells (ATCC) were routinely maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (both from Life Technologies) and a 1% penicillin-streptomycin solution (Invitrogen). All cells were maintained in a 5% CO2/95% air atmosphere at 37°C unless indicated otherwise. Primary mouse fibroblasts were isolated from dorsal dermal explants of 2- to 4-wk-old male wild-type, M1-mmp+/+ or Cartoon mice (C57BL/6J background) as described (35,36) and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin-fungizone solution (GIBCO). Bone marrow-derived mesenchymal stem cells were isolated from wild-type and M1-mmp+/+ mice and cultured in 3-dimensional type I collagen hydrogels as described (41). In brief, bone marrow cells were isolated from mouse hind limbs and cultured in DMEM supplemented with 10% heat-inactivated FBS. Adherent colonies were sorted by flow cytometry with antibodies to Sca-1, CD29, CD45 and CD116 as described (Tang et al., 2009). The sorted cells were cultured and used for up to five passages (Tang et al., 2009). For 3-D culture, 5x10^5 cells/mL were embedded in 2.2 mg/mL rat tail-derived type I collagen (36) and cultured in Transwell dishes with 0.4µm pore size. Where indicated, cells were cultured in the presence or absence of 10 µM BB-94 (Abcam). All cell lines and cultured cells were mycoplasma-negative. All mouse work was performed with IACUC approval and in accordance with protocols approved by the University of Michigan Institutional Animal Care and Use Committee.
**Construction of expression plasmids and transfection**

Subcloning of HA-tagged mouse or human MT1-MMP cDNA, as well as cDNAs encoding mutant human MT1-MMP with a cytosolic tail-deletion (M1–R563; MT1-ΔCYT) or a hemopexin domain deletion (MT1ΔPEX; deleted Cys318-Gly535) was performed as described previously (32). A FLAG-tagged variant of human MT1-MMP cDNA was generated wherein the epitope tag was inserted directly downstream of the C-terminus of the proprotein recognition motif at R111 by using overlapping primer sets containing the FLAG sequence; forward, 5'-TACCCATACGATGTTCCAGATTACGCTGA GGGGACTGGAGGAG-3' and reverse, 5'-AGCGTAATCTGGAACATCGTATGGGTAA TCGGGCCGCCCCC-3'. HA- and FLAG-tagged versions of human and mouse MT1-MMP Cartoon mutation (Ser466→Pro466) were generated using site-directed mutagenesis produced with polymerase chain reaction (PCR) primers; forward, CCCAGAGGGCCATTCATGG-3 and reverse, TGCCCATGAATGGCCCTCTG-3. Each mutant was sequenced to verify the generation of the desired mutation. COS-1 cells were transiently transfected with either a control vector (pCR3.1; Invitrogen) or with the indicated expression vectors using FUGENE6 (Roche) according to the manufacturer's instructions. In selected experiments, COS-1 cells were co-transfected with a Lifeact expression vector (Addgene) to visualize F-actin.

**Gelatin zymography**

Pro-MMP-2 was transiently expressed in COS-1 cells and the conditioned medium harvested after 18 h. Aliquots of the conditioned medium containing recombinant proMMP-2 were then incubated with COS-1 cells overexpressing each of the indicated expression vectors for 24 h. Aliquots of conditioned medium were then subjected to gelatin zymography after a 12 h incubation period (32). Gelatinolytic activity is linear with incubation time over this period (data not shown).

**Immunoblotting and immunofluorescence**

For Immunoblotting cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing the Complete H protease inhibitor cocktail (Roche). Protein contents of the lysates were determined by bicinchoninic acid protein assay (Sigma-Aldrich). Equivalent quantities of protein were size-fractionated by gradient (4–20%) SDS-PAGE followed by transfer to nitrocellulose membranes. Immunoblot analyses were performed with the following antibodies; mouse monoclonal anti-HA-11 (Clone 16B12; Covance), rabbit monoclonal anti-β-actin (Cell Signaling), Calnexin (BD Biosciences), rabbit monoclonal anti-GM130, anti-EEA1, anti-elf2α/p-elf2α, anti-JNK/p-JNK, anti-ERK/p-ERK (Cell Signaling), and FLAG-M1 (Sigma-Aldrich). Primary antibodies were detected with horseradish peroxidase-conjugated species-specific secondary antibodies (Santa Cruz Biotechnology) using the Super Signal Pico system (Pierce).

For immunofluorescence, cells were fixed in 4% paraformaldehyde, washed in PBS and permeabilized with Triton X-100 (Sigma-Aldrich). Following blocking with 3% goat serum and 1% BSA in PBS, samples were incubated with primary antibodies overnight at 4°C. Alexa fluor-488 and -594 conjugated secondary antibodies (Molecular Probes) were used for protein detection.

**Cell surface-biotin labeling**

Cell surface biotinylation was performed as described (32). Briefly, cells were rinsed twice with PBS and incubated with Triton X-100 (Sigma-Aldrich). Following blocking with 3% goat serum and 1% BSA in PBS, samples were incubated with primary antibodies overnight at 4°C. Alexa flour-488 and -594 conjugated secondary antibodies (Molecular Probes) were used for protein detection.

**Type I collagen degradation**

Acid extracted type I collagen was prepared from rat tail tendons and dissolved in 0.2% acetic acid to a final concentration of 2.7 mg/ml. To generate matrix-coated surfaces, collagen was mixed with 10X MEM and 0.34 N NaOH in an 8:1:1 ratio with 25 mM Heps at 4°C, and 100 µL of the
mixture uniformly spread over the surface of 2 cm² Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL). Fibrillogenesis was induced by incubating the collagen-coated slides for 45 min at 37°C and collagen films were labeled with Alexa Fluor 488 (Molecular Probes). Post-fibrillogenesis labeling does not alter the sensitivity of type I collagen to collagenolytic attack relative to unlabeled type I collagen (data not shown). Fibroblasts or COS-1 cells (0.5 x10⁴) were seeded at low density atop collagen or gelatin films and incubated for 3 d in DMEM/10% FBS at 37°C. Degradation rates increase in linear fashion between 1 d and 3 d under these culture conditions. Fluorescence images were captured by laser confocal microscopy. Collagen degradation was quantified by the area of zones without fluorescent signal with results expressed as the mean ± SEM of 3 experiments.

**Microscopy**

Confocal imaging of collagen degradation and cellular immunofluorescence was performed with a spinning disc Nikon Eclipse Ti confocal microscope using a 20X objective lens, NA 0.75, or a 100X objective lens, NA 1.45. Images of Alexa Fluor-488 and Alexa Fluor-594 signals were captured at 25°C with a Yokogawa CSU-W1 camera using Micromanager MM Studio (Version 1.4.23) software and Image J for image processing. Cellular immunofluorescence signals were also imaged using a Leica DM IRB spinning-disc confocal microscope with a 63X objective lens, NA 1.4, and images were captured with a Perkin Elmer UltraView Vox system camera using Volocity 4.0 software. Equal photomultiplier tube intensity and gain settings were used in acquiring images. All other fluorescence and bright-field images were captured using a Spot digital camera (Diagnostic Instruments, Inc.) through a Leica upright microscope. Image-processing software (Photoshop 7; Adobe) was used to overlay images and to enhance equally image color and clarity.

**Structural analysis**

The Ser(466)→Pro mutation was modeled into the structure of the MT1-MMP hemopexin domain using the COOT program and then subjected to structural idealization using the REFMAC5 program as described (71). Contacts were analyzed using COOT.

**Statistical analysis**

Statistical analyses were performed using unpaired Student’s t-test. All experiments were performed 3 or more times.
**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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FOOTNOTES
Funding was provided by the National Institutes of Health [AR065524 and CA071699 to S.J.W; HL071818 and HL122416 to J.J.G.T]; and the Breast Cancer Research Foundation.
Figure 1. Cartoon and MT1-MMP-deleted mice display comparable phenotypes. (A) Two week-old MT1-MMP*+ and Cartoon mice exhibit a pattern of stunted growth, kyphosis and rounded skulls. Bar = 5 mm. (B) H&E-stained femur cross-sections of MT1-MMP*+ and Cartoon mice display a triad of increased cartilage, decreased trabecular bone and the absence of the secondary ossification zone. (C) H&E-stained skin cross-sections highlighting the complete loss of dermal adipocyte layer in MT1-MMP*+ and Cartoon mice. Bar = 200 µm. Secondary ossification zone, bone, cartilage and growth plate are marked as SOZ, B,C and G, respectively. Double-headed arrows mark the dermal fat pad.
Figure 2. Cartoon mouse fibroblasts are devoid of pericellular collagenolytic activity. (A) Adherent cultures of dermal fibroblasts isolated from wild-type, Cartoon or MT1-MMP<sup>−/−</sup> mice were stained with phalloidin and DAPI to highlight F-actin networks and nuclei, respectively (upper panels). Dermal fibroblasts recovered from wild-type, Cartoon or MT1-MMP<sup>−/−</sup> mice were cultured atop a bed of fluorescently-labeled type I collagen fibrils for 72 h. Zones of collagen degradation appear as ‘black holes’ (lower panels). (B) Wild-type, Cartoon or MT1-MMP<sup>−/−</sup> fibroblasts were lysed and MT1-MMP protein levels assessed by Western blot analysis using a rabbit anti-MT1-MMP monoclonal antibody (mAb) relative to β-actin controls. Under these conditions, the pro- and mature forms of MT1-MMP are not completely resolved (39) and appear as a single band. Results are representative of 3 or more experiments. (C) Quantification of collagen degradation areas mediated by wild-type, Cartoon or MT1-MMP<sup>−/−</sup> dermal fibroblasts as shown in Fig. 2A. Cartoon mouse fibroblasts degrade only minimal quantities of type I collagen relative to wild-type controls. ** p<0.05. (D) wild-type or mutant fibroblasts were cultured atop 3-dimensional type I collagen hydrogels for 72 h with invasion triggered by the addition of PDGF-AA. Hydrogels were fixed and sectioned with invading fibroblasts identified in H&E-stained samples. The invasive activity of wild-type fibroblasts is inhibited completely by the broad-spectrum MMP inhibitor, BB-94. Neither Cartoon nor MT1-MMP<sup>−/−</sup> fibroblasts display invasive activity. Arrowhead marks the fibroblast monolayer while arrows highlight invading cells. (E) The number of invading fibroblasts invading type I collagen gels power high power field (HPF) (as shown in panel D) was quantified in 3 experiments (mean ± SEM).
Figure 3. MT1-MMP<sub>S466P</sub> displays multiple defects in proteolytic processing and activity. (A) COS-1 cells were transiently transfected with mouse wild-type MT1-MMP or MT1-MMP<sub>S466P</sub> and MT1-MMP protein expression in cell lysates as well as MMP-2 zymogen processing in cell-free supernatants assessed by Western blot using rabbit anti-MT1-MMP mAb and gelatin zymography, respectively. Wild-type MT1-MMP or MT1-MMP<sub>S466P</sub> were expressed at similar levels. Only wild-type MT1-MMP processed the pro-MMP-2 zymogen (arrowhead) to its mature, active form (arrow). (B) COS-1 cells were transiently transfected with HA-tagged wild-type MT1-MMP or HA-tagged MT1-MMP<sub>S466P</sub> and MT1-MMP products determined in cell lysates following Western blotting with a mouse anti-HA mAb. Wild-type MT1-MMP-transfected cells expressed both pro-/mature MT1-MMP (arrowhead) as well as the membrane-anchored ~46kDa autoproteolytic degradation product (arrow). MT1-MMP<sub>S466P</sub>-transfected cells fail to generate the autoproteolytic fragment. (C) COS-1 cells were transiently transfected with HA-tagged human MT1-MMP or MT1-MMP<sub>S466P</sub> and protein expression and proMMP-2 activation assessed as described in panel A. (D) COS-1 cells, transiently expressing HA-tagged MT1-MMP and MT1-MMP<sub>S466P</sub>, were cultured alone or in the presence of BB-94 for 24 h and cell lysates prepared for Western blot analysis using a mouse anti-HA mAb. The ability of wild-type MT1-MMP-transfected cells to generate the autoproteolytic fragment (arrow) is blocked in the presence of BB-94. MT1-MMP<sub>S466P</sub>-transfected cells fail to generate the MT1-MMP fragment in the absence or presence of BB-94. (E,F) Mock, MT1-MMP and MT1-MMP<sub>S466P</sub>-transiently transfected cells were cultured atop a bed of fluorescently-labeled type I collagen fibrils for 72 h and...
collagen degradation imaged and quantified. Results are expressed as mean ± SEM (n=3). **p<0.05. Bar = 100 µm for all panels.
Figure 4. Defective cell surface trafficking of MT1-MMP<sub>S466P</sub>. (A) Mock-, mouse HA-tagged MT1-MMP- or HA-tagged MT1-MMP<sub>S466P</sub>- transiently transfected COS-1 cells were cultured alone or with BB-94 for 24 h. Cell surface MT1-MMP was biotin-labeled and cell lysates prepared for Western blot analysis using mouse anti-HA mAb before and after streptavidin pulldown. Wild-type MT1-MMP-transduced COS-1 cells expressed both MT1-MMP (arrowhead) and the MT1-MMP autoproteolytic fragment (arrow) on the cell surface in the absence of BB-94, but only the pro-/mature forms of MT1-MMP on the cell surface in the presence of BB-94. Neither pro-/mature nor the MT1-MMP fragment were expressed on the surface of MT1-MMP<sub>S466P</sub>-transfected cells in the absence or presence of BB-94. (B) COS-1 cells were transiently transfected with HA-tagged mouse MT1-MMP or HA-tagged MT1-MMP<sub>S466P</sub> and cell surface MT1-MMP detected with anti-HA antibodies in non-permeabilized cells. (C) Cell surface MT1-MMP was detected in wild-type, but not Cartoon fibroblasts, as assessed by Western blotting of cell lysates with rabbit anti-MT1-MMP mAb following biotin-labeling and streptavidin capture. (D) COS-1 cells were transiently transfected with HA-tagged wild-type MT1-MMP, cytosolic tail-deleted MT1-MMP (MT1<sub>∆CT</sub>), MT1-MMP<sub>S466P</sub> or cytosolic tail-deleted MT1-MMP<sub>S466P</sub> (MT1<sub>∆CT</sub>S466P). After a 48 h culture period, MT1-MMP expression levels, cell surface-associated MT1-MMP and proMMP-2 activation in cell-free supernatants were assessed.
by Western blot using mouse anti-HA mAb and zymography, respectively, as described in Fig. 3. Relative to wild-type MT1-MMP, MT1ΔCT displayed markedly increased levels of the cell-surface-associated proteinase with attendant increases in MMP-2 activation (arrowhead marks position of proMMP-2 while arrow indicates mature MMP-2). By contrast, MT1-MMP$_{S466P}$ could not be detected on the cell surface and failed to significantly process proMMP-2 when expressed as a cytosolic tail-deleted mutant. (E) Cell surface distribution of HA-tagged MT1ΔCT and MT1ΔCT$_{S466P}$ as assessed by anti-HA staining in non-permeabilized, transiently transfected COS-1 cells. (F,G) MT1ΔCT-expressing COS-1 cells (transiently transfected) display a trend towards increased type I collagenolytic activity relative to MT1-MMP-transiently transfected cells as assessed by immunofluorescence following transient transfection (F) and quantification of degraded area (G). By contrast, MT1ΔCT$_{S466P}$-transiently transfected COS-1 cells fail to display collagenolytic activity. Results are expressed as mean ± SEM (n=3). **p<0.05. Bars = 100 µm.
Figure 5. Hemopexin-deleted MT1-MMP retains proteolytic function and activity. (A) COS-1 cells were transiently transfected with HA-tagged wild-type MT1-MMP, MT1-MMP<sub>S466P</sub> or hemopexin domain-deleted MT1-MMP(MT1ΔPEX), and total MT1-MMP expression in cell lysates, cell surface MT1-MMP expression (following cell surface biotinylation and streptavidin capture of cell lysates) and proMMP-2 activation assessed by Western blot and gelatin zymography, respectively. By contrast with MT1-MMP<sub>S466P</sub>, MT1ΔPEX undergoes autoproteolytic processing and is displayed on the cell surface as both pro-/mature and autoproteolytic forms (pro-/mature MT1-MMP marked by arrowhead, autocatalytic product marked by arrows, pro-/mature forms of MT1-ΔPEX are indicated by arrowhead/asterisk and MT1ΔPEX processed form by arrow/asterisk). MT1ΔPEX, but not MT1-MMP<sub>S466P</sub>-transfected COS-1 cells processed proMMP-2 to its mature/active form. (B,C) Mock-, MT1-MMP, MT1-MMP<sub>S466P</sub> or MT1ΔPEX- transiently transfected cells were cultured atop fluorescently-labeled type I collagen fibrils and degradation monitored by immunofluorescence microscopy (B) and quantified (C). (D) MT1-MMP<sup>-/-</sup> MSCs were either mock-transfected or transiently transfected with MT1-MMP<sub>S466P</sub>, MT1ΔPEX or wild-
type MT1-MMP expression vectors and embedded in 3-dimensional type I collagen hydrogels in media supplemented with a cocktail of adipogenic and osteoblastogenic growth factors. Following a 5 d culture period, cultures were stained with Oil Red O or assessed for alkaline phosphatase activity to monitor adipocyte and osteoblast commitment, respectively. Mock-transfected MT1-MMP\(^{-/}\) or MT1- MMP\(_{5466P}\)-transfected MT1-MMP\(^{-/}\) MSCs preferentially undergo adipogenesis. MT1\(\Delta\)PEX-transfected and wild-type MT1-MMP-transfected MT1-MMP\(^{+/}\) stem cells differentially underwent osteoblastogenic commitment. (E,F) Mock-, MT1- MMP\(_{5466P}\)-, MT1\(\Delta\)PEX- or MT1-MMP- transiently transfected MT1-MMP-null mesenchymal cells were cultured as described in panel D for 5 d and adipogenic (E) versus osteoblastogenic (F) commitment assessed by qPCR of transcripts normally associated with adipogenesis (i.e., \(AP2\), adiponectin and \(PPAR\)) or osteoblastogenesis (i.e., \(RUNX2\), \(SP7\), \(Alp\), \(Bglap\)). Results are expressed on the mean ± SEM of 3 experiments. **\(p<0.05\). Bars = 100 µm.
Figure 6. Cartoon mouse mutation induces structural changes in the MT1-MMP hemopexin domain. Without structural rearrangements, a Ser466→Pro substitution in the MT1-MMP hemopexin domain is predicted to be destabilizing as it generates unfavorable steric contacts between the Pro466 and Ala417 side chains (distances between clashing atoms are drawn as dashed lines) as well as force the backbone residues surrounding Pro466 into a configuration incompatible with their native position in a β-sheet. The model shown is derived from the coordinates of the native MT1-MMP domain (PDB entry 3C7X) (see Experimental Procedures) (23).
Figure 7. MT1-MMP<sub>S466P</sub> mutation disrupts its intracellular trafficking. (A, B) COS-1 cells were transiently transfected with HA-tagged wild-type MT1-MMP or MT1MMP<sub>S466P</sub> and proteinase distribution in the ER, cis-Golgi and early endosome compartments assessed by their co-localization with calnexin, GM130 and EEA1, respectively, in fixed permeabilized cells. COS-1 cells were then imaged by confocal laser microscopy. While wild-type MT1-MMP is found in each of the 3 compartments, MT1-MMP<sub>S466P</sub> is confined to the ER. (C) COS-1 cells were transiently transfected with wild-type MT1-MMP or MT1MMP<sub>S466P</sub> expression vectors that contain a FLAG sequence (DYKDDDK) inserted downstream of the MT1-MMP RRKR(111) proprotein convertase-recognition sequence. Cells were then lysed and MT1-MMP expression assessed Western blotting using either an anti-HA monoclonal antibody or a FLAG-M1 antibody that only recognizes the FLAG sequence when displayed at the N-terminus. Under these conditions, the wild-type MT1-MMP construct containing both FLAG and HA inserts can be resolved into a closely migrating doublet representative of proMT1-MMP and active MT1-MMP as well as its autocatalytic degradation product (39). By contrast, MT1-MMP<sub>S466P</sub> is primarily expressed as the proenzyme alone. Following immunoblotting with the FLAG-M1 antibody, the processing of wild-type MT1-MMP to its prodomain-deleted mature/active form is detected. (D) COS-1 cells were transfected with MT1-MMP or MT1-MMP<sub>S466P</sub>, lysed 24 h later and levels of p-ERK/total ERK, p-elf2α/total elf2α, and p-JNK/total JNK as well as β-actin assessed by Western blotting. Bars = 20 µm.
Figure 8. MT1-MMP\textsubscript{S466P} is a temperature-sensitive mutant. (A) HA-tagged MT1-MMP\textsubscript{S466P}-transiently transfected COS-1 cells were cultured at 37°C or at 25°C for 12h prior to returning the cells to 37°C for an additional 12h culture period (25°C→37°C) and MT1-MMP trafficking to the \textit{cis}-Golgi compartment assessed following anti-HA and anti-GM130 staining of fixed, permeabilized cells. (B) HA-tagged MT1-MMP\textsubscript{S466P} transiently transfected COS-1 cells or Cartoon fibroblasts were either cultured at 37°C or at 25°C→37°C as described in panel A. Cell surface-associated MT1-MMP was detected by Western blotting with a mouse anti-HA mAb following biotin-labeling and streptavidin capture of cell lysates. Total MT1-MMP levels were assessed in whole cell lysates. (C) COS-1 cells were transiently co-transfected with HA-tagged MT1-MMP\textsubscript{S466P} and Lifeact, and either cultured at 37°C or 25°C→37°C. Cells were then fixed, but not permeabilized, and cell surface-associated MT1-MMP assessed following anti-HA staining (green) by confocal laser microscopy. Red fluorescence indicates F-actin. Intracellular MT1-MMP is detected in permeabilized cells (insets). (D) COS-1 cells transiently transfected with MT1-MMP\textsubscript{S466P}...
were cultured at 37°C or 25°C→37°C and then allowed to adhere to fluorescently-labeled gelatin (upper panels) or type I collagen-coated (lower panels) surfaces. Following a 72h culture period, gelatin/type I collagen degradation was assessed by confocal laser microscopy and quantified. Results are expressed as mean ± SEM (n=3). ** p<0.05. Bars in panels A/C = 20 µm and in panel D = 100 µm.
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J. Biol. Chem. published online April 11, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001503

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