Supplementary Information

Target-activated transcription for the amplified sensing of protease biomarkers

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**Amino acid of PR\textsubscript{Thr}** ....................................................................................................................... 19
Experimental Section

Materials and Instruments

MMP-2, RNase and thrombin were purchased from Sigma Aldrich (St. Louis, MO, USA). MMP-3 was purchased from PeproTech Inc. (Rocky Hill, NJ, USA) MMP-8, and MMP-9 were purchased from Sino Biological Inc, China. ARP 100 was purchased from Santa Cruz Biotechnologies, USA. GM 6001 was purchased from MedChemExpress (NJ, USA). rATP, rCTP, rGTP, rUTP, SYBR Green II and DNase I were purchased from Thermo Scientific (Thermo Scientific, Waltham, MA). Furin was purchased from New England Biolabs (Beijing, China) Other protein expression reagents including Tris and imidazole were purchased from Sangon (Shanghai, China). Cell culture medium (DMEM and RPMI-1640), trypsin and fetal bovine serum (FBS) were purchased from Invitrogen (Gibco, USA). The primary antibody for MMP-2 (EPR1184) was obtained from Abcam (Cambridge, MA). The α-tubulin primary antibody (DM1A, #3873) was purchased from Cell Signaling Technology. The secondary antibody goat anti-rabbit (926-68071) and goat anti-mouse (926-32210) were obtained from Li-cor. MMP Colorimetric Assay Kit (Catalog # 72095) and 390 MMP FRET Substrate I (Catalog#27076) were purchased from AnaSpec (USA). Sodium chloride (NaCl), zinc chloride (ZnCl2), monopotassium phosphate (KH2PO4), disodium hydrogen phosphate (Na2HPO4), magnesium chloride (MgCl2), and potassium chloride (KCl) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 30% Acrylamide-N, N’-methylenebisacrylamide was purchased from ApexBio (Boston, MA, USA). All chemical reagents were of analytical grade and used without further purification, and all solutions were prepared with ultra-pure water (18.25 MΩ·cm) from the Millipore system. The tissue samples were obtained from the Second Xiangya Hospital, Central South University (China), and used with the approval of the local institutional review boards.

To measure the DNA concentration, absorption intensities at 260 nm were recorded using a Beckman DU-800 spectrophotometer (USA). Fluorescence spectroscopy was performed on a Synergy™ Mx multimode microplate reader Receives (BioTek, USA). For plasmid DNA and template, PCRs were performed on a S1000™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA). Ultrafiltration operations were carried out using ultrafilter centrifuge tubes (Amicon Ultra,10K, Millipore, USA). Western blot images were acquired on an odyssey clx imaging system (Li-cor, USA). The dual labeled-peptide (Dadcyl–GPLGVRG-FITC) is synthesized and purified by KareBayBiochem (Zhejiang, China), which was used in the peptide-based assay. All oligonucleotides sequences were synthesized and purified by Sangon Biotech (Shanghai, China). The oligonucleotides were all dissolved in DEPC water (Sangon, China) at a concentration of 100 μM and stored at -20 °C for further use.

Construction, Expression and Purification of PRMMP-2 and PRthr

To construct PRMMP-2, primers were reasonably designed to use polymerase chain reaction (PCR) to amplify T7 RNA polymerase (T7 RNAP) fragments from BL21 (DE3) and T7 lysozyme (T7 LYS) fragments from BL21 (DE3) pLysS with a point mutation (391T>A), respectively. The PCR products of T7 RNAP and T7 LYS were both containing linker regions. Then the PCR products of T7 RNAP and T7 LYS were simultaneously ligated to the linear vector pET28a by homologous recombination. A pET28a
recombinant plasmid containing an MMP-2 substrate linker (pET28a-PR_{MMP-2}) was obtained. Based on the same principles and procedures with slightly altered primer design, we obtained the pET28a recombinant plasmid containing a thrombin substrate linker (pET28a-PR_{Thr}).

Then the plasmids (pET28a-PR_{MMP-2} and pET28a-PR_{Thr}) were transformed into *E. coli* BL21 (DE3) by heat shock separately for protein expression. The cells were cultured in LB media with 25 μg/mL of kanamycin in 500 mL at 37 °C until OD600 ≈ 0.6, and then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. Cultures were then grown at 16 °C for another 20 h. Then the cells were collected by centrifugation at 5000 rpm for 8 min and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol and pH 7.5). The resultant cell suspension was broken using a SCIENTZ-IID ultrasonic cell disruption system and the soluble lysate was obtained by centrifugation at 10000 rpm for 15 min. The His-tagged proteins were purified under Ni-NTA agarose chromatograph (ÄKTA, GE) by washing and eluting the resin with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 5% glycerol and pH 7.5 ) and elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 300 mM imidazole, 5% glycerol and pH 7.5). And then the buffer was exchanged into desalination buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DDT and pH 7.5) by desalination chromatography (ÄKTA, GE). The purified proteins were then stored at -80°C for further use. The purified protein PR_{MMP-2} and PR_{Thr} were both quantified by BCA protein determination reagent and validated by SDS-PAGE.

**MALDI-TOF Characterization**

MALDI-TOF mass datas were collected on a Bruker ultraflXtreme MALDI-TOF mass spectrometer (Germany). Sinapinic acid (SA) was used as a matrix and the concentration of PR_{MMP-2} and MMP-2 are 7.5 μM and 500 nM, respectively. The matrix solution SA and reaction solution were mixed in a 1:1 volume ratio to maximize the mass signal detection. Then the sample-matrix mixture was spotted onto a MALDI plate. After the sample was completely dried on the plate, the plate was loaded into the mass spectrometer for characterization.

**PRA and Peptide-based Assay of MMP-2 and Thrombin Activities**

PRA was carried out in 30 µL reaction buffer composed by 40 mM Tris (pH 7.5), 9 mM MgCl₂, 10 mM NaCl, 100 mM KCl, 2 mM spermidine, 10 mM DTT, 5 mM rNTP, 1 μM DNA template, 2 μM ThT, PR and protease (MMP-2 or thrombin). All components were mixed together except for the PR sensor and protease. To initiate the reaction, the PR was added to the solution, after which the protease was added and fluorescence measurements were immediately initiated. The reactions were incubated at 37 °C and detected by fluorescence (excitation: 425/9.0 nm, emission: 450-600/9.0 nm) reading taken in a Synergy™ Mx multi-mode microplate reader.

For sensitivity experiments, 5 mM rNTP, 1 μM DNA template, 2 μM ThT, and 1 μM PR_{MMP-2} were added into Tris buffer (pH 7.5, 9 mM MgCl₂, 10 mM NaCl, 100 mM KCl, 2 mM spermidine, 10 mM DTT). Then different concentrations of MMP-2 or thrombin were added into the reaction solution. Samples were incubated at 37 °C for 40 min, and the fluorescence spectra was recorded.
For selectivity experiments, 5 mM rNTP, 1 µM DNA template, 2 µM ThT, and 1 µM PR_{MMP-2} were added into Tris buffer (pH 7.5, 9 mM MgCl$_2$, 10 mM NaCl, 100 mM KCl, 2 mM spermidine, 10 mM DTT), then different interfering agents were added into the reaction solution. Samples were incubated at 37 °C for 40 min, and the fluorescence spectra was taken. For the enzyme inhibition experiment, MMP-2 was incubated with ARP 100 (selective MMP-2 inhibitor, 100 µM) and GM 6001 (broad-spectrum metalloprotease inhibitor, 100 µM) at 37 °C for 12 h before added into the reaction solution.

Peptide-based assay was carried out in 30 µL MMP-2 buffer composed by 40 mM Tris, 10 mM calcium chloride, 10 mM NaCl, pH 7.5, 0.75 µM peptide and protease (MMP-2 or thrombin). All components were mixed together except for the peptide and protease. To initiate the reaction, the peptide was added to the solution, after which the protease was added and fluorescence measurements were immediately initiated. The reactions were incubated at 37 °C and detected by fluorescence (excitation: 485/9.0 nm, emission: 500-650/9.0 nm) reading taken in a Synergy™ Mx multi-mode microplate reader.

Analysis the activities of MMP-3, MMP-8, MMP-9, and furin

For the test of MMP-3 activity, a commercial colorimetric assay kit (Catalog#72095) was used and procedures were followed according to the kit protocol. Briefly, dilute MMP substrate (1:50) in assay buffer. Then 50 nM MMP-3 was mixed with 50 µL diluted substrate and shook the plate gently for 30 sec. Then immediately start measuring absorbance reading at 412 nm continuously and record data every 10 min for 60 min.

For the test of MMP-8 and MMP-9 activities, a commercial assay kit (Catalog#27076) was used and procedures were followed according to the kit protocol. Briefly, 10 nM MMP-8 or 2.5 nM MMP-9 in a test tube were mixed with the fluorogenic substrate respectively. The reactions were incubated at 37 °C and detected immediately at the emission wavelength of 393 ± 20 nm, with excitation wavelength of 325 ± 20 nm. Data is recorded every 20 s for a total of 300 s.

The activity of furin was measured according to a method reported previously by our group (Sun et al., Chem. Commun., 2019, 55. 2218). The substrate probe (10 µM) was co-incubated with furin (0.25 U/µL) at 37 °C in 10 µL of the assay buffer (100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM CaCl$_2$, and 1 mM 2-mercaptoethanol). Then the emission spectrum was measured immediately by the fluorescence spectrophotometer with the excitation wavelength of 470 nm.$^1$

Preparation of Cell Supernatant

HepG2 and LO2 cells were seeded in 35 mm dishes with DMEM and RPMI-1640 with 10 % FBS, respectively. When the cells reached 80% confluence, they were washed 3 times with PBS and starved for 36 h in DMEM and RPMI-1640 without FBS. After the starvation, conditioned media was cleared of cells and cell debris via centrifugation (12000 rpm, 10 min) to obtain secreted MMP-2. Then the extracted total proteins were enriched by 2.5 times via freeze drying and resuspended in water saved at -20 °C before use.
Preparation of SDS-PAGE samples

For SDS-PAGE, the reactions were performed in 1 × reaction and then stopped with 5 × loading buffer. The reaction systems were heated at 100 °C for 5 m to fully denature the protein. After cooling to room temperature, the reactions were analyzed by gel-shift assay on 12% denaturing polyacrylamide gel (Bio-Rad Stain-Free gels).

Western Blot Assay

The protein samples were separated by 8% SDS-PAGE electrophoresis, and then transferred to nitrocellulose membrane by semi-dry electrophoretic transfer unit for 12 min. After blocking with 5% BSA-PBST (1× PBS with 0.1% Tween-20) solution for 1 h, the membrane reacted with primary antibody (1:1000 dilution) overnight at 4 °C and secondary antibody (1:5000 dilution) for 1 h at room temperature. After washing three times, images were obtained using odyssey clx imaging system.

Extracting MMP-2 from Clinical Osteosarcoma Tissue Samples

All the clinical samples were obtained from the Second Xiangya Hospital, Central South University (China, Hunan). Approval was obtained the local institutional review boards, and informed consents for tissue sampling were obtained from all subjects. Six tissues were collected by standard surgical procedures (see Table S2 for details). The tissues were cut into tiny pieces and homogenized by grinding with liquid nitrogen. Then the powder was transferred to EP tubes with 400 µL of working buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl$_2$, 0.05% (w/v) Brij-35, and 0.02% (w/v) NaN$_3$) supplied with 1% (v/v) Triton X-100. The homogenate was centrifuged at 12000 rpm at 4 °C for 15 min, and the supernatant was enriched via freeze drying and resuspended in water. Total protein content of homogenate was measured by Synergy™ Mx multi-mode microplate reader. Then the activities of MMP-2 were profiled by PRA as previously described.
# Tables

**Table S1** Sequence of the oligonucleotides used in this study.

| Strand | Sequence |
|--------|----------|
| P T7.0T | TAATACGACTCACTATAGGGAGGGTTGGGTTGGG |
| P T7.3T | TAATACGACTCACTATAGGGTTAGGTTGGGTTGGG |
| P T7.5T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| P T7.7T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| P T7.9T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| P T7.11T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| P T7.13T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| P T7.15T | TAATACGACTCACTATAGGGTTAGGGTTGGGTTGGG |
| T T7.0T | CCCACCCAACCCACCCTCCCTCTATAGTGAGTGCTATTA |
| T T7.3T | CCCACCCAACCCACCCTACCCCTATAGTGAGTGCTATTA |
| T T7.5T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |
| T T7.7T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |
| T T7.9T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |
| T T7.11T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |
| T T7.13T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |
| T T7.15T | CCCACCCAACCCACCTAAAAACCCCTATAGTGAGTGCTATTA |

* The blue text is the sequence of T7 promoter.

* The green text is the sequence of NG17.

* The red text is the spacer sequence.
Table S2 Detail information of clinical tissue samples.

| Patient ID | Sex | Age | Diagnosis   |
|------------|-----|-----|-------------|
| 1          | M   | 16  | osteosarcoma|
| 2          | M   | 48  | osteosarcoma|
| 3          | M   | 33  | osteosarcoma|
| 4          | F   | 17  | osteosarcoma|
| 5          | M   | 19  | osteosarcoma|
| 6          | M   | 37  | chondrosarcoma|
Figures

![Figure S1](image)

**Figure S1** Root-mean-square deviation (RMSD) plots of simulated trajectories of PR\textsubscript{MMP-2}. 


Figure S2  Vector schematic for expression vector of PR\textsubscript{MMP-2} and PR\textsubscript{Thr} used in this study.
**Figure S3** SDS-PAGE (10%) analysis of the cleavage of $\text{PR}_{\text{MMP-2}}$ by MMP-2. MMP-2, 1 µM (lane 1) or 50 nM (lanes 3 and 4); $\text{PR}_{\text{MMP-2}}$, 1.0 µM; and ARP 100, 100 µM; Bovine Serum Albumin (BSA), 1µM.

**Figure S4** CD spectra of the transcribed RNA under different conditions. $\text{PR}_{\text{MMP-2}}$, 1.0 µM; MMP-2; 100 nM; DNA template, 1.0 µM and rNTP, 10 mM. All the samples were treated with DNase I (5.0 U) to remove template DNA.
**Figure S5** Fluorescence emission spectra of PRA in the presence and absence of MMP-2 (50 nM).

**Figure S6** Background fluorescence of each components in the PRA assay. The reaction solution contains $\text{PR}_{\text{MMP-2}}$, 0.75 μM; MMP-2, 10 nM; DNA template, 1.0 μM; rNTP, 5.0 mM; and ThT, 2.0 μM.
**Figure S7** Fluorescence intensity (a) and S/B (b) of the PRA at various concentrations of PR_{MMP-2}. The reaction solution contains 1× reaction buffer (with 9 mM Mg^{2+}); MMP-2, 10 nM; rNTP, 5.0 mM; DNA template, 1.0 µM; and ThT, 2.0 µM.

**Figure S8** Fluorescence intensity (a) and S/B (b) of the PRA at various concentrations of Mg^{2+}. The reaction solution contains 1× reaction buffer (without Mg^{2+}); PR_{MMP-2}, 0.75 µM; MMP-2, 10 nM; rNTP, 5.0 mM; DNA template, 1.0 µM; and ThT, 2.0 µM.
**Figure S9** Fluorescence spectra of PRA in response to various concentrations of MMP-2. The experiments have been performed at 37 °C, in 30 µL solution of 1× reaction buffer (with 9 mM Mg²⁺); PRₘₚ₂, 0.75 µM; rNTP, 5.0 mM; DNA template, 1.0 µM; and ThT, 2.0 µM and signal detection was performed at 40 min.

**Figure S10** Fluorescence spectra of peptide-based assay in response to various concentrations of MMP-2. The experiments have been performed at 37 °C, in 30 µL solution of 1× MMP-2 buffer; peptide, 0.75 µM and signal detection was performed at 70 min.
Figure S11 (a) Time-dependent absorbance change in the assay of AnaSpec (Catalog#72095) kit with the presence of 50 nM MMP-3. Time-dependent fluorescence intensity changes in the assays of AnaSpec (Catalog#27076) kit with the presence of (b) 10 nM MMP-8 and (c) 2.5 nM MMP-9.

Figure S12 Specificity of PRA for MMP-2 detection. The experiments have been performed at 37 °C, in 30 µL solution of 1× reaction buffer; PMMP-2, 0.75 μM; rNTP, 5.0 mM; DNA template, 1.0 μM; and ThT, 2.0 μM and different biological samples. MMP-2, 10 nM; MMP-3, 50 nM; MMP-8, 50 nM; MMP-9, 50 nM; ARP 100, 100 µM; GM 6001, 100 µM.
Figure S13 Sequences of the flexible linkers containing the substrate sequence of target protease used for PR\textsubscript{MMP-2} and PR\textsubscript{Thr}, respectively.

| Enzyme   | Substrate peptide                  | Length |
|----------|-----------------------------------|--------|
| MMP-2    | GSGPLGVRGKL                       | 19.1A  |
| Thrombin | GSGGGASGGAGLVPVRCS AGGSAGSGAGKL  | 26.0A  |

Figure S14 Time-dependent fluorescence ratio changes of $F_{\text{GFP}}/F_{\text{TAMRA}}$ in the presence of 0.25 U/μL furin. The substrate of furin is GFP-RDHMVLLHESVNAAGITGGSGGSRVRR|SVK-TAMRA, 10 μM.
Figure S15 Determination of the PRA specificity for thrombin detection. The experiments have been performed at 37 °C, in 30 µL solution of 1× reaction buffer; PRThr, 0.75 µM; rNTP, 5.0 mM; DNA template, 1.0 µM; and ThT, 2.0 µM and different biological samples (thrombin, 10 nM; MMP-2, 50 nM; MMP-3, 50 nM; MMP-8, 50 nM; MMP-9, 50 nM; furin, 50 nM).

Figure S16 Detection of MMP-2 in cell culture secretomes of LO2 and HepG2 cells using the peptide-based assay. Peptide, 0.75 µM and signal detection was performed at 70 min.
Figure S17 MMP-2 in the extraction of the osteosarcoma (OS) tissues and paracancerous tissues determined by western blotting.

**Figure S18** Normalized fluorescent signals of samples extracted from OS tissues with and without pretreatment of ARP 100 (100 μM). Fluorescence signal detection in PRA was performed at 70 min. The concentration of total protein is 10 mg/mL.
Amino acid of PR<sub>MMP-2</sub>

T7 Lysozyme-linker-T7 RNAP

MARVQFKQRESTDAIFVHSATKPSQNVGREIRQWHKEQGWLDVGYHFIIKRDTGVEAGRDEMAVGSHAKGYNHINSIGCVLGVGIDDKGKFANFTPAQMQSLRSLLVTTLAKYEGAVLRHVEHAVPKASPSDLKRWKEKVELVTSRGSGGPLGVRGKLMNTINIAKNDFSIAELAPFNTLADHYGRELAREQLALEHESYEMGEARKFMRQFLKAGEVADNAAAKPLITTLPKMIARINDFEEVKAARKRGKPTAFQFLQIEKPEAVAYITIKTTLACLTSAWNNTQAVASAIGRAIEDEARFGRIDLEAKHFKNVEQLNKVRGHYKKAFCMVQVVEDAMLKSLGGGEAWSSWHKESDHIVGHRVCIEMLIESTGMVSLHRQNMARVQDSTELAPEYAEAAIATRAGALAGISPMFQCVVPKPTGVITGGYWANGRRLPLAVRTHSSKAKLMRYEDVYMPEYVKAINTAWKINKVLAVANVITKWKCPCPEDAIREELMKPEDIMNPALTAIAAAAYVRDKARKRSSRISLEFLMEQANKFANHKAIFPWYVNMDWRGVRVYAVSMFNQGDNTMKGLTLLLAKGKGKEGYYWLKIHGANCAGKDVPFPERPFRKFIEHENIMACAKSPLENTWWAEPQSFCSFCSQIQHSFSAMLRDVEGRVANLPSQDMRDFA

Amino acid of PR<sub>Thr</sub>

T7 Lysozyme-linker-T7 RNAP

MARVQFKQRESTDAIFVHSATKPSQNVGREIRQWHKEQGWLDVGYHFIIKRDTGVEAGRDEMAVGSHAKGYNHINSIGCVLGVGIDDKGKFANFTPAQMQSLRSLLVTTLAKYEGAVLRHVEHAVPKASPSDLKRWKEKVELVTSRGSGGPLGVRGKLMNTINIAKNDFSIAELAPFNTLADHYGRELAREQLALEHESYEMGEARKFMRQFLKAGEVADNAAAKPLITTLPKMIARINDFEEVKAARKRGKPTAFQFLQIEKPEAVAYITIKTTLACLTSAWNNTQAVASAIGRAIEDEARFGRIDLEAKHFKNVEQLNKVRGHYKKAFCMVQVVEDAMLKSLGGGEAWSSWHKESDHIVGHRVCIEMLIESTGMVSLHRQNMARVQDSTELAPEYAEAAIATRAGALAGISPMFQCVVPKPTGVITGGYWANGRRLPLAVRTHSSKAKLMRYEDVYMPEYVKAINTAWKINKVLAVANVITKWKCPCPEDAIREELMKPEDIMNPALTAIAAAAYVRDKARKRSSRISLEFLMEQANKFANHKAIFPWYVNMDWRGVRVYAVSMFNQGDNTMKGLTLLLAKGKGKEGYYWLKIHGANCAGKDVPFPERPFRKFIEHENIMACAKSPLENTWWAEPQSFCSFCSQIQHSFSAMLRDVEGRVANLPSQDMRDFA