# Chemical Tools for Selective Activity Profiling of Endogenously Expressed MMP-14 in Multicellular Models

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ABSTRACT: Matrix metalloproteases (MMPs) are a large family of zinc-dependent endopeptidases involved in a diverse set of physiological and pathological processes, most notably in cancer. Current methods for imaging and quantifying MMP activity lack sufficient selectivity and spatiotemporal resolution to allow studies of specific MMP function *in vivo*. Previously, we reported a strategy for selective targeting of MMPs by engineering a functionally silent cysteine mutation that enables highly specific covalent modification by a designed activity-based probe. Here, we describe the translation of that technology into a mouse model of breast cancer and subsequent demonstration of the utility of the approach for studies of MMP-14 activation in the tumor microenvironment.

Using this approach, we find that MMP-14 is active in late stage tumors and is predominantly associated with stromal cell populations that have been activated by specific signaling molecules (e.g., TGFβ) produced by tumor cells. Our data demonstrate the applicability of this approach for studies of MMP function in whole organisms and identify important regulatory mechanisms for MMP-14 activity in the tumor microenvironment.
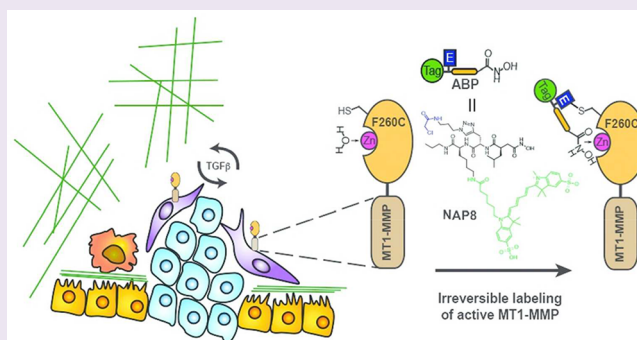
Matrix metalloproteases (MMPs) are a family of zinc-dependent endopeptidases, consisting of more than 25 members in humans.[1] This large family of proteases is responsible for the degradation of most extracellular proteins and has long been associated with many types and stages of cancer.[2,3] Proteolysis by MMPs is essential for a variety of physiological activities[4] and signaling events[5,6] and is tightly regulated at several levels. In addition to regulation at the transcriptional level, all MMPs are produced as latent pro-enzymes that require enzymatic activation for catalytic function.[1] Their activity is regulated by compartmentalization and trafficking and is inhibited by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs).[7]

MMPs promote tumor progression and regulate the tumor microenvironment through degradation of the extracellular matrix (ECM) as well as through additional signaling functions.[6,8] However, attempts to target MMPs for therapeutic purposes have been hampered by a general poor understanding of the specific roles of individual MMP members and inadequate target validation.[3,9] Contradicting roles have emerged for MMPs both as tumor promoters and tumor suppressors.[10] Depending on the circumstances, different members of the MMPs regulate tumorigenesis during the process of microenvironment remodeling. Despite mounting evidence of elevated MMP activity at the interface of a tumor and its stroma,[2,8] our understanding of the specific roles, physical location, and dynamics of proteolytic activation of individual MMPs remains elusive. Current methods to visualize MMP activity such as fluorogenic substrates,[11,12] radiolabeled inhibitors,[13,14] or fluorescent matrix proteins[15] lack the specificity and spatiotemporal precision required to map the activity of individual MMPs in the context of a native tissue during tumor development.

Our group previously reported the design and validation of a methodology to specifically image and selectively inhibit individual MMPs.[16] This approach makes use of a combination of protein engineering and a corresponding activity-based probe (ABP) to selectively label active forms of MMPs in intact cells (Figure 1a). A proximity-induced interaction between an engineered cysteine and an electrophilic warhead on the ABP results in irreversible labeling and selective inhibition of the engineered "probe-sensitive" MMP, while any nonspecific interaction of the probe with wild type (WT) MMPs remains reversible and results in weak, reversible inhibition (Figure 1b). Because ABP binding relies on the accessibility of the catalytic zinc ion and the active site, this strategy provides a means to distinguish active enzyme from pro-enzyme forms or inhibited complexes in the context of live cells or intact tissues with high spatial resolution.[16]

The membrane-anchored MMP-14 (MT1-MMP) is thought to be one the main proteases involved in the process by which
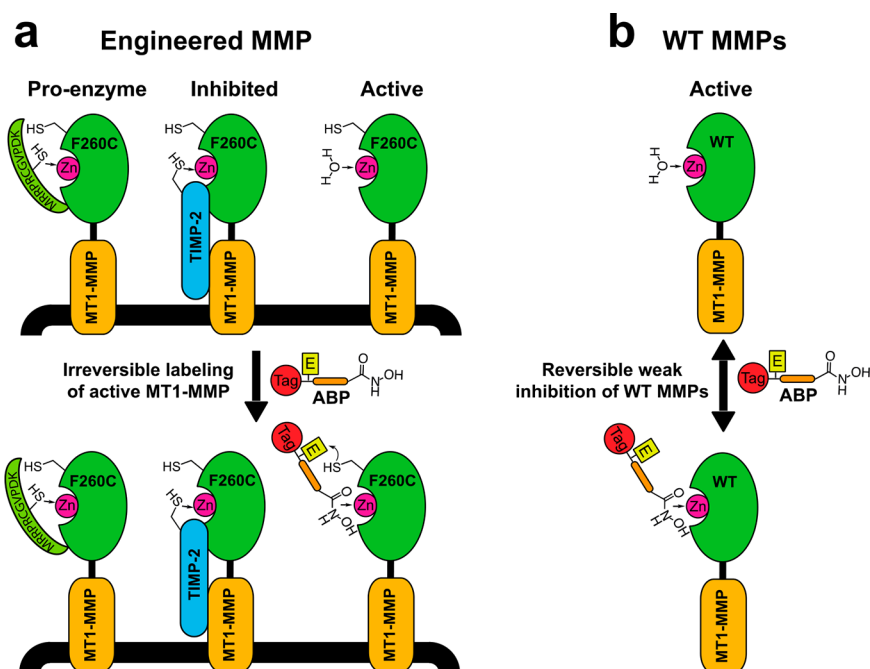
Figure 1. Specific labeling of active MMP-14 using protein engineering coupled with ABPs. (a) Engineered MMP-14 contains a cysteine nucleophile that is irreversibly labeled by the ABP. Covalent binding occurs only with the active form of MMP-14 following affinity targeting of the catalytic zinc by the hydroxamate-containing ABP but not with the proenzyme or the inhibited form in which the zinc is inaccessible. (b) ABP labeling is selective to the probe-sensitive MMP, while any interactions with native MMPs result in reversible weak inhibition.
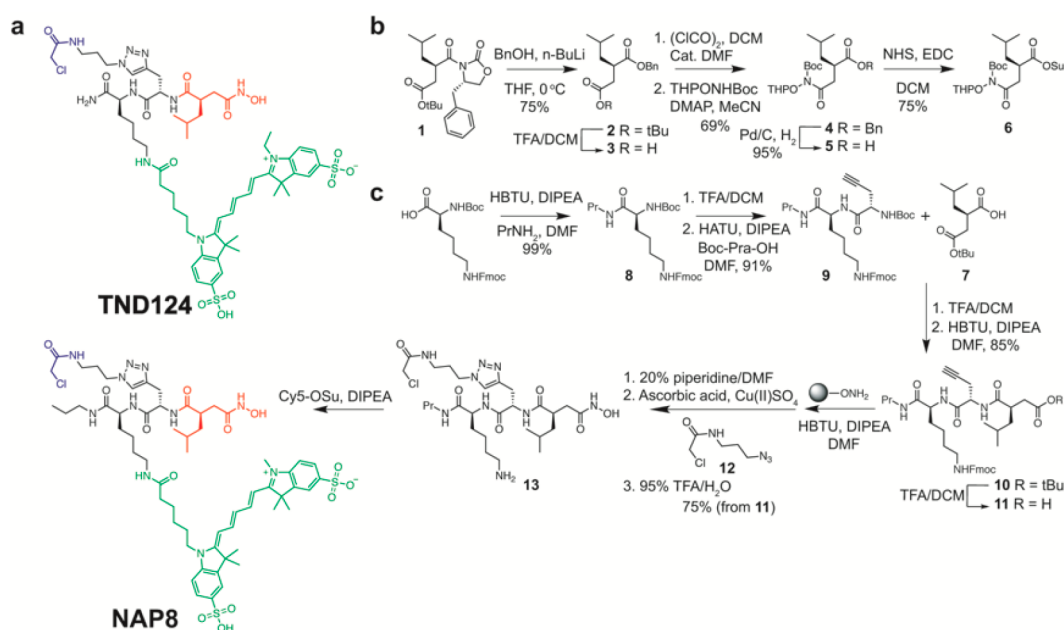

Figure 2. Chemical structure and optimized synthesis of an ABP that targets the cysteine mutant MMP-14. (a) ABPs TND124 and NAP8 are designed based on the core scaffold of GM6001 and comprise its high affinity isobutylsuccinylhydroxamic acid motif (red) with the addition of a chloromethyl amide electrophilic warhead (blue) and a Cy5 fluorescent tag (green). NAP8 has an additional propylamide to improve its metabolic stability. (b) Optimized synthesis strategy to prepare double-protected hydroxylamine derivative **6**; synthesis procedures were adopted from Overkleeft et al.[28] (c) Improved synthesis scheme for NAP8, employing an on-resin click reaction to add the electrophile **12** in the penultimate reaction step.

tumor cells penetrate the basement membrane, a process that marks the transition to invasive carcinoma.[2,17,18] High expression levels of MMP-14 by tumor cells and cells within the tumor stroma are associated with rapid tumor progression and increased invasiveness.[19−21] The coordinated compartmentalization of MMP-14 in invadopodia or podosomes,

membrane protrusions specialized for ECM adhesion and degradation,[22] is evident for both the invading tumor cells and stromal cells in the tumor microenvironment such as cancer-associated fibroblasts[18,23] (CAFs), macrophages,[24,25] and other myeloid cells.[26] However, the identity of soluble signaling molecules produced by malignant cancer cells and the
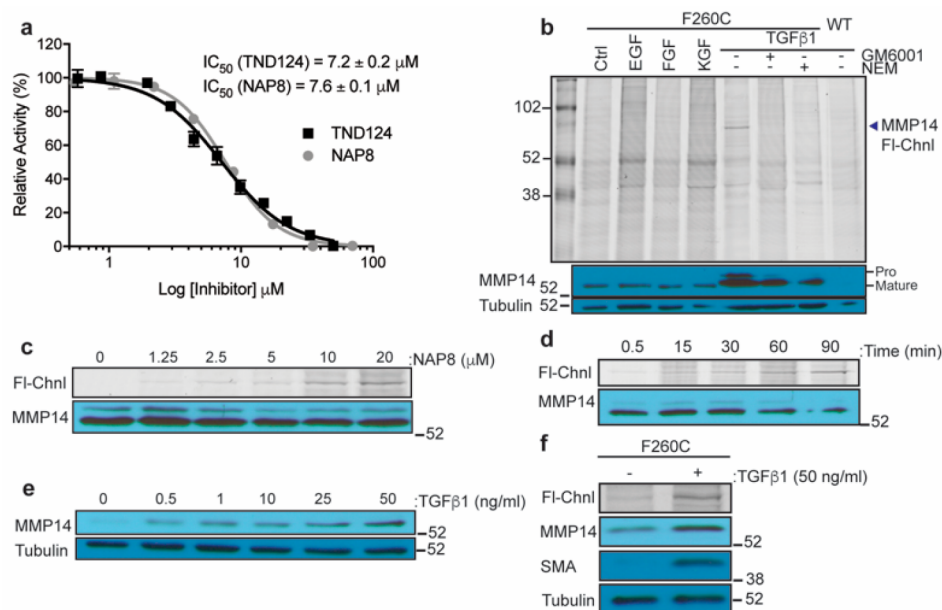
Figure 3. Active MMP-14 was selectively labeled by ABPs in stimulated fibroblasts. (a) Recombinant MMP-14 F260C catalytic domain was incubated with TND124 or NAP8 over a range of concentrations. Enzyme activity was measured with the MMP FRET substrate MCA-PLGL-Dap(Dnp)-AR-NH$_2$ (Anaspec). Activity is relative to a DMSO control. Each data point represents an average of three replicates, error bars ± SD. (b) Primary mouse fibroblasts derived from the MMP14-F260C knock-in mice and immortalized WT fibroblasts were serum starved for 12 h followed by 24 h incubation in the presence or absence of growth factors (all at 10 ng/mL), GM6001 (100 μM), and NEM (100 μM). Cell cultures were then incubated with NAP8 (10 μM) for 60 min and washed. Cell were lysed in sample buffer and resolved on SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α-tubulin. Locations for pro-MMP14 (60 kDa) and mature MMP14 (57 kDa) are marked. (c) Serum starved primary mMmp14-F260C fibroblasts were incubated with TGFβ1 for 24 h then NAP8 over a range of concentrations as indicated. Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with MMP-14 antibody. (d) Same as (c) except cells were labeled with 10 μM NAP8 for the indicated incubation times. (e) Serum starved WT fibroblasts were incubated with TGFβ1 over a range of concentrations for 24 h. Western blot analysis was performed with antibodies for MMP-14 and α-tubulin. (f) Serum starved primary mMmp14-F260C fibroblasts were incubated in the presence or absence of TGFβ1 (50 ng/mL) for 24 h and then treated with NAP8 (10 μM). Cell lysates were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with antibodies for MMP-14, α-smooth muscle actin (SMA), and α-tubulin.

surrounding stroma that induces expression and activation of MMP-14 remain largely undefined. Understanding the regulation, activity, and function of MMP-14 is essential for defining the cell invasion programs of specific cancers. Here, we describe the construction of a new mouse model in which the native MMP-14 gene is engineered to express a cysteine mutant MMP-14 that can be targeted with a corresponding ABP through a covalent labeling reaction. This mutation was also crossed with MMTV-PyMT mice to generate a breast cancer model in which all cells express the probe-sensitive MMP-14. Using an activity-based probe, we were able to visualize MMP-14 activity patterns and cellular localization within components of the tumor microenvironment. We found elevated levels of expression and activation of MMP-14 primarily in stromal cells, while MMP-14 is expressed at the protein level within the tumor cells but remains largely inactive within the tumor bulk. The induction of MMP-14 activity within the stroma is orchestrated by soluble signals produced by cancer cells. We identify TGF-β1 as one of the potential cytokines that regulates this activity. Overall, our data demonstrate the applicability of this methodology to visualize and quantify the activity of a specific protease in the context of the complex tumor microenvironment. It also shows that, while MMP-14 is expressed at the protein level by both tumor and stromal derived immune cells, it is primarily activated in

the immune cell populations as the result of response to tumor cell-derived cytokines.

■ RESULTS AND DISCUSSION

Optimization of the Synthesis of Probes for Cysteine Mutant MMP-14. The ABP TND124 (Figure 2a) was previously designed and synthesized by our group to enable specific, activity-dependent labeling of a mutant MMP-14 containing a functional silent cysteine mutation near the active site. It is capable of irreversibly labeling engineered MMP mutants MMP-12 T184C and MMP-14 F260C. These point mutations do not interfere with enzymatic activity, substrate specificity, or protein stability as we have previously reported.[16] Its design was based on the scaffold of the broad-spectrum MMP inhibitor Ilomastat (GM6001) that employs an isobutylsuccinylhydroxamic acid motif as the affinity recognition element. Difficulties with the chemical stability of the isobutylsuccinylhydroxamic acid and several low yielding steps prompted us to modify the synthesis route. Specifically, our focus turned to issues arising from (1) low yields for the formation of the N-terminal amide, (2) low chemical stability of the isobutylsuccinylhydroxamic acid intermediate, and (3) considerable loss of material due to the nucleophilic potential of the hydroxamic acid and its cross-reactivity with the electrophilic warhead that is introduced in a click reaction. Replacing the terminal amide of TND124 with a propyl amide
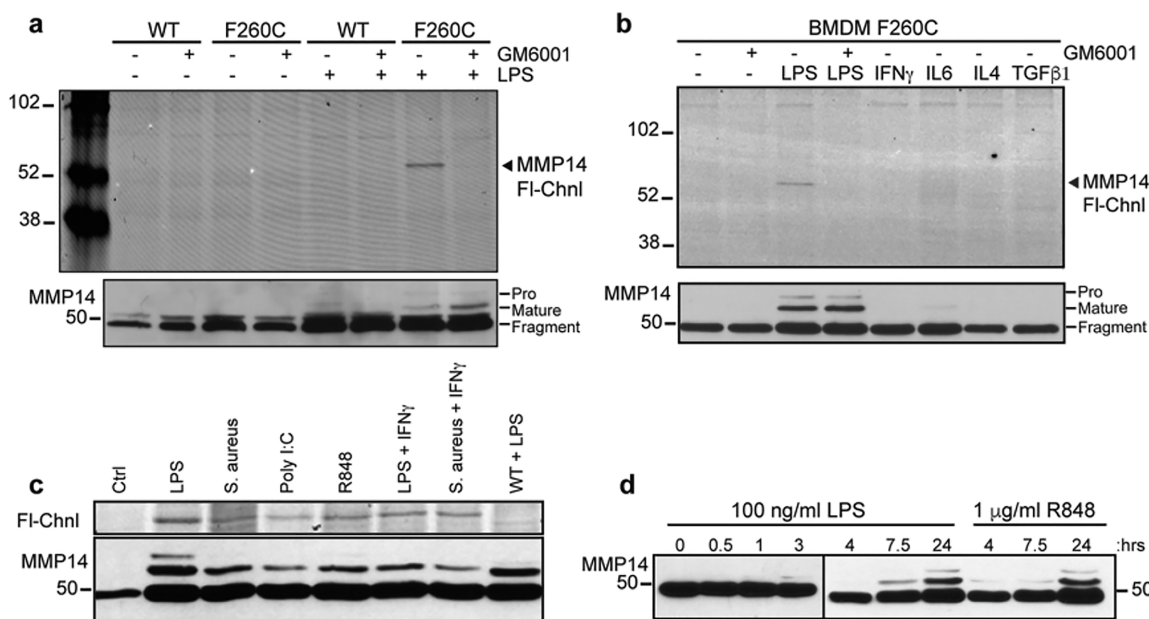
Figure 4. Active MMP-14 is detected only in M1 activated macrophages. Primary BMDMs from the WT or mMmp14-F260C knock-in mice were primed in the presence of (a) LPS (100 ng/mL) and (b) other pro-inflammatory cytokines; IFNγ (40 ng/mL), IL6 (50 ng/mL), IL4 (10 ng/mL), and TGFβ1 (10 ng/mL) for 16 h, and treated with GM6001 (10 μM) or DMSO vehicle control. Cell cultures were treated with TND124 (10 μM), and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α-tubulin. Locations for pro-MMP-14, mature MMP-14, and an inactive fragment are marked. (c) Primary mMmp14-F260C and WT BMDMs were primed with different TLR agonists; LPS (100 ng/mL), S. aureus (heat inactivated, 1 × 10⁷ cells/ml), Poly I:C (1 μg/mL), R-848 (1 μg/mL), and IFNγ (50 ng/mL) for 16 h. Cell cultures were treated with TND124 (5 μM) and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with MMP-14 antibody. (d) Western blot analysis for BMDMs primed with LPS (100 ng/mL) or R-848 (1 μM) over 24 h.

in NAP8 (Figure 2), using a solution phase amidation reaction instead of solid support, increased the yields for this step, and potentially improves its metabolic stability. The precursor t-butyl (R)-2-isobutylsuccinate 7 used in the synthesis of TND124 is intrinsically unstable with a high propensity to form a cyclic anhydride, leading to the formation of multiple byproducts in both basic and acidic reaction conditions. To get around instability of 7 and avoid the cross-reactivity of the unprotected hydroxamate toward the electrophilic warhead of the probe, we pursued a strategy that replaces 7 with a protected hydroxamate. Thus, we synthesized precursor 5, adopting a published strategy,[27,28] but quickly discovered that its instability was even greater than that of 7 (Figure 2b). Preparing the activated ester derivative 6 slightly improved stability but still resulted in considerable byproduct formation and low reaction yields in the consequent coupling step. We determined that a hydroxylamine Wang resin could be used to both produce the final hydroxamate product and also serve as a protecting group while introducing the electrophilic warhead (Figure 2c). To improve reaction yields for the coupling of 7, we used HBTU and DIPEA because it balances mild activation with fast reaction time, minimizing byproducts and increasing reaction yields. To facilitate the click reaction on the solid support (CuAAC-SP), we used anhydrous CuSO₄ dissolved in DMF as the source of Cu(II) ions along with ascorbic acid as the reducing agent. An overnight reaction with incubation at 37 °C with agitation increased the yields from 18 to 75% over four steps from 11 to 13. Collectively, these modifications of the synthesis route enable the synthesis of NAP8 at multimilligram scale.

Generation of a Mouse Line Endogenously Expressing Probe-Sensitive MMP-14. To confirm that the binding

and inhibition properties of NAP8 do not differ from those of TND124 we incubated recombinant MMP-14 F260C active domain with each probe and compared the IC₅₀ inhibition values (Figure 3a). As expected, both TND124 and NAP8 had nearly identical IC₅₀ values toward the recombinant enzyme, roughly 100-fold higher than that of GM6001.[29] MMP-14 is tightly regulated by various mechanisms, including compartmentalization and trafficking, enzymatic activation, inhibition by small molecules or TIMPs, degradation, and clearance.[7,30] Therefore, it is essential to achieve expression of the cysteine mutant MMP-14 from the endogenous gene locus to be able to make relevant conclusions about its activation and regulation. Using clustered regularly interspaced short palindromic repeats (CRISPR) technology, we generated a knock-in mouse in which the native mMmp-14 gene was replaced with the gene containing the F260C mutation. This was achieved by changing thymidine to guanine at the 6688th nucleotide of mMmp-14 cDNA (Figures S1a−c), followed by pronuclear microinjection of guide RNA (gRNA), single stranded oligo donor nucleotide (ssODN), and Cas-9 mRNA into a C57BL/6 embryo (conducted by Applied StemCell Inc., Milpitas, CA). After implantation and isolation of offspring, we identified heterozygous founders by PCR screening. These were used to generate homozygous mMmp14-F260C F1 mice using standard breeding schemes (Figure S1d). Importantly, we observed no detectable phenotypes associated with the presence of the homozygous mutation with growth and breeding indistinguishable from that of the WT mice. This confirms that the cysteine mutation on MMP-14 is functionally silent, as shown in our prior studies.[16]

Engineered ABPs Specifically Label Probe-Sensitive MMP-14 in Activated Fibroblasts. Using primary fibro-
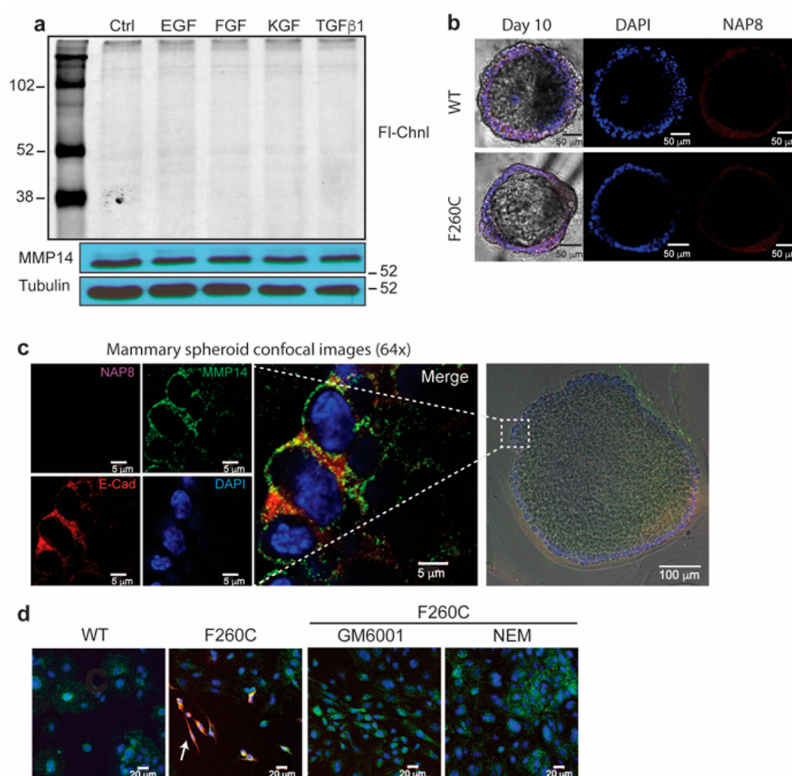
Figure 5. Active MMP-14 is not detected on the surface of primary epithelial cells. (a) Primary mMmp14-F260C epithelial cells were serum starved for 12 h followed by 24 h incubation in the presence or absence of growth factors (10 ng/mL). Cell cultures were treated with NAP8 (10 μM), and lysed cells were analyzed by SDS-PAGE followed by in-gel fluorescence scanning for Cy5 (Fl-Chnl). Western blot analysis was performed with antibodies for MMP-14 and α-tubulin. (b) Confocal microscopy of mammary spheroids in 3D cultures. Primary organoids isolated from WT and mMmp14-F260C 14-week virgin females were cultured on Matrigel for 10 days. At day 10, spheroids were treated with NAP8 (10 μM), fixed, stained with the molecular marker DAPI, and visualized by confocal microscopy (scale bar 50 μm). (c) Magnified images (64×) of sections of mMmp14-F260C spheroids treated with NAP8 (10 μM, purple) and stained with antibodies for MMP-14 (green), E-cadherin (red), and DAPI (blue). The magnified section is marked with a white box (scale bar 5 μm) on the white light image of the spheroid (bottom right). (d) Crude primary organoid extract, grown in 2D cultures were pretreated with GM6001 (10 μM), NEM (100 μM), or DMSO and then treated with NAP8 (10 μM). Cells were fixed and stained with anti-MMP-14 and DAPI and visualized by confocal microscopy. NAP8 (red), MMP14 (green), DAPI (blue); cells that have active MMP14 are marked with a white arrow (scale bar 20 μm).

blasts generated from the mMmp14-F260C mice, we performed labeling studies with the NAP8 probe to assess its overall selectivity and determine the levels of MMP-14 activity in those cells. We first labeled WT and mMmp14-F260C fibroblasts after incubation with multiple paracrine factors such as epithelial growth factor (EGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), and transforming growth factor β1 (TGFβ1) over 24 h (Figure 3b). We then treated intact cells with NAP8 and resolved lysates by SDS-PAGE. Surprisingly, in the absence of growth factors, no specific labeling was observed. However, labeling of fibroblasts that were stimulated with TGFβ1 showed a selective fluorescent band that corresponds to the active MMP-14. The labeled protein was only observed in the cells expressing the mutant MMP-14 F260C and was blocked by preincubation with excess of the nonspecific hydroxamate inhibitor Ilomastat (GM6001) or the general cysteine reactive molecule N-ethylmaleimide (NEM), suggesting that it results from labeling of the probe-sensitive MMP-14 by the NAP8 probe. Consistent with this finding, Western blot of MMP-14 protein levels showed increased expression in fibroblasts only when treated with TGFβ1 and not with other cytokines such as EGF, FGF, and KGF. Notably, the labeled band for active MMP-14 in Figure 3b appears to migrate slightly higher than the

expected ~57 kDa for the mature active protein. The intensity of labeling was dependent on both probe concentration (Figure 3c) and labeling time (Figure 3d) as expected for a covalent modifier. In addition, incubation with TGFβ1 increased the expression of MMP-14 in a dose dependent manner (Figure 3e). This increase in MMP-14 activity corresponds with the TGFβ-induced differentiation program of fibroblasts into myofibroblasts[31] that is accompanied by the production of α smooth muscle actin (SMA; Figures 3f and S2).[32]

MMP-14 is Active in Primary Macrophage Cells. We next assessed the labeling of MMP-14 in bone marrow derived macrophages (BMDMs) isolated from either WT or mMmp14-F260C mice. As observed for primary fibroblasts, we found no probe labeling of MMP-14 in unstimulated BMDMs (Figure 4a). When we stimulated the cells with a range of cytokines, including TGFβ1, IFN-γ, IL6, and IL4, we again found no labeling by the NAP8 or TND124 probes. However, we did observe labeling of active MMP-14 in BMDMs treated with LPS, and this labeling was blocked by pretreatment with GM6001 (Figure 4b). Western blot analysis revealed that the mature 57 kDa form of MMP-14 accumulated only upon stimulation by LPS. Unstimulated cells and cells treated with the panel of cytokines showed
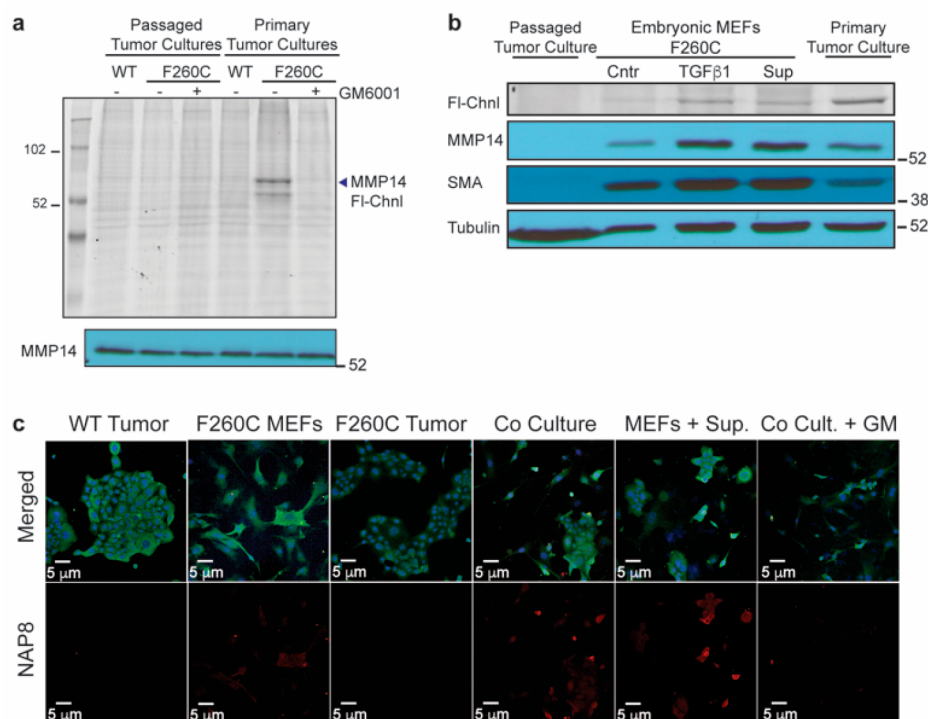
Figure 6. The majority of MMP-14 activity originates from stromal cells in the microenvironment of late-stage carcinoma that respond to stimulation by tumor cells. (a) Tumors harvested from the mammary glands of mMmp14(F260C)^{WT}MMTV-PyMT$^+$ and mMmp14-(F260C)$^{+/+}$MMTV-PyMT$^+$ mice were cultured in 2D cultures and treated with NAP8 (10 μM) at the first passage or after 10 passages. Whole cell lysates were analyzed by SDS-PAGE and scanned for in-gel fluorescence of Cy5 (Fl-Chnl). Western blot analysis was performed with MMP-14 antibody. (b) Primary tumor cultures, passaged tumor cultures (neoplastic epithelial cells), and primary fibroblasts from mMmp14-(F260C)$^{+/+}$MMTV-PyMT$^+$ mice were incubated with TGFβ1 (50 ng/mL) or supernatant of neoplastic epithelial cells (24 h, serum-free medium) for 24 h and then treated with NAP8 (10 μM). Whole cell lysates were analyzed by SDS-PAGE and scanned for in-gel fluorescence of Cy5 (Fl-Chnl). Western blot analysis was performed with antibodies for MMP-14, SMA, and α-tubulin. (c) 2D cultures of mMmp14(F260C)^{WT}MMTV-PyMT$^+$ and mMmp14(F260C)$^{+/+}$MMTV-PyMT$^+$ passaged tumor cells, fibroblasts, and cocultures of passaged tumor cells and fibroblasts were pretreated with DMSO or GM6001 (10 μM) or preincubated with supernatant of neoplastic epithelial cells (24 h, serum-free medium) and then treated with NAP8 (10 μM). Cells were fixed and stained with anti-MMP-14 and DAPI and visualized by confocal microscopy. NAP8 (red), MMP14 (green), DAPI (blue); each experiment was performed in three independent biological repeats (scale bar 20 μm).

accumulation of only a 44 kDa fragment. Previous studies have identified the 44 kDa fragment as the product of autocatalytic processing that takes place in the absence of TIMP-2. Sequential cleavages at Ala255 and Gly284 of the 57 kDa species generates an inactive 18 kDa soluble species and a membrane bound 44 kDa species, missing the entire catalytic domain.[30] LPS activates macrophages to their pro-inflammatory, M1 functional state[33] and results in MMP-14 activation. Stimulation by immune-regulatory cytokines such as IFNγ and IL-6 (M1 activation) or IL-4 and TGFβ1 (M2 activation) does not result in increased activity of MMP-14 (Figure 4b). To test if this is an LPS-specific response, we tested the effect of different agonists of toll-like receptors (TLRs). Stimulation of macrophages with multiple different pathogen-associated molecular patterns (PAMPs) triggered a uniform response, resulting in activation of MMP-14 (Figure 4c). Sensing of PAMPs is a general response that leads to the pro-inflammatory M1 activation, a state that has been implicated with generally higher proteolytic activity.[31,34] Priming of macrophages with PAMPs, either LPS or R-848, results in an increase in levels of the mature form of MMP-14 over 24 h (Figure 4d), suggesting that activation of pro-inflammatory response through TLRs leads to an increase in levels of active MMP-14.

MMP-14 Activity Is Tightly Regulated on the Surface of Mammary Epithelial Cells. After determining that MMP-14 activity is found predominantly on activated fibroblasts and macrophage cells, we wanted to examine how the activity of this protease may be regulated on mammary epithelial cells during normal cell growth and morphogenesis. The development of the mammary gland is an ideal model to study the physiologic roles of MMPs. This complex organ undergoes continuous changes in structure and function from initial development through cycles of lactation and involution.[35] MMPs play a crucial role in ECM remodeling that takes place during ductal growth and morphogenesis.[6,36] Mammary epithelial cells or epithelial organoids can be grown in three-dimensional *ex vivo* cultures (3D cultures). These 3D cultures recapitulate numerous features of glandular epithelium *in vivo*, including differentiation of myoepithelial and luminal cells and the formation of polarized spheroids with a hollow lumen.[37,38] We therefore generated mammary organoids by isolation of cells from the inguinal fat pads of WT or mMmp14-F260C virgin females. We treated organoid cultures with NAP8 and lysed cells for analysis by fluorescence scanning. Surprisingly, while the mature 57 kDa MMP-14 species is present as confirmed by Western blot, we could not detect labeling of active MMP-14 in these cells with our probe (Figure 5a). This suggests that while mature MMP-14 is present, its activity may

be kept in check by endogenous inhibitors. Incubation with different growth factors, among these TGFβ1, for 24 h did not increase the expression of MMP-14 nor change its activation state. To understand if chemical cues or stimuli are needed for MMP-14 activation, we seeded mammary organoids on collagen matrix (Matrigel) and allowed development and maturation of the spheroids over the course of 10 days. At day 10, we incubated spheroids with NAP8 to label active MMP-14 and then performed fluorescence microscopy (Figure 5b). Active MMP-14 was not detected on the surface of the mature spheroids, and no significant differences in fluorescence were observed between the WT spheroids and the mMmp14-F260C spheroids. To further assess the presence and specific localization of MMP-14 on mature spheroids, we fixed probe labeled spheroids on slides for immunofluorescence analysis. Anti-MMP-14 staining showed its localization in small microdomains on the apical membrane, where it colocalizes with E-cadherin, forming tight junctions between neighboring cells (Figure 5c). The lack of probe fluorescence indicates that MMP-14 is inactive on the surface of the spheroids, suggesting that growth and expansion of the spheroid into the matrix is independent of MMP-14, or alternatively, MMP-14 activity may be contributed from cell populations present in the stroma that are not present in our 3D cultures. It is important to note that Matrigel contains an undefined mixture of proteins and can include hormones, growth factors, or enzymes that may influence the activation or inhibition of MMP14 on the surface of the spheroids. When organoids were plated and cultured in 2D cultures, prior to separation from populations of stromal cells, probe labeling revealed that active MMP-14 is present on subpopulations of cells other than the epithelial cells (Figures 5d and S3). These labeled cells have spindle morphology resembling that of mesenchymal cells such as fibroblasts or myeloid cells that reside in the stroma. Probe labeling of these cell populations was specific to the probe-sensitive MMP-14 in the mMmp14-F260C organoids. Preincubation with excess of Ilumastat (GM6001) or NEM blocked labeling by NAP8.

Construction of a Cancer Model to Study the Pathophysiologic Role of MMP-14. To better understand the role of MMP-14 in regulation of the tumor microenvironment, we crossed the mice expressing the probe-sensitive MMP-14 with mice that express the Polyoma Virus middle T antigen under the direction of the mouse mammary tumor virus promoter (MMTV-PyMT). MMTV-PyMT+ females develop mammary tumors that become palpable typically at around 140 days of age and metastasize to the lung. This allowed us to harvest tumors from the mammary glands of mMmp14^WT^MMTV-PyMT+ and mMmp14-F260C^+/+^MMTV-PyMT+ females. After the initial digestion and dissociation of tumor tissue, we filtered cells and plated them for culture. Primary cell cultures treated with NAP8 show specific labeling of MMP-14 only in the cells derived from tumors from the mice expressing MMP14-F260C but not from mice expressing WT MMP-14, confirming the presence of active MMP-14 in the tumor microenvironment (Figure 6a). However, after multiple passages of the cells in culture, the MMP-14 activity was completely lost. While primary, non-neoplastic cells (i.e., stromal cells) reach replicative senescence and perish after 4−5 passages, transformed cancer cells proliferate and dominate the culture. After 10 passages, the entire cell population consists of neoplastic epithelial cancer cells. This population of cells, although clearly expressing MMP-14, shows no active MMP-14 as assessed by probe labeling (Figure 6a). We therefore

concluded that the observed labeled active MMP-14 from the primary tumor cultures originates from non-neoplastic cells that normally exist in the tumor microenvironment. These cell populations include fibroblasts, bone marrow derived cells, leukocytes, and endothelial cells.[26,39] Activated fibroblasts, also known as cancer associated fibroblasts (CAFs), are myofibroblast populations present at the tumor−stroma interface.[31]

To test our hypothesis, we compared the expression and activity profile of MMP-14 in primary tumor cultures to that of TGFβ1-stimulated myofibroblasts (Figure 6b). An increase in MMP-14 expression as well as probe labeling was observed in primary tumor cultures, similar to TGFβ1-stimulated fibroblasts. The expression of SMA in the primary tumor culture confirmed the presence of myofibroblasts. Although the total expression of MMP-14 was lower than that of fibroblasts, primary tumor cultures consists primarily of cancer cells that express lower levels of MMP-14; thus, the observed increase results from a minority population of cells. Nevertheless, MMP-14 activity is highest in the primary tumor cultures as evidenced by probe labeling, presumably by stimulation through TGFβ1 signaling or in conjunction with additional factors. A similar enhancement in expression of MMP-14 was achieved by incubation of fibroblasts with tumor cell culture supernatant. Stimulation through soluble factors also resulted in increased activity of MMP-14, although not to the extent seen in the primary tumor cultures.

While TGFβ1 produced by tumor cells may act on fibroblasts to increase MMP-14 expression and activation, additional signals such as physical contact of tumor cells with the surrounding CAFs may be needed for substantial activation of MMP-14. To recapitulate the interactions between tumor cells and fibroblasts in the tumor microenvironment, we cocultured tumor cells with embryonic fibroblasts on cover slides followed by incubation with NAP8 to label active MMP-14 before cells were fixed and stained with an MMP-14 antibody and DAPI (Figure 6c). Similar to the biochemical results, epithelial cancer cells, although expressing MMP-14, do not show labeling by the probe. Embryonic fibroblasts that are not stimulated express high levels of MMP-14, but virtually all of this is in an inactive state. On the other hand, fibroblasts that are cocultured with cancer cells have substantial levels of active MMP-14, as assessed by NAP8 labeling. This increase is likely due to signaling events that involve soluble factors given that fibroblasts that are cultured with the supernatant of cancer cells exhibit a similar induction of MMP-14 activity that can be blocked by excess GM6001.

◼ CONCLUSIONS

MMP-14 is associated with multiple human cancers and is considered the major cell-associated protease necessary to confer invasive properties to normal or neoplastic cells.[18] Its role in breaching the basement membrane during the early stages of invasion is well-established, both in the physiologic and pathologic states.[18,40] However, its role in infiltration and migration through the interstitial tissue at the later stages of tumorigenesis is still subject to debate, as are the molecular cues that stimulate its activity. MMP-14 has been implicated in tissue-invasive activity, but mounting evidence suggests that the majority of MMP-14 is expressed at the tumor−stroma interface rather than within the bulk of the tumor.[8,19,21,41,42] Significant proteolytic activity is attributed to the stromal cell populations that are stimulated through the cross talk between the tumor and its microenvironment.[26,39,43−45] While many of

these studies make use of expression of mutants or deletion of MMP-14, there remains an insufficient understanding of its activation pattern in the context of complex multicellular tissues.[3,46,47] Using an engineered probe-sensitive MMP-14 and a corresponding ABP, we were able to profile the activity levels of this protease *in vivo* in several cell types as well as in the components of the microenvironment of late stage breast cancer tumors.

Our findings support the notion that MMP-14 activity is tightly regulated in the absence of stimulatory signals. This regulation is well documented in the literature with TIMP-2 as the major endogenous inhibitor of MMP-14.[6,7,36] The interaction between MMP-14 and TIMP-2 has been suggested to increase stability of MMP-14 and prevent autoproteolytic degradation.[30,48] While the autoproteolytic degradation product was the predominant form of MMP-14 observed in nondifferentiated (M0) or alternatively activated (M2) macrophages, classically activated (M1) macrophages had increased levels of mature MMP-14 and active MMP-14 was detected by our probe only in classically activated M1 macrophages. This finding is consistent with previous findings that M1 macrophages exhibit enhanced matrix degradation capabilities and increased localization of MMP-14 in filopodia.[24] The compartmentalization of MMP-14 in filopodia or invadopedia has also been associated with increased stability of MMP-14 and elevated collagenase activity.[22] Interestingly, the activation of MMP-14 in M1 macrophages requires stimulation through TLRs with pro-inflammatory cytokines such as IFNγ and IL6 not eliciting MMP-14 activation.

MMP-14 activity was also low in nonstimulated fibroblasts. We identified TGFβ1 as a paracrine factor that not only increases expression[49] but also induces the activation of MMP-14. This activation correlates with the initiation of the myofibroblast differentiation program, which is accompanied by elevated MMP-14 protein levels. Interestingly, we found that labeled MMP-14 migrated as a slightly higher molecular weight species in fibroblasts compared to macrophages. This difference in migration could be due to modifications of the enzyme that alter its regulatory activities in different cell types. Consistent with this hypothesis, various post translational modifications and protein–protein interactions have been attributed to the short cytoplasmic tail of MMP-14, which could result in the observed differences in migration in the gel.[50−54] While additional studies will be required to confirm such modifications and their biological significance, these data highlight the value of the active site probe for identifying the specific active form a target protease.

In primary tumor cultures, we observed high activity of MMP-14; however, this activity originated from non-neoplastic fibroblasts and other stromal cell populations and not from transformed cancer cells. We found that MMP-14 activation is stimulated by soluble factors secreted by the tumor cells. One such effector is TGFβ1, which acts to differentiate fibroblasts in the stroma and stimulate expression and activation of MMP-14. TGFβ1 signaling has been suggested to promote tumor growth, progression, and migration at later stages of multiple carcinomas.[55−58] Santibanez et al. describe TGFβ signaling in the tumor microenvironment as a regulatory loop, where TGFβ is overproduced by late stage cancer cells causing increased expression of MMPs, which in turn mediate the activation of more latent TGFβ.[59] Thus, through balancing levels of MMPs and TIMPs, both TGFβ and MMPs contribute to tumor progression by the release of cytokines sequestered by the ECM. Similarly, TGFβ increases the expression of furin, a protease known to activate Pro-MMP-14 as well as Pro-TGFβ but not the expression of TIMPs, thus potentially enhancing the proteolytic response.[32,60] Fibroblasts that were cocultured with tumor cells show similar patterns of MMP-14 activity, suggesting that CAFs similarly stimulated by tumor cells not only express more MMP-14 but also increase levels of active MMP-14. TGFβ1 and perhaps additional soluble factors are responsible for the activation of MMP-14 in fibroblasts that are cultured with supernatants of tumor cells. As tumor cells invade through the basement membrane and make contact with the three-dimensional collagenous matrix of the interstitial tissue, paracrine signaling leads to the recruitment of stromal cells, mostly fibroblasts and macrophages. These cells within the invasion front support tumor progression, help evade the immune system, and sustain the flow of nutrients.[2,17,61,62] Our data support this model in which the invading cancer cells mobilize and activate MMP-14 in these recruited stromal populations to negotiate the ECM barrier.[39,43]

Herein, we demonstrate the utility of a methodology that involves the coupling of protein engineering with ABP design to specifically and selectively label active MMP-14 in the context of live cells. Generation of mice endogenously expressing the probe-sensitive cysteine mutant MMP-14 F260C followed by crossing of the mice into a model of breast cancer provides a means to selectively image MMP-14 in complex cellular environments. Specifically, it enables the dissection of specific roles of MMP-14 at various stages of tumorigenesis as well as the contribution of specific cell populations within the tumor microenvironment. We showed that signaling cascades initiated by TGFβ1 or through TLRs lead to the activation of MMP-14 on fibroblasts and macrophages and furthermore that these are the cells that predominantly express active MMP-14. Further studies may elucidate additional signaling pathways that lead to the activation of MMP-14 on other cell types. A clear understanding of the activity profile of MMP-14 is paramount to determine specific cell populations to be targeted for therapeutic applications. In addition, this general methodology is applicable to additional MMP members as well as other large enzyme families that lack selective inhibitors and dynamic reporters of enzyme activity levels.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00562.

> Supplemental figures; detailed synthetic procedures, materials, and methods; and compound characterizations (PDF)

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Notes

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