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

Received for publication, December 15, 2017, and in revised form, March 23, 2018. Published, Papers in Press, April 11, 2018, DOI 10.1074/jbc.RA117.001503

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Edited by Amanda J. Fosang

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 M1-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 WT 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 nearly normal collagenolytic activity. Alternatively, when MT1-MMPS466P–expressing cells are cultured at a permissive temperature of 25 °C that depresses misfolding, the mutant successfully traffics from the ER to the trans-Golgi network, 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→trans-Golgi network 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). Although 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 (MMPs), 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, whereas 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 six discrete regions: an N-terminal promdomain, 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

3 The abbreviations used are: MMP, matrix metalloproteinase; ER, endoplasmic reticulum; HA, hemagglutinin; MSC, mesenchymal stem cell; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; H&E, hematoxylin and eosin; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p-, phosphorylated.
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 (http://mutagenetix.utsouthwestern.edu/, allele: Cartoon) 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/, allele: Cartoon). Genome sequencing subsequently identified a single Ser446 → Pro substitution in the MT1-MMP hemopexin domain, a finding seemingly consistent with the myriad functions previously assigned to this domain. However, the impact of this point mutation on MT1-MMP function remains undefined, and the mechanism by which this mutant hemopexin domain regulates MT1-MMP activity in intact cell systems has not been established. To more specifically characterize the impact of the Cartoon mouse mutation on MT1-MMP function, we have compared and contrasted the phenotype of WT, MT1-MMP−/−, and Cartoon mice and defined the effect of the Ser446 → Pro mutation on MT1-MMP activity in intact cell systems. Herein, we report that whereas the Cartoon mouse mutation recapitulates key in vivo features of the Mt1-mmpnull phenotype, changes in MT1-MMP function do not arise as a consequence of a direct loss of proteolytic activity. Rather, the S466P substitution effects a loss-of-function phenotype by conferring the hemopexin domain with new properties wherein the Cartoon mutant is retained in the endoplasmic reticulum (ER) and fails to traffic to the cell surface. By contrast, by either deleting the entire hemopexin domain or culturing Cartoon mutant-expressing cells at lower temperatures that are permissive for ER → trans-Golgi network trafficking of the misfolded MT1-MMP5466P mutant, both the hemopexin domain–deleted MT1-MMP and Cartoon proteinase are displayed on the cell surface, where they retain collagenolytic activity and function.

Results

Cartoon mice recapitulate an Mt1-mmp−/− phenotype

Cartoon mice display an overall phenotype similar to Mt1-mmp−/− mice with stunted growth and craniofacial anomalies characterized by a shortened head and snout as well as dwarfism (Fig. 1A) (http://mutagenetix.utsouthwestern.edu/, allele: Cartoon)4 (9, 10). Indeed, Cartoon mice, like their MT1-MMP–null counterparts, also exhibit an osteopnenotypic 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 where 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 WT, Cartoon, and Mt1-mmp−/− 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 WT and Cartoon mouse fibroblasts (Fig. 2B). However, when cultured atop a bed of fluorescently labeled type I collagen fibrils, only WT, and not Cartoon or MT1-MMP−/− fibroblasts, display a collagenolytic phenotype (Fig. 2, B and C). Likewise, collagenolytic activity is a prerequisite for supporting invasive activity through native type I collagen hydrogels (35–37), Cartoon fibroblasts, like MT1-MMP−/− fibroblasts, are unable to mount an invasive response (Fig. 2, D and 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-MMP5466P activity

To define the role of the hemopexin point mutation in regulating MT1-MMP activity, mouse WT 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 WT and mutant protein (i.e. MT1-MMP5466P) 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 WT 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 mAb used here is directed toward an epitope localized near the catalytic domain, a 46-kDa autocatalytic 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 WT proteinase generated the autocatalytic degradation product, the ∼46-kDa fragment is not detected in MT1-MMP5466P-transfected COS cells (Fig. 3B). These results are not confined to mouse MT1-MMP; as similar findings are found when COS-1 cells are transfected with a human MT1-MMP5466P mutant carrying an HA-epitope tag (Fig. 3C). As expected, the generation of the 46-kDa degradation product derived from WT MT1-MMP is blocked in the presence of the pan-specific MMP inhibitor, BB-94 (35), whereas 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-MMP5466P–transfected COS-1 cells in either the absence or presence of BB-94 (but not in Cartoon fibro-

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blasts) and appears to reflect a degradation product of the overexpressed mutant protein. In any case, the inabilities of MT1-MMPS466P to activate the MMP-2 zymogen or undergo autocatalytic degradation are both consistent with a presumed loss of hemopexin-dependent MT1-MMP activity. Indeed, whereas COS-1 cells transfected with WT MT1-MMP readily degrade subjacent collagen, MT1-MMPS466P mutant–transfected cells are predictably devoid of detectable collagenolytic activity (Fig. 3, E and F).

Cell-surface trafficking of Cartoon mouse MT1-MMP
Whereas MT1-MMP-dependent defects in pro-MMP-2 processing and collagenolytic activity are consistent with activities assigned previously to the hemopexin domain, MT1-MMP must undergo trafficking to the cell surface to function as a pericellular proteinase (3, 11). To directly assess MT1-MMP routing to the cell surface, COS cells were transfected with either WT or the MT1-MMPS466P mutant, and surface proteins were biotinylated and then captured by streptavidin-affinity chromatography before Western blotting. As expected, in cells expressing WT 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-MMPS466P mutant cannot be detected on the cell surface in either the absence or presence of BB-94 by surface biotinylation or immunostaining (Fig. 4, A and 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-MMPS466P 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 WT MT1-MMP is expressed as a tail-deleted mutant (i.e. MT1-MMPΔCT), cell-surface expression is marginally increased in tandem with increased MMP-2 activation (Fig. 4, D and E). By contrast, deleting the tail of the MT1-MMP_S466F mutant yields only barely detectable levels of the enzyme at the cell surface (Fig. 4, F and G).
Figure 3. MT1-MMP<sub>566P</sub> displays multiple defects in proteolytic processing and activity. A, COS-1 cells were transiently transfected with mouse WT MT1-MMP or MT1-MMP<sub>566P</sub>, and MT1-MMP protein expression in cell lysates as well as MMP-2 zymogen processing in cell-free supernatants were assessed by Western blotting using rabbit anti-MT1-MMP mAb and gelatin zymography, respectively. WT MT1-MMP or MT1-MMP<sub>566P</sub> was expressed at a similar level. Only WT 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 WT MT1-MMP or HA-tagged MT1-MMP<sub>566P</sub>, and MT1-MMP products were determined in cell lysates following Western blotting with a mouse anti-HA mAb. WT MT1-MMP–transfected cells expressed both pro-/mature MT1-MMP (arrowhead) as well as the membrane-anchored ~46-kDa autoproteolytic degradation product (arrow). MT1-MMPS466P–transfected cells fail to generate the autoproteolytic fragment. C, COS-1 cells were transiently transfected with HA-tagged human MT1-MMP or MT1-MMPS466P, and protein expression and pro-MMP-2 activation were assessed as described in A. D, COS-1 cells, transiently expressing HA-tagged MT1-MMP and MT1-MMPS466P, were cultured alone or in the presence of BB-94 for 24 h, and cell lysates were prepared for Western blot analysis using a mouse anti-HA mAb. The ability of WT MT1-MMP–transfected cells to generate the autoproteolytic fragment (arrow) is blocked in the presence of BB-94. MT1-MMPS466P–transfected cells fail to generate the MT1-MMP fragment in the absence or presence of BB-94. E and F, mock, MT1-MMP, and MT1-MMPS466P–transfected cells were cultured atop a bed of fluorescently labeled type I collagen fibrils for 72 h, and collagen degradation was imaged and quantified. Results are expressed as mean ± S.E. (error bars) (n = 3). **, p < 0.05. Bar, 100 μm for all panels.
Dysregulated trafficking of Cartoon mouse MT1-MMP

Recent studies suggest that the 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 before 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. 5, A–C). Although the level of collagenolytic activity displayed by MT1ΔPEX is modestly depressed relative to that of the WT 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 3D type I collagen hydrogels, MT1-
MMP promotes osteogenic commitment and differentiation, whereas in the absence of MT1-MMP, bone marrow–derived MSCs engage a default adipogenic program (41). As such, when cultured in a mixture of adipogenic and osteogenic factors, MT1-MMP−/−MSCs preferentially commit to an adipogenic pathway, as assessed by the formation of Oil Red-O positive adipocytes or gene expression (i.e. AP2, adiponectin, or PPARγ) (Fig. 5, D and E). Similarly, following transduction of MT1-MMP−/−MSCs with an MT1-MMPS466P expression vector, 3D collagen-embedded MSCs remain locked in an adipogenic lineage commitment program (Fig. 5, D and E). By contrast, when MT1-MMP−/−MSCs are transduced with the WT protease or MT1ΔPEX, adipogenesis is inhibited, whereas lineage commitment is redirected toward osteoblastogenesis (Fig. 5, D–F). Although MT1ΔPEX is less active than WT MT1-MMP in terms of either collagenolytic or MSC differentiation–inducing activity (Fig. 5, C–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 the MT1-MMP structure by first interrogating the recently solved crystal structure of the MT1-MMP hemopexin domain (23). In WT MT1-MMP, Ser466 is characterized as a buried moiety within the mid-region of a β-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-MMPS466P. As expected, WT MT1-MMP co-localized with markers for the ER and cis-Golgi compartments (i.e. calnexin and GM130, respectively) (Fig. 7, A and B). By contrast, MT1-MMPS466P is confined almost entirely to the ER (Fig. 7, A and B). Further, whereas WT MT1-MMP internalized from the cell surface co-localizes with the early endosomal marker, EEA1, MT1-MMPS466P cannot be detected in this compartment (Fig. 7, A and 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-MMPS466P to the ER raises the possibility that the protease remains locked in its zymogen form as a proenzyme. To monitor MT1-MMP processing in situ, a FLAG sequence was inserted into both WT MT1-MMP and MT1-MMPS466P downstream of the RXKR111 proprotein convertase-recognition sequence (39). Using this approach, as WT 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 mAb. Indeed, whereas the M1-positive product of processed MT1-MMP is readily detected in WT-transfected cells, the MT1-MMPS466P–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-MMPS466P to the ER might be predicted to trigger an unfolded protein stress response (48, 49), no significant changes in p-ERK, p-eIF2α,
p-JNK are detected in WT versus mutant-transfected COS cells (Fig. 7D).

**MT1-MMP$_{5466P}$ is a temperature-sensitive mutant that retains pericellular collagenolytic activity**

The inability of MT1-MMP$_{5466P}$ to traffic to the cell surface precludes efforts to assess its proteolytic activity as a membrane-anchored protease. Regardless of whether the intracellular confinement of the mutant protease 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$_{5466P}$-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$_{5466P}$-transfected COS cells fail to traffic the mutant to the GM130$^+$ cis-Golgi network (Fig. 8A). In marked contrast, the 25 → 37 °C switch allows the mutant protease to bypass the ER block, traffic to the cis-Golgi network, and then move to the cell surface (Fig. 8, A–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$_{5466P}$ mutant to express pericellular proteolytic activity. To this end, transfected COS cells were again cultured under nonpermissive conditions at 37 °C or, alternatively, allowed to undergo the 25 → 37 °C switch, before plating the cells atop fluorescently labeled gelatin- or type I collagen-coated surfaces at 37 °C. Whereas MT1-MMP$_{5466P}$-transfected COS cells precultured under standard 37 °C conditions predictably failed to display gelatinolytic or collagenolytic activity, the 25 → 37 °C switch allowed mutant-expressing cells to display both proteolytic activities.
Hemopexin domain control of MT1-MMP

**A**

| 37°C | MT1<sub>1-sgap</sub> | GM130 | Merge |
| --- | --- | --- | --- |
| 29°C—37°C | MT1<sub>1-sgap</sub> | GM130 | Merge |

**B**

| kDa | Surface | Total |
| --- | --- | --- |
| 64- | MT1-MMP | MT1-MMP |
| 40- | GAPDH | GAPDH |

**C**

| 37°C | MT1<sub>1-sgap</sub> | MT1<sub>Cartoon</sub> |
| --- | --- | --- |

**D**

| Collagenolytic Zone Area | 37°C | 25—37°C |
| --- | --- | --- |

Figure 8. MT1-MMP<sub>S466P</sub> is a temperature-sensitive mutant. A, HA-tagged MT1-MMP<sub>1-sgap</sub>-transiently transfected COS-1 cells were cultured at 37 °C or at 25 °C for 12 h before returning the cells to 37 °C for an additional 12-h culture period (25 → 37 °C), and MT1-MMP trafficking to the cis-Golgi compartment was assessed following anti-HA and anti-GM130 staining of fixed, permeabilized cells. B, HA-tagged MT1-MMP<sub>1-sgap</sub>-transiently transfected COS-1 cells or Cartoon fibroblasts were either cultured at 37 °C or at 25 → 37 °C as described in 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<sub>1-sgap</sub> and Lifeact and cultured at either 37 °C or 25 → 37 °C. Cells were then fixed, but not permeabilized, and cell surface–associated MT1-MMP was 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<sub>1-sgap</sub> were cultured at 37 °C or 25 → 37 °C and then allowed to adhere to fluorescently labeled gelatin (top panels) or type I collagen-coated (bottom panels) surfaces. Following a 72-h culture period, gelatin/type I collagen degradation was assessed by confocal laser microscopy and quantified. Results are expressed as mean ± S.E. (error bars) (n = 3). **, p < 0.05. Bars, 20 μm (A and C) and 100 μm (D).

(Fig. 8D). Hence, MT1-MMP<sub>S466P</sub> 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<sup>−/−</sup> mice display an almost bewildering array of phenotypic abnormalities, including major defects in bone and cartilage formation, angiogenesis and lymphangiogenesis, and 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, 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 pro-teolytic and nonproteolytic 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 heterooligomeric 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<sub>S466P</sub> Cartoon mouse mutant ([http://mutagenetix.utsouthwestern.edu/](http://mutagenetix.utsouthwestern.edu/), allele: Cartoon). 4 Whereas 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/](http://mutagenetix.utsouthwestern.edu/), allele: Cartoon).

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 pro-MMP-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. Whereas this outcome is consistent with a report 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 glycosylphosphatidylinositol-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 with the
MT4-MMP hemopexin domain swap most likely arose as a consequence of unanticipated domain clashes. Indeed, as opposed to the Cartoon mouse mutation, the MT1-MMPΔPEX deletion mutant retains not only its ability to activate pro-MMP-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 et al. (31) reported that the ability of secreted, WT MT1-MMP to hydrolyze triple-helical substrates (as defined by $k_{\text{cat}}/k_m$ values) is decreased when the hemopexin domain is deleted, but only by 3-fold. Nevertheless, it should be stressed that although 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 protease 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.

Although 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–dependence processing to its active form (39, 47). The C-terminal hemopexin domain of MT1-MMP is composed 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). Whereas 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 S466P 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 S466P 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 or trafficking to the cell surface or preclude the expression of type I collagenolytic activity. Direct kinetic analyses of the collagenolytic activity of WT versus mutant MT1-MMP cannot be readily determined, but we note that a recombinant MT1-MMP S466P 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 a modest increase in longevity with partial morbidity observed by 3.5 weeks with no mice surviving beyond 6 weeks). Whereas it remains possible that small amounts of MT1-MMP S466P 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 2–4-week-old male WT, MT1-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 WT and MT1-mmp−/− mice and cultured in 3D 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 directed against Sca-1, CD29, CD45, and CD116, and the harvested cells were cultured and used for up to five passages as described (72). For 3D culture, 5 × 10⁵ cells/ml were embedded in 2.2 mg/ml rat tail-derived type I collagen (36) and cultured in Transwell dishes with 0.4-μm 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 institutional animal care and use committee 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 (Met1−Arg563; MT1-ΔCYT) or a hemopexin domain deletion (MT1ΔPEX; Cys318−Gly535 deleted) 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 Arg111 by using overlapping primer sets containing the FLAG sequence: forward, 5'-TACCCATAAGATTTCCAGATTACGCTGAGGGACTGAGGAG-3'; reverse, 5'-AGCGTAATCTGGAA-

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and cell lysates were recovered for pulldown with streptavidin-

Cartoon mutation (Ser466 → Pro466) were generated using site-
directed mutagenesis produced with PCR primers: forward, 

CCCCAGAGGGCCATTCATGGGTAATCGGGCCGGCCCCC-3’ (39). 

HA- and FLAG-tagged versions of human and mouse MT1-MMP Cartoon mutation (Ser466 → Pro466) were generated using site-
directed mutagenesis produced with PCR primers: forward, 

CCCAGAGGGCCATTCATGGGTAATCGGGCCGGCCCCC-3’; reverse, TGCCCCATGA-

ATGGCCCTCTG-3’. Each mutant was sequenced to verify the generation of the desired mutation. COS-1 cells were tran-
siently transfected with either a control vector (pCR3.1; Invitro-
gen) or with the indicated expression vectors using 

FUGENE6 (Roche Applied Science) according to the manufa-
turer’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 was harvested after 18 h. Aliquots of the conditioned medium containing recombinant pro-MMP-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 lin-

ear with incubation time over this period (data not shown).

**Immunoblotting and immunofluorescence**

For immunoblotting, cells were lysed with radioimmune pre-

cipitation assay buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) con-
taining the Complete H protease inhibitor mixture (Roche 

Applied Science). Protein contents of the lysates were deter-

mined by a bicinchoninic acid protein assay (Sigma-Aldrich).

Equivalent quantities of protein were size-fractionated by gra-
dient (4–20%) SDS-PAGE followed by transfer to nitrocellu-

lose membranes. Immunoblot analyses were performed with the following antibodies: mouse monoclonal anti-HA-11 

(Clone 16B12; Covance); rabbit monoclonal anti-MT1-MMP 

(GM130, anti-EEA1, anti-elf2, anti-JNK/p-JNK, and 

anti-ERK/p-ERK (Cell Signaling); and FLAG-M1 (Sigma-Al-

drich). Primary antibodies were detected with horseradish 

peroxidase–conjugated species-specific secondary antibodies 

(Santa Cruz Biotechnology, Inc.) using the Super Signal Pico 

system (Pierce).

For immunofluorescence, cells were fixed in 4% parafo-

dehyde, 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 antibi-

dies 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 0.5 

mg/ml Sulfo-NHS-SS-Biotin (Pierce) in PBS at 4 °C for 1 h. The reaction was terminated by washing with 150 mM glycine/TBS, 

and cell lysates were recovered for pulldown with streptaviden-

agarose (Pierce). The captured material was resolved by SDS-
PAGE, and surface-labeled MT1-MMP was assessed by West-

ern blotting.

**Type I collagen degradation**

Acid-extracted type I collagen was prepared from rat tail ten-

dons 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 10× Eagle’s minimum essential media and 0.34 N 

NaOH in an 8:1:1 ratio with 25 mM Hepes at 4 °C, and 100 μl of the mixture was uniformly spread over the surface of 2-cm² 

Lab-Tek II chamber slides (Nalge Nunc International, Naper-

ville, IL). Fibrillogenesis was induced by incubating the colla-

gen-coated slides for 45 min at 37 °C, and collagen films were 

labeled with Alexa Fluor 488 (Molecular Probes). Post-fibrillo-
genesis 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 × 10⁴) were 

seeded at low density atop collagen or gelatin films and incu-

bated for 3 days in DMEM, 10% FBS at 37 °C. Degradation rates increase in linear fashion between 1 and 3 days 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 ± S.E. of three experiments.

**Microscopy**

Confocal imaging of collagen degradation and cellular immu-

nofluorescence was performed with a spinning disc Nikon 

Eclipse Ti confocal microscope using a ×20 objective lens, 

numerical aperture 0.75, or a ×100 objective lens, numerical 

aperture 1.45. Images of Alexa Fluor 488 and Alexa Fluor 594 

signals were captured at 25 °C with a Yokogawa CSU-W1 cam-

era using Micromanager MM Studio (version 1.4.23) software and ImageJ for image processing. Cellular immunofluorescence signals were also imaged using a Leica DM IRB spinning-disc confocal microscope with a ×63 objective lens, numerical aper-

ture 1.4, and images were captured with a PerkinElmer Ultra-

View Vox system camera using Velocity version 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 (Diag-

nostic Instruments, Inc.) through a Leica upright microscope. 

Image-processing software (Photoshop version 7, Adobe) was 

used to overlay images and to enhance equally image color and 

clarity.

**Structural analysis**

The Ser466 → Pro mutation was modeled into the structure of 

the MT1-MMP hemopexin domain using the COOT pro-

gram 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 three or more times.
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