



The intranuclear PEX domain of MMP involves proliferation, migration, and metastasis of aggressive adenocarcinoma cells

Journal:	<i>Journal of Cellular Biochemistry</i>
Manuscript ID	JCB-17-1144.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
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Keywords:	nuclear MMP, PEX domain, non-proteolytic MMP, cancer metastasis, tumor stroma

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5 **The intranuclear PEX domain of MMP involves proliferation,**
6 **migration, and metastasis of aggressive adenocarcinoma cells**
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45 **Running title:** PEX domain of MMPs involves cancer metastasis
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50 **Keywords:** nuclear MMP, PEX domain, non-proteolytic MMP, cancer metastasis, tumor
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1
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5 Number of figures: 7
6

7 Number of tables: 2
8

9 Number of supplemental items: 3
10

11 Number of references: 25
12

13 Word count: 5220 (introduction to discussion)
14
15
16
17

18 **FUNDING.** This work was supported by JSPS KAKENHI, grant numbers JP17K17895 (to
19 Y.O.), JP17K11642 (to T.E.), JP26293067 (to K.K.), JP26670815 (to K.K.), Start-up Grant for
20 Young Scientists at Okayama Univ. (to Y.O.), Molecular Imaging Micro Dose Phase 0 Grant at
21 Okayama Univ. (to Y.O. and K.K.), Gender Equality Grant at Okayama Univ. (to Y.O.), and a
22 Ryobi Teien Memorial Foundation Grant (to T.E.).
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30 **Abbreviations:** PEX, hemopexin-like repeat; MMP, matrix metalloproteinase; RNAi, RNA
31 interference; siRNA, small interfering RNA; HSP, heat shock protein; CTGF, connective tissue
32 growth factor; IHC, Immunohistochemistry.
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Abstract (< 150 words)

Members of matrix metalloproteinase (MMP) family promote cancer cell migration, invasion, and metastasis through alteration of the tumor milieu, intracellular signaling pathways, transcription. We examined gene expression signatures of colon adenocarcinoma cell lines with different metastatic potentials and found that rapidly metastatic cells powerfully expressed genes encoding MMP3 and MMP9. The non-proteolytic PEX isoform and proteolytic isoforms of MMPs were significantly expressed in the metastatic cells *in vitro*. Knockdown of MMP3 attenuated cancer cell migration and invasion *in vitro* and lung metastasis *in vivo*. Profound nuclear localization of MMP3 / PEX was found in tumor-stroma marginal area. In contrast, MMP9 was localized in central area of subcutaneous tumors. Overexpression of the PEX isoform of MMP3 promoted proliferation and migration of the rapidly metastatic cells *in vitro*. Taken together, the non-proteolytic PEX isoform of MMPs locating in cell nuclei involves proliferation, migration, and subsequent metastasis of aggressive adenocarcinoma cells.

INTRODUCTION

Metastasis is a critical phenomenon in cancer. Over time, malignant cancer cells undergo clonal evolution, which promotes their diversity and heterogeneity, as well as therapy resistance [Greaves and Maley, 2012; Kreso and Dick, 2014; Nowell, 1976]. In addition, it has been proposed that highly evolved clones of cancer cells are resistant to selective pressure by the immune, chemo- and radiation therapies. Although previously considered to be mutually exclusive models, it is recently considered that genetic evolution and cancer stem cell models can be harmonized in three related biological fields; tumor genetics, epigenetics and pathways, and microenvironment [Kreso and Dick, 2014]. Matrix metalloproteinases (MMPs) represent the most prominent family of proteinases associated with tumorigenesis and regulators of the tumor microenvironment [Kessenbrock et al., 2013; Kessenbrock et al., 2010]. MMPs have also been reported to be potent biomarkers of tumor progression as well as one of the causal factors that promote multiple processes of tumorigenesis, including oxidative stress-dependent DNA damage and chromosomal instability, epithelial-to-mesenchymal transition (EMT) [Radisky et al., 2005], migration and invasion of cancer cells [Sato H et al., 1994], angiogenesis, and metastasis [Kessenbrock et al., 2013; Kessenbrock et al., 2010; Vandenbroucke and Libert, 2014]. Canonical proteolytic roles for MMPs is to cleave substrate proteins at extracellular space. Proteolysis of extracellular matrix (ECM) and intercellular adhesion molecules by MMPs enable cells to migrate and invade and to release cytokines, chemokines, and growth factors that activate their receptors and intracellular signaling pathways. In addition to those indirect activations of the extracellular factors, MMPs also directly alter activities of growth factors, cytokines, and chemokines by proteolysis. For example, MMPs are able to cleave connective tissue growth factor (CTGF/CCN2) and then release VEGF leading to angiogenesis [Hashimoto et al., 2002]. Another study showed that MMPs and a disintegrin and metalloproteinases cleave

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5 membrane-bound heparin-binding EGF-like growth factor (HB-EGF) and release soluble
6 HB-EGF, which binds to cells and stimulates EGFR/ERBB signaling [Prenzel et al., 1999].
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10 Intracellular and nuclear roles for MMPs have been recently discovered. We showed
11 that MMP3 possesses nuclear localization signals and can translocate into cellular nuclei, in
12 which MMP3 can bind to chromatin proteins and DNA leading to transcriptional regulation of
13 *CTGF/CCN2* gene [Eguchi et al., 2008]. MMP3 is composed of a protease domain, a hinge
14 region, and a C-terminal hemopexin-like repeat (PEX) domain, which is non-proteolytic (see
15 later figure). We recently showed that a PEX isoform of MMP3 activate some members of heat
16 shock protein (HSP) genes [Eguchi et al., 2017], which can contribute to anti-apoptosis and
17 drug resistance. MMP3 can interact with heterochromatin proteins, members of the chromobox
18 protein family that involve transcriptional and chromosomal control [Eguchi et al., 2017;
19 Eguchi et al., 2008; Eguchi et al., 2010]. MMPs also involve oxidative stress, DNA damage, and
20 chromosome instability in cell nuclei. Intranuclear MMP2 and MMP9 activities have been
21 shown to cleave PARP-1 and XRCC1, nuclear matrix proteins, thus promoting oxidative DNA
22 damage and apoptosis in an ischemic injury model [Yang et al., 2010]. MMP3-induced EMT
23 and genomic instability have been shown to be mediated by small GTPase Rac1b and a reactive
24 oxygen species in breast adenocarcinoma and pancreatic cancer [Mehner et al., 2014; Radisky et
25 al., 2005], indicating potent roles for MMPs in proteotoxic and genotoxic stress.
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44 MMPs appear to be appropriate target molecules in treatments of aggressive types
45 of cancers. Although more than 50 MMP inhibitors have been investigated in clinical trials for
46 various cancers, all of those trials failed [Vandenbroucke and Libert, 2014]. However, the
47 involvement of intracellular and non-proteolytic roles for MMPs in cancer have not
48 well-investigated yet.
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54 In the present study, we first investigated gene expression of MMPs correlated with
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5 metastatic phenotype of colon cancer cells. We then assessed nuclear localization of the PEX
6 domain of MMP3 and MMP9 in primary and lung-metastatic tumors and their roles in
7 proliferation, migration, invasion and metastasis of the aggressive cancer cells.
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11 12 13 14 **MATERIALS AND METHODS**

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16 **Cells.** Colon26 (also known as colon26), LuM1, and NM11 cells were maintained in RPMI1640
17 with 10% FBS supplemented with penicillin, streptomycin, and amphotericin B [Hyuga S et al.,
18 1994; Sakata K et al., 1996; Tsuruo et al., 1983].
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24 **Comprehensive gene expression analysis and bioinformatics.** Cells were cultured for 3 days
25 and total RNA was extracted from cultured cells using the AGPC method with Trizol
26 (Molecular Research Center, Cincinnati, OH). Concentration and quality (A260/A280) of total
27 RNA were determined. cDNA was synthesized from 0.1 µg of total RNA using a Low Input
28 Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA), then hybridized to probes of
29 a SurePrint G3 Mouse GE 8x60 K v.2 Microarray system (Agilent Technologies, Santa Clara,
30 CA). Gene expression and ratios were analyzed using MeV 4.0 software
31 (<http://www.Tm4.org/mev.html>). Functional annotation clustering and pathway analysis were
32 performed with LuM1 genes expressed at a 10-fold or higher level as compared to the NM11
33 and Colon26 cells ($p < 0.05$) using DAVID Bioinformatics Resources 6.8. Relative expression
34 levels in LuM1 or NM11 to the levels in Colon26 were shown in heat maps and bar graphs.
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50 **RNAi.** Cells were transfected with small interfering RNA (siRNA) targeting mRNA of *Mmp3* or
51 *Mmp9* (ON-TARGETplus SMARTpool siRNA; Thermo Fisher Scientific, Rockford, IL), or
52 non-targeting double strand RNA (dsRNA) (siGENOME Control Pool Non-Targeting siRNA) at
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5 a final concentration of 50 nM using DharmaFECT 1 (Thermo Fisher Scientific, Rockford, IL).
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7 Cells were cultured for 48 hours before RT-qPCR, Western blot, migration assay, invasion assay
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9 and subcutaneous injection and for 72 hours before zymography. For analysis of culture
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11 supernatant, cells were cultured for 48 hours in serum-containing medium and subsequently 24
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13 hours in serum-free medium. To examine mRNA levels in tumors, total RNA was extracted
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15 from subcutaneous tumors on the day 10 after subcutaneous injection. Lung metastasis assay
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17 and immunohistochemistry (IHC) were performed on the day 20 after subcutaneous injection as
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19 depicted in Fig. S2A.
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24 **Real-time RT-qPCR.** Total RNA preparation and RT-qPCR was carried out as described
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26 previously [Eguchi et al., 2017; Eguchi et al., 2008]. The miRNeasy mini kit (Qiagen) was used
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28 with DNase (Qiagen). Total RNA concentration was measured by using a microspectrometer
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30 K2800 (Beijing Kaiiao, Beijing, China). cDNA synthesis was carried out by using iScript™
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32 cDNA Synthesis Kit (Bio-Rad). Specific primer pairs for *Mmp3*, *Mmp9*, *Actb*, and *Gapdh* were
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34 used as follows: mMMP3Fw, 5'-ACC AAC CTA TTC CTG GTT GCT GCT and mMMP3Rv,
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36 5'-ATG GAA ACG GGA CAA GTC TGT GGA [Ma et al., 2009]; mMMP9Fw, 5'-CAG CCG
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38 ACT TTT GTG GTC TT and mMMP9Rv, 5'-GCT TCT CTC CCA TCA TCT GG [Sousa et al.,
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40 2014]; mGapdhFw, 5'-ACC ACA GTC CAT GCC ATC AC and mGapdhRv, 5'-TCC ACC ACC
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42 CTG TTG CTG TA [You et al., 2012]; mActb-Fw, 5'-AAC GAG CGG TTC CGA TG and
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44 mActb-Rv, 5'-GGA TTC CAT ACC CAA GAA GGA [Sousa et al., 2014]. Realtime PCR was
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46 carried out using iQ SYBR Green PCR mixture (Bio-Rad). Relative mRNA levels to *Actb* or
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48 *Gapdh* mRNA levels were quantified by the $\Delta\Delta C_t$ method using the formula as follows: fold
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50 change = $2^{-\Delta\Delta C_t}$. PCR was carried out in triplicate and mean values were calculated with the
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5 mean \pm S.D. of biological triplicates.
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10 **Migration and invasion assays.** Migration and invasion assays were performed as previously
11 described [Sakata K et al., 1996]. Uncoated and Matrigel-coated culture systems (Becton
12 Dickinson) were used for *in vitro* migration and invasion assays, respectively. For knockdown
13 experiments, cells were transfected with siRNA and then cultured for 48 hours before re-seeding
14 to upper chambers. Cells were seeded at concentrations of 5×10^4 per an upper chamber of a
15 Transwell® 24-well (Corning) for the migration assay and of 2.5×10^4 per an upper chamber of
16 a Transwell® 24-well for the invasion assay. Cells transfected with PEX-overexpressing or
17 control plasmids were seeded at concentrations of 5×10^4 per an upper chamber of a Transwell®
18 24-well for the migration assay and of 1.6×10^4 per an upper chamber of a Transwell® 24-well
19 for invasion assay. Cells that migrated or invaded through the pores to the lower surface of filter
20 were fixed, stained using Diff-Quick stain (Sysmex) and counted after 6 hours of migration
21 period or 19 hours of invasion period for knockdown experiments, and after 0, 12 and 24 hours
22 for comparison of different cell lines. To evaluate the effects of the transfection, percentages of
23 migrated and invaded cells relative to the control were calculated.
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41 **Cell proliferation.** Cells were transfected with siRNA or plasmid DNA and then seeded at a
42 concentration of 3.8×10^3 cells / well in a 96-well plate on the day 2 after transfection. Number
43 of cells at 1 to 4 days post-transfection period were counted by using Countess automated cell
44 counter (Invitrogen). Flويد® cell imaging station (ThermoFisher Scientific) was used for
45 photomicrography.
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54 **Allogeneic transplantation.** All animal experiments were performed according to the
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5 guidelines for care and use of laboratory animals approved by Okayama University and the
6 Japanese Pharmacological Society. Subcutaneous allogeneic transplantation was performed as
7 previously described [Sakata K et al., 1996], with 5.0×10^5 cells transplanted subcutaneously
8 into a side abdominal wall of each 6 to 7-week-old BALB/c mouse. Twenty days after
9 transplantation, subcutaneous tumors and lungs were resected, then fixed with Bouin's fluid.
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11 Nodules larger than 0.5-mm-diameter were counted.
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20 **Casein and gelatin zymography.** For zymography of non-transfected cells, cells were
21 inoculated at a concentration of 2×10^6 cells into 60-mm culture dishes in 4 ml of RPMI1640
22 containing 10% FBS and cultured overnight, then incubated in serum-free medium for 24 hours.
23 Cell culture supernatants were concentrated 20 times using an Amicon filtration tube (for MW
24 10,000). Cells were washed with PBS, collected using a cell scraper, and lysed in RIPA buffer
25 containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), then further lysed by
26 homogenization (25-gauge needle, 10 strokes) and incubated for 30 minutes on ice. Lysates
27 were centrifuged at $12,000 \times g$ for 20 minutes to remove debris and supernatants were used as
28 cell lysates. Twenty microgram of cell lysates or $4 \mu\text{g}$ of cell culture supernatants were mixed
29 with an SDS sample buffer and CaCl_2 (5 mM final concentration), then applied to 10%
30 acrylamide gel containing copolymerized 0.1% gelatin (Cosmo Bio, Tokyo, Japan). For
31 zymography with knockdown experiments, cells were cultured in 6-well plates. At 48 hours
32 after transfection, medium was replaced with serum-free medium and cells were cultured for 24
33 hours, then the supernatants were ultra-filtrated and concentrated as described above. Following
34 a protein assay, $9 \mu\text{g}$ of the $>10\text{-kDa}$ cell culture supernatant was mixed with a 2x sample buffer
35 (10 μl) and 1 μl of 50 mM CaCl_2 , then separated on 10% acrylamide gels containing
36 copolymerized 0.1% gelatin or 0.5% casein (CosmoBio, Tokyo, Japan). The gels were washed
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5 with wash buffer for 30 minutes at RT and incubated for 40 hours in enzyme reaction buffer at
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7 37°C. Coomassie Brilliant Blue staining was performed and photographs of the gels were
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9 obtained, then quantitative densitometric analysis was performed using Image J.
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14 **Western blotting analysis.** Cells were seeded into 6-well plates and transfected with siRNA
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16 using the procedure described above, then lysed in RIPA buffer as described above. Next, 30- μ g
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18 protein samples were separated by SDS-PAGE on 10% poly-acrylamide gels and transferred to
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20 PVDF membranes. The membranes were blocked in Tris-buffered saline containing 0.05%
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22 Tween 20, 2.5% skim milk, and 2.5% BSA for 30 minutes at room temperature (RT). Each
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24 membrane was incubated with the primary antibody (1/1000) overnight at 4°C and subsequently
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26 incubated with horseradish peroxidase (HRP)-conjugate secondary antibodies against anti-rabbit
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28 IgG (GE Healthcare) (1/2000) for 1 hour at RT in the blocking solution. Blots were visualized
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30 with a Clarity Western ECL Substrate (Bio-Rad).
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35 **Antibodies.** Rabbit monoclonal anti-PEX domain MMP3 antibody (EP1186Y, ab52915) and a
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37 rabbit polyclonal anti-MMP9 antibody (ab38898, full-length protein corresponding to mouse
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39 MMP9 was immunized) were purchased from Abcam.
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44 **Immunohistochemistry.** Excised tumor tissues were immediately fixed in Bouin's fluid, then
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46 dehydrated by passage through an ethanol series and embedded in paraffin. Samples were cut
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48 into 4- μ m serial sections, which were then placed onto saline-coated slides. Deparaffinized
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50 sections were treated with methanol containing 0.3% H₂O₂ for 30 minutes to block endogenous
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52 peroxidase activity. Sections were then autoclave heated in 10 mM of citrate buffer solution (pH
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54 6.0) for 10 minutes at 120°C for antigen retrieval. Blocking was performed using a Dako Chem
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6 Mate Envision Kit/HRP. Sections were incubated with the anti-MMP3 antibody (ab52915;
7 1/100) overnight at 4°C and subsequently with a secondary antibody against anti-rabbit IgG
8 HRP-conjugated (K400211-2, EnVision+ Single Reagents, Dako) for 1 hour at 4°C. IHC
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10 reaction products were visualized by use of a diaminobenzidine chromogen substrate. Samples
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12 were then counterstained with hematoxylin and observed with a BZ-X700 (Keyence, Osaka,
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14 Japan). For a negative control, sections were incubated with omission of the primary antibody
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16 under the same protocol.
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22 **Immunofluorescence and confocal microscopy.** For immunofluorescence staining, excised
23 tumor tissues were immediately fixed in Bouin's fluid, then dehydrated by passage through an
24 ethanol series and embedded in paraffin. Samples were cut into 4- μ m serial sections, which
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26 were then placed onto saline-coated slides. Blocking was performed using a Dako Chem Mate
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28 Envision Kit. Sections were incubated with the anti-MMP3 antibody (ab52915; 1/100) and the
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30 anti-MMP9 antibody (ab38838; 1/500) overnight at 4°C and subsequently with a secondary
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32 antibody against anti-rabbit IgG (ab150076; 1/5000) for 1 hour at 4°C. For a negative control,
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34 sections were incubated with omission of the primary antibody under the same protocol. After
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36 three washes with TBS, the mounting and DNA staining were performed by using ProLong gold
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38 AntiFade reagents with 4',6-diamino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen).
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40 Fluorescence images were taken using a confocal laser scanning microscopy LSM780 (Carl
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42 Zeiss). We defined four areas of a subcutaneous tumor as follows: the stromal as an outside of
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44 the tumor margin, the marginal as an area 0 to 30 μ m apart from the tumor margin, the interior
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46 as an area 30 to 80 μ m apart from the outside of the marginal area. According to this definition,
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48 MMP3/9 and/or DAPI positive cells in each area were counted using Image J. The nuclear
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50 MMP positive rate was calculated as follows: MMP3/9 and DAPI double positive nuclei per
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5 DAPI positive nuclei.
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10 **Plasmid constructs and transfection.** pFlag3-PEX-myc and pFlag3-myc were described
11 previously [Eguchi et al., 2018]. These plasmid constructs were transfected using NEPA21
12 electroporator (NEPA Gene, Ichikawa, Japan). To optimize transfection condition and efficiency
13 for LuM1, ten different conditions of electroporation was tested. An optimized electroporation
14 condition with 150V and 5 ms pulse twice was then used for plasmid transfection.
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22 **Statistical analysis.** Statistical significance was calculated using Microsoft Excel. Three or
23 more mean values were compared using one way analysis of variance (ANOVA), while
24 comparisons of 2 were done with an unpaired Student's *t*-test. $p < 0.05$ was considered to
25 indicate statistical significance.
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31 32 33 **RESULTS**

34 35 **Gene expression signatures and gene screening of high- and low-metastatic cancer cell** 36 **lines.** 37

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39 We first examined gene expression signatures of colon adenocarcinoma cell lines with different
40 metastatic potentials in high-metastatic LuM1, low-metastatic NM11 and the parental Colon26
41 cells. To characterize these cell types with different invasive and metastatic potentials at gene
42 expression levels, microarray analysis was carried out. (Raw data was submitted to the Gene
43 Expression Omnibus (GEO) database repository; accession ID: GSE97166; Colon26,
44 GSM2553008; LuM1, GSM2553009; NM11, GSM2553010.) Among the 62,976 genes tested,
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479 genes were strongly expressed at a greater than 10-fold higher level in the metastatic LuM1
as compared to the low-metastatic lines (Fig. 1A; Fig. S1). Functional annotation clustering and

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5 pathway analysis then revealed that the gene expression signature of LuM1 involved particular
6 biological events in the extracellular microenvironment, cell adhesion, immunology, and protein
7 interactions (Table 1, 2). In addition, functional classification of those LuM1-specific genes
8 indicated that these 104 genes specifically expressed in LuM1 involved cancer cell invasion,
9 EMT, and metastasis (Fig. 1B). These results indicated that more than a hundred
10 metastasis-related genes involved metastatic, invasive and transforming potential of the LuM1
11 cells. Among the 104 metastasis-related genes expressed specifically in LuM1 cells, several
12 members of *Mmp* gene family, were profoundly expressed. We therefore examined *Mmp*
13 expression signatures in the LuM1, NM11, and Colon26 cells. Of note, the gene expression
14 level of *Mmp3* in the metastatic LuM1 was the highest among all *Mmp* gene family members
15 and higher than that in Colon26 and NM11 (2400.9-fold and 4.6-fold, respectively, Fig. 1C, D;
16 Fig. S1C) . *Mmp9* was also expressed in the LuM1 at a high level, as compared to the
17 low-metastatic Colon26 and NM11 (512.1-fold and 237.8-fold, respectively, Fig.1D).

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33 These results indicate that *Mmp3* and *Mmp9* were profoundly co-expressed in the
34 metastatic adenocarcinoma cells.
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39 **Targeted knockdown of MMP3 attenuates proliferation, migration and invasion of the** 40 **metastatic adenocarcinoma cells.** 41

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43 We next examined migration and invasion activities of metastatic LuM1 cells and
44 low-metastatic NM11 cells, as well as of their parental low-metastatic adenocarcinoma cell line
45 Colon26. LuM1 showed the highest levels of migration and invasion as compared to the other
46 low metastatic lines (Fig. 2A-C). We then examined siRNA-mediated knockdown of MMP3 and
47 MMP9. Both MMP3 and MMP9 at mRNA levels were specifically and efficiently reduced by
48 their targeted siRNA (Fig. 2 D, E). (Later figures show knockdown at protein levels). Yet the
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MMP3 siRNA increased the MMP9 mRNA level (Fig. 2E) and, in contrast, the MMP9 siRNA increased the MMP3 mRNA level (Fig. 2D), suggesting compensatory expression system of these essential MMPs. (Some mutual compensation was also seen in Western blotting and zymography as shown in later figures).

We next examined roles of MMP3 and MMP9 in regard to proliferation of LuM1 cells. The MMP3 siRNA (50 nM) tended to attenuate proliferation of the LuM1 cells whereas the MMP9 siRNA (50 nM) and MMP9/MMP3 siRNA mixture (each 25nM) did not (Fig. 2F, Fig. S2B). The MMP3 siRNA and MMP3/MMP9 siRNA attenuated the migration of LuM1 cells to 46.9% and 51.3%, respectively, of the level of the control (Fig. 2G), and attenuated invasion to 71.0% and 59.7%, respectively (Fig. 2H). The MMP9 siRNA attenuated migration of the LuM1 to 69.6%, which was a lesser effect as compared to MMP3-targeting, and did not alter invasion (Fig. 2G, H).

These results indicate that MMP3 is a key protein in the proliferation, migration, and invasion of the aggressive adenocarcinoma cells.

Targeted reduction of MMP3 and MMP9 attenuates primary and lung-metastatic tumorigenesis of the aggressive adenocarcinoma cells.

We next examined whether knockdown of MMP3 and MMP9 could alter primary tumor development and lung metastasis of the LuM1 cells in the allogeneic transplantation experiments. As pilot studies, we tested sustainability of siRNA-mediated knockdown as follows. The RNAi effect on MMP9 was sustained for 96 hours after transfection to LuM1 cells, as shown by gelatin zymography (Fig. S2C). Of note, RNAi effects on *Mmp3* and *Mmp9* at mRNA levels were sustained in tumors *in vivo* even at day 10 after subcutaneous injection (Fig. S2D). Using this knockdown system, we examined whether MMP3- and MMP9-targeting

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6 siRNA alter tumor development and metastasis. The weights of primary tumors were reduced by
7 the MMP3 siRNA and the MMP9 siRNA on day 20 after the transplantation, as compared to the
8 control (Fig. 3A, B).
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11 The number of metastatic nodules in lungs were significantly reduced by the MMP3
12 siRNA, the MMP9 siRNA and the MMP3/MMP9 siRNA, as compared to the control (Fig. 3C,
13 D). The weights of lungs bearing the metastatic nodules were significantly reduced by the
14 MMP3 siRNA, the MMP9 siRNA and the MMP3/MMP9 siRNA, as compared to the control
15 (Fig. 3E).
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22 These results suggest that targeted knockdown of MMP3 and MMP9 reduce primary
23 tumor development and subsequent metastasis of aggressive adenocarcinoma cells.
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29 **The intracellular non-proteolytic PEX domain of MMP3 and the short MMP9 were**
30 **expressed in the metastatic adenocarcinoma cells.**
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32 We next examined expression and proteolytic activities of several isoforms of the MMPs
33 produced from the metastatic LuM1 cells, and examined siRNA-mediated knockdown of these
34 *in vitro*, based on that MMPs had been historically demonstrated as extracellular proteases,
35 whereas non-proteolytic and intracellular roles for MMPs have been recently shown [Eguchi et
36 al., 2017; Eguchi et al., 2008]. Therefore, prior to detection, we organized structures of MMP3
37 and MMP9 (Fig. 4A, B). We examined isoforms of MMP3 produced in the LuM1 cells at by
38 performing siRNA-mediated knockdown and subsequent Western blotting analysis and
39 zymography. (Full images of Western blot and zymography were shown in supplemental Fig.
40 S3.) The anti-PEX domain MMP3 antibody detected both full-length 54-kDa (arrow) and short
41 25-kDa PEX domain (arrowhead) of MMP3 (Fig. 4C). The MMP9 siRNA increased the PEX of
42 MMP3 and decreased full-length MMP3 (Fig. 4C, compare lane 1 and lane 3), indicating
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5 crosstalk between MMP3 and MMP9. We next examined MMP3 proteolytic activity in the
6 culture supernatant of the LuM1 cells in casein zymography. The 54-kDa and 100-kDa
7 proteinases lysed casein, and these were thought to be proteolytic MMP3 monomer and dimer,
8 respectively (Fig. 4 D, control si). The proteolytic MMP3 monomer was reduced by the MMP3
9 siRNA (arrow), whereas the proteolytic putative MMP3 dimer was not reduced by the MMP3
10 siRNA (arrow), whereas the proteolytic putative MMP3 dimer was not reduced by the MMP3
11 siRNA, but reduced by MMP3/MMP9 siRNA (arrowhead) (Fig. 4D), indicating crosstalk
12 between MMP3 and MMP9. As expected, the non-proteolytic 25-kDa PEX domain was not
13 detected in the casein zymography.
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22 We next examined isoforms of MMP9 produced from the LuM1 cells. Intracellular
23 gelatinase activities of approx. 90-kDa and 250-kDa proteinases were significantly found in
24 both lysate (Fig. 4E) and culture supernatant (Fig. 4F) of the metastatic LuM1 cells, but at much
25 lower levels in the other low-metastatic cell lines. The 90-kDa gelatinase was thought to be
26 MMP9 and the 250-kDa gelatinase was thought to be oligomers of MMP9.
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33 We next examined knockdown of the intracellular and extracellular MMP9 produced
34 by LuM1 cells. The anti-MMP9 antibody detected full-length (approx. 90-kDa, arrow),
35 processed forms (64- and 67-kDa, arrowhead), and putative oligomer (250-kDa, asterisk) of
36 MMP9 in the lysate of LuM1 cells (Fig. 4G). Gelatinase activities of the 90-kDa MMP9 in the
37 cell culture supernatant of LuM1 cells treated with the MMP9 siRNA was lower than that
38 treated with the control siRNA or the MMP3 siRNA (Fig. 4H).
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46 These results indicate that the non-proteolytic PEX domain of MMP3 and the short
47 MMP9 were intracellularly produced in the metastatic adenocarcinoma cells. The siRNAs
48 targeting MMP3/9 successfully reduced proteolytic and non-proteolytic intracellular and
49 extracellular isoforms of MMPs.
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6 **Expression and nuclear localization of MMP3 in primary tumors and in metastatic lungs**
7 **in allogeneic transplants.**
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10 MMP3 was initially found as the first member of stromelysin, which lyse ECMs in stroma.
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12 Later studies have shown intranuclear localization and roles of MMP3 [Eguchi et al., 2017;
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14 Eguchi et al., 2008]. In the present study, *Mmp3* was notably expressed in the metastatic LuM1
15
16 *in vitro*. We next examined expression and subtumoral and subcellular localization of MMP3 in
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18 primary tumors and in lungs with metastatic tumors in an allogeneic transplant model. As it
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20 have been shown that the PEX domain of MMP3 has an essential role in transcriptional
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22 regulation [Eguchi et al., 2017], we used an anti-PEX MMP3 antibody in
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24 immunohistochemistry. MMP3 was expressed at relatively high levels at marginal zone of
25
26 tumor parenchyma (P) to stroma (S) in primary tumors, but at lower levels in central area of the
27
28 parenchyma (Fig. 5A, B). Notably, intranuclear MMP3 was found in both marginal
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30 parenchymal cells (arrowheads) and stromal cancer-associated fibroblast (CAF)-like cells
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32 (arrow) (Fig. 5C).
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36 We next examined subtumoral and subcellular localization of MMP3 in the metastatic
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38 lungs in the same model. MMP3-positive cells were found throughout the metastatic tumor
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40 nodules and in pulmonary alveolar locations (Fig. 5E). MMP3 was found inside nuclei (arrow)
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42 and the nuclear margins (arrowhead) of cells with nuclear polyploidy in metastatic tumors in the
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44 lung (Fig. 5F).
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47 These results indicate that extra- and intracellular MMP3 involve CAF-related
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49 invasion, nuclear polyploidy, and metastasis of the aggressive adenocarcinoma cells.
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52 **Nuclear PEX domain / MMP3 localized in tumor-stroma marginal area and MMP9**
53 **localized in central area of tumors.**
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5 To more precisely investigate the nuclear localization of MMP3/9 in tumors, we next used
6 immunofluorescence and confocal laser scanning microscopy and examined nuclear MMP3/9
7
8 positive rate in each tumor area. We defined four areas of a tumor as follows: central (Fig. 6A,
9 left), interior, marginal and stromal areas (Fig. 6A, right). The rate of cells with nuclear MMP3
10 was most significant in the stromal area (17.6 %) and next in the tumor marginal area (9.4 %) as
11 compared to those in the interior (0.80 %) and central areas (0.73%) with the lower rate (Fig. 6B
12 - D). On the other hand, nuclear MMP9 positive cells were found notably in the central area
13 (2.5 %) as compared to the other areas (Fig. 6B, D).
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22 We next assessed localization of MMP3 and MMP9 in a lung lesion with metastatic
23 tumors. Tumor cells with nuclear MMP3/9 appeared to scatter throughout the metastatic cancer
24 nodule in the lung and in pulmonary alveolar locations (Fig. 6E), consisting with IHC data
25 shown in Fig. 5.
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30 Of note, DNA stained with DAPI appeared to be dot-like condensation in cell
31 nuclei in both primary and metastatic tumors (Fig. 6C-E), indicating chromosomal condensation
32 involving gene regulation. MMP3 and MMP9 appeared to co-localize with DNA (Fig. 6C-E,
33 arrows), suggesting DNA-binding and gene regulation. MMP3 and MMP9 appeared to localize
34 out of nuclei- cytoplasm or extracellular region as well.
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41 These results indicate that nuclear PEX domain / MMP3 localized in primary
42 tumor-stroma marginal area and lung-metastatic tumor nodules. Nuclear MMP9 localized in
43 central area of primary tumors and in lung-metastatic tumor nodules.
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50 **Overexpression of the PEX-domain promotes proliferation and migration of the metastatic**
51 **cancer cells.**
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53 We next asked whether the non-proteolytic PEX domain could alter proliferation, migration,
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5 and invasion of the aggressively metastatic cancer cells. Overexpression of the PEX of MMP3
6 significantly increased proliferation of LuM1 cells (Fig. 7A). Of note, overexpression of the
7 PEX increased migration of LuM1 cells to 204% level of the control (Fig. 7B). Overexpression
8 of the PEX tended to increase invasion of LuM1 cells to 127.1% level of the control (Fig. 7C),
9 suggesting that protease activities of MMPs might promote invasion rather than the
10 non-proteolytic PEX.
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18 These results indicate that the PEX-domain has key roles in abilities of proliferation
19 and migration of the metastatic adenocarcinoma cells.
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24 **DISCUSSION**

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26 MMP family members are consist of secretory types (collagenases, gelatinases, stromelysins)
27 and membrane types (MT-MMPs) and have crucial roles in the progression of diseases,
28 including cancer, arthritis, and periodontitis. Most metalloproteinase inhibitors had been
29 designed and, indeed, to block catalytic pockets that possess a zinc ion and proteinase activity.
30
31 However, any metalloproteinase inhibitor has been approved for clinical application. Recent
32 studies, including the present study, have shown intracellular and non-proteolytic roles for
33 MMPs in arthritis and cancer [Eguchi et al., 2017; Eguchi et al., 2008; Eguchi et al., 2010]. In
34 addition to in an arthritis model and in a chondrosarcoma-derived cell line [Eguchi et al., 2008],
35 nuclear MMP3 was significantly found in primary tumors and in metastatic sites in allogeneic
36 transplant model of the aggressive adenocarcinoma cells (Fig. 5, 6). Both the PEX isoform and
37 full-length MMP3 were significantly found in the metastatic LuM1 cells (Fig. 4). The siRNA
38 targeting MMP3 reduced both 25-kDa PEX isoform and 54-kD full-length MMP3 (Fig. 4C) and
39 simultaneously attenuated proliferation and migration of the aggressive adenocarcinoma cells
40 (Fig. 2 F, G). On the other hand, overexpression of the PEX isoform promoted proliferation and
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5 migration of the LuM1 cells (Fig. 7). Therefore, the PEX isoform and domain are responsible
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7 for MMP-driven cell proliferation, migration and subsequent metastasis of the aggressive
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9 adenocarcinoma cells.
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11 MMP3 and MMP9 colocalized with DNA in subcutaneous tumors *in vivo* (Fig. 6).
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13 These data indicated potential roles for MMP3 and MMP9 in gene regulation in primary and
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15 metastatic tumors. We showed that MMP3 localized in cell nuclei in stroma-tumor marginal
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17 cells in subcutaneous tumors (Fig. 5B, 5C, 6B, 6C), suggesting that MMP3 involves potential
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19 tumor-stromal interaction such as induction of CAFs, migration, invasion and tumor
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21 angiogenesis, tumor-immunology interaction through the stroma. In contrast, MMP9 was
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23 localized in central area of subcutaneous tumors (Fig. 6 B, D). Central area of tumors are often
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25 necrotic and/or hypoxic where hypoxia-inducible transcription factor (HIF1) controls
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27 transcription of target genes, including MMP genes [Kondo, 2002] and stem cell genes [Eguchi
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29 et al., 2018; Keith et al., 2011; Keith and Simon, 2007; Semenza, 2016]. We previously showed that
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31 MMPs and CTGF/CCN2 were inducible upon hypoxia through distinctive mechanisms [Kondo,
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33 2002]. MMP9 is one of key proteins induced by HIF1 in tumors whereas CTGF could be
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35 increased through mRNA stabilization [Kondo, 2002]. Thus, HIF1, MMP9, and CTGF can be
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37 co-increased at central area of tumors and the induced MMP9 and CTGF can promote tumor
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39 angiogenesis [Kondo, 2002]. CTGF may be increased by transcriptional role for MMP9 in
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41 central area of tumors (Fig. 6D), in addition to the role for MMP3 in transcription [Eguchi et al.,
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43 2017; Eguchi et al., 2008] . Indeed, mRNA levels of CTGF were altered in tumors *in vivo* upon
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45 knockdown of MMP3 and MMP9 (data not shown). Thus, we are further investigating
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47 mechanisms underlying MMPs regulation of CTGF and HSPs that may mediate tumor
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49 progression and metastasis.
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54 In addition to the intracellular and non-catalytic roles for MMP3 as shown above,
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5 intracellular and non-catalytic MMP9 was suggested in the present study. The approx. 60-kDa
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7 MMP9 isoforms were significantly detected in the lysate of the metastatic LuM1 cells (Fig. 4G).
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9 However, any intracellular proteolytic activity of the 60-kDa MMP9 was not detected in the
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11 lysate of LuM1 cells, even though the gelatinase activities of 90-kDa and 250-kDa MMP9 were
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13 found (Fig. 4F) and extracellular proteinase activity of 60-kDa MMP9 was detected in the cell
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15 culture supernatant (Fig. 4H). Thus, the intracellular 60-kDa MMP9 does not possess any
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17 proteolytic activity or some intracellular factors such as DNA/chromatin might mask the
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19 proteinase domain.
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22 Of note, the non-proteolytic MMPs cannot be directly targeted by metalloproteinase
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24 inhibitors that block proteolytic pockets. Moreover, the intranuclear MMPs might be difficult to
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26 be accessed by small molecule drugs, which can be excreted from cancer cells, and are much
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28 more difficult by molecularly targeted antibody drugs, which are too large to enter into cells and
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30 nuclei. The siRNA-mediated knockdown tested in the present study significantly and
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32 sustainably reduced intracellular and extracellular, non-catalytic and catalytic MMPs (Fig. 4,
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34 S3). These efficient knockdown of various isoforms of MMPs might involve their efficient
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36 effects to attenuate migration, invasion and metastasis. The RNAi approaches are able to target
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38 mRNAs that fundamentally encode proteins that are post-translationally modified, processed
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40 and localized to specific regions. Thus, the RNAi approaches might be more fundamentally
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42 effective in disease therapies.
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45 It was recently reported that MMP3 functions in a coordinated manner with a
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47 chemokine, IL-8/CXCL8, expressed in melanoma via direct regulation by NFAT1, thus
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49 promoting tumor progression and metastasis [Shoshan et al., 2016]. Another recent study found
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51 that interleukin-regulatory transcription factor 8 (IRF-8) directly repressed the *Mmp3* gene in
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53 relatively mild mammary tumor cells, whereas loss of IRF-8 in coordination with gain in *Mmp3*
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5 expression promoted tumor progression and metastasis [Banik et al., 2015.]. These studies were
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7 done using xenotransplantation of human cancer cells into immunodeficient mice. We consider
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9 that the present protocol of allogeneic transplantation into an immunologically genetically
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11 relatively normal host will be useful for further studies of tumor microenvironment and
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13 immunology.
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16 Thus, intracellular and non-proteolytic MMPs have crucial roles in cancer progression.
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18 The versatile RNAi approach is able to reduce intracellular and extracellular, non-proteolytic
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20 and proteolytic MMPs at once and thus appears to be significantly effective to attenuate various
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22 aspects of cancer progression, including proliferation, migration and invasion of cancer cells,
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24 tumor development and metastasis.
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28 **ACKNOWLEDGEMENTS.** This paper is dedicated to the memory of one of the coauthors,
29
30 Professor Ken-ichi Kozaki, who passed away on May 29, 2016. The authors thank Toshifumi
31
32 Fujiwara, Kisho Ono, Tomoko Yamamoto and Kazumi Ohyama for their technical assistance
33
34 and useful discussion.
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39 **CONFLICTS OF INTEREST.** The authors have no competing financial interests to declare.
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27 **FIGURE LEGENDS**

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29 **Figure 1. Transcriptome analysis, gene screening, *Mmp* genes expressed in colon cancer**
30 **cells with different metastatic potential.** (A) Flow chart of gene screening. Microarray
31 screened 62976 genes. The 479 genes were expressed at more than 10-fold higher level in
32 LuM1 as compared to NM11 and Colon26 cells. (B) Venn diagram extracted 104 genes
33 involved in invasion, EMT, and metastasis. (C) Heat map analysis of *Mmp* gene family. The
34 gene expression levels relative to the levels in Colon26 were shown. (D) Expression levels of
35 *Mmp* genes in LuM1, NM11 and Colon26 cells. The gene expression level of *Mmp3* in LuM1
36 was the highest among all *Mmp* gene family members, as well as compared to that in NM11 and
37 Colon26 cells.
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50 **Figure 2. The roles of MMP3 and MMP9 in proliferation, migration, and invasion of the**
51 **metastatic adenocarcinoma cells.** (A) Differential *in vitro* migration activities. Migrated cells per
52 well was shown. The migration activity of LuM1 was higher than that of NM11 and Colon26 cells.
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5 * $p < 0.05$ as compared with Colon26 (one way ANOVA). $n = 3$. (B) Differential *in vitro*
6 invasion activities. * $p < 0.05$ as compared with Colon26 (one way ANOVA). $n = 3$. (C) Staining
7 of invaded cells. Diff-Quick staining was carried out after invasion assay. Scale bars: 200 μm .
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11 (D) The mRNA levels of MMP3 in the LuM1 cells transfected with siRNA. Relative mRNA
12 levels normalized to *Gapdh* mRNA levels were shown. * $p < 0.05$ as compared with control si
13 group. † $p < 0.05$ as compared with Mmp9 si group (one way ANOVA). $n = 3$. (E) The mRNA
14 levels of MMP9 in the LuM1 cells transfected with siRNA. Relative mRNA levels normalized
15 to *Gapdh* mRNA levels were shown. * $p < 0.05$ as compared with control si group. † $p < 0.05$ as
16 compared with MMP3 si group (one way ANOVA). $n = 3$. (F) Growth curves of LuM1 cells
17 transfected with MMP-targeting or control siRNA. (G, H) *In vitro* migration (G) and invasion
18 (H) activities altered by MMP3 and/or MMP9 knockdown. Upper panels, quantified migration
19 (G) and invasion (H) activities of LuM1 cells transfected with siRNA-targeting MMP3, MMP9,
20 MMP3/MMP9 or the control dsRNA. $n = 5$. * $p < 0.05$ as compared to the control si group (one
21 way ANOVA). Lower panels, migrated (G) and invaded (H) cells stained with Diff-Quick. Scale
22 bars: 200 μm . Control si group, $n = 5$; MMP3 si group, $n = 5$; MMP9 si group, $n = 5$; MMP3/9
23 si group, $n = 6$. * $p < 0.05$ as compared to the control si group. † $p < 0.05$ as compared to the
24 MMP9 si group (one way ANOVA). Lower panel, Invaded cells stained with Diff-Quick. Scale
25 bars: 200 μm .
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46 **Figure 3. Targeting MMP3 and MMP9 attenuated tumor development and metastasis of**
47 **the aggressive colon cancer cells.** LuM1 cells transfected with siRNA targeting MMP3 and/or
48 MMP9 or non-targeting dsRNA were subcutaneously injected to abdominal walls in mice. The
49 primary tumors and the metastatic tumors in lungs were analyzed 20 days post-injection period.
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54 (A) Representative images of mice bearing subcutaneous primary tumors. (B) Weights of
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5 subcutaneous primary tumors. (C) Representative images of lungs with metastatic tumor
6 nodules. (D) Numbers of metastatic tumor nodules in lungs. (E) Weights of lungs with
7 metastatic tumor nodules. (C-E) Control si group, n = 5; MMP3 si group, n = 5; MMP9 si group,
8 n = 5; MMP3/9 si group, n = 6. * $p < 0.05$, as compared to the control si group (one way
9 ANOVA).
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18 **Figure 4. Knockdown of non-proteolytic and proteolytic, intracellular and extracellular**

19 **MMP3 and MMP9.** (A) Schematic structures of murine MMP3. The structures of protease
20 domain-containing 54-kDa MMP3 and non-proteolytic 25-kDa PEX domain of MMP3 are
21 shown. The anti-PEX MMP3 antibody EP1186Y recognizes the epitope 450 to 477 position,
22 which both the 54-kDa MMP3 and the 25-kDa PEX isoform contain. PG, peptide glycan
23 binding domain. PEX, hemopexin-like repeat. Asterisks are positions of nuclear localization
24 signals. (B) Schematic structures of approx. 92-95-kDa MMP9 and 64- and 67-kDa MMP9. Fn,
25 fibronectin type II domain. PT, PT repeat composed on the tetrapeptide xPTx. (C) Western blot
26 showing intracellular isoforms of MMP3 in the LuM1 cells and those knockdown. An arrow
27 indicates full-length MMP3 (54-kDa). An arrowhead indicates the intracellular 25-kDa PEX
28 isoform of MMP3. WCL, whole cell lysate. (D) Casein zymography of extracellular isoforms of
29 MMP3 and those knockdown in the LuM1 cells. An arrow indicates full-length MMP3
30 (54-kDa). An arrowhead indicates proteolytic MMP3 monomer or dimer. M, molecular weight
31 maker. (E) Intracellular gelatinase activities in the LuM1 cells. The WCLs prepared from LuM1,
32 Colon26 and NM11 were examined in zymography. Gelatinase activities at approx. 90-kDa
33 (arrow) and 250-kDa (asterisk) were seen. (F) Extracellular gelatinase activities in the culture
34 supernatant of the LuM1 cells. Gelatinase activities at approx. 90-kDa (arrow) and 250-kDa
35 (asterisk) were seen. (G) Western blotting analysis of intracellular isoforms of MMP9 in the
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5 lysate of LuM1 cells and those knockdown. An arrow indicates approx. 90-kDa MMP9. An
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7 arrowhead indicates intracellular short MMP9 (64- and 67-kDa). An asterisk indicates putative
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9 intracellular oligomer of MMP9. (H) Gelatin zymography of extracellular MMP9 and those
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11 knockdown in the LuM1 cells. Gelatinase activities were seen at approx. 90-kDa (arrow, the
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13 major band), 60-kDa (arrowhead), and 250-kDa (asterisk). M, molecular weight maker.
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18 **Figure 5. The subtumoral and subcellular localization of the PEX / MMP3 in primary**
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20 **tumors and in metastatic tumors in lungs.** LuM1 cells were subcutaneously injected into
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22 abdominal walls in a mouse. A developed subcutaneous primary tumor (A-C) and a lung lesion
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24 (D-F) with metastatic tumor nodules were prepared at day 20 after the injection and then
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26 immunohistochemistry of the PEX / MMP3 was performed. (A) A whole tumor. Scale bar: 500
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28 μm . (B) A magnified view of the box shown in A. Scale bar: 50 μm . (C) A magnified view of
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30 the box shown in B. Scale bar: 10 μm . An arrow indicates MMP3 immunostained in a
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32 cancer-associated fibroblast (CAF)-like cell. Arrowheads indicate nuclear localization of MMP3.
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34 (D) A whole lung with metastasis. Scale bar: 500 μm . (E) A magnified view of the box shown in
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36 D. Scale bar: 50 μm . (F) A magnified view of the box shown in E. Scale bar: 10 μm . An arrow
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38 indicates MMP3 accumulated in the nucleus. An arrowhead indicates MMP3 localization to the
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40 nuclear margin.
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46 **Figure 6. The nuclear PEX / MMP3 found in tumor-stroma marginal area and nuclear**
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48 **MMP9 in tumor central area.** For immunostaining, an anti-PEX MMP3 antibody and
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50 anti-MMP9 full-length were used. (A) The scheme of definition of the central area (shown in
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52 the left photomicrograph), interior, marginal and stromal areas (shown in the right
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54 photomicrograph) of a tumor. Scale bars, 500 μm (left) and 10 μm (right). The area in the
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5 rectangle in the left image was shown with a high power magnification in the right image. (B)
6 The positive rate of MMP3 and MMP9 in the nuclei in individual area. $n = \text{random } 3 \text{ fields}$. $*p$
7 < 0.05 as compared to the nuclear MMP3 positive rate in the stromal area. (C, D) Confocal
8 microscopy of subtumoral and subcellular localization of MMP3 (C) and MMP9 (D). Red,
9 MMP3 or MMP9. Blue, DNA stained with DAPI. Arrows indicate the MMP3 or MMP9
10 localization in the nuclei. Scale bars, 10 μm (C) and 20 μm (D). (E) Confocal microscopy of
11 subcellular localization of MMP3/9 in the lung lesion with metastatic cancer cells. Arrows
12 indicate MMP3 (left) and MMP9 (right) positive region in the nuclei. Scale bars, 5 μm .

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24 **Figure 7. The potent roles of the PEX-domain in proliferation and migration of the**
25 **metastatic cancer cells.** (A) Growth curves of the metastatic LuM1 cells transfected with
26 pFlag3-PEX-myc or the control vector. $*p < 0.05$ as compared with control vector. (B) *In vitro*
27 migration activities altered by overexpression of the PEX domain. Upper panels, staining of the
28 migrated cells. Scale bars: 200 μm . Lower panel, ratio of migrated cells upon overexpression of
29 the PEX domain or the control vector. $n = 3$. $*p < 0.05$ as compared with control vector. (C) *In*
30 *vitro* invasion activities. Upper panels, staining of the invaded cells. Scale bars: 100 μm , Lower
31 panel, ratio of the invaded cells upon overexpression of the PEX domain or the control vector. n
32 = 3.

33 SUPPORTING INFORMATION LEGENDS

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47 **Figure S1. Expression signatures of *Mmp* gene family in rapidly metastatic LuM1, slowly**
48 **metastatic NM11 and the parental Colon26 cells.** (A) Differential migration activities of the
49 tested cells after the treatment with trypsin or EDTA. LuM1 cells treated with trypsin migrated
50 more than other cell lines or EDTA-treated LuM1 cells. EDTA is a chelator for metal. MMPs
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5 coordinate a zinc ion at their activity pockets. EDTA chelates zinc ions coordinated in proteins,
6 including MMPs. The chelation of zinc ion in MMPs may alter their structures and functions.
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8 Trypsin cleaves and alters cell surface proteins, possibly including receptor-bound MMPs and
9 MT-MMPs. Scale bars: 200 μm . (B) Quality check of the total RNA by agarose gel
10 electrophoresis. The quality of RNA samples were confirmed before microarray and RT-qPCR
11 analysis. (C) Heat map showing relative expression levels of all *Mmp* gene family members.
12 The gene expression level of *Mmp3* in LuM1 was the highest among all *Mmp* gene family
13 members, as well as compared to that in NM11 and Colon26 cells.
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24 **Figure S2. Targeted knockdown of MMP3 and/or MMP9.** (A) A flowchart of knockdown
25 studies. Cells were cultured for 48 hours after transfection of siRNA. We then performed
26 RT-qPCR (Fig. 2D, E), Western blotting analysis (Fig. 4C, G), migration assay (Fig. 2G),
27 invasion assay (Fig. 2H), and subcutaneous injection (Fig. 3). Cells transfected with siRNA
28 were cultured for 48, 72 or 96 hours for zymography while the media were replaced to
29 serum-free media 24 hours prior to harvest of culture supernatant. To analyze mRNA levels in
30 tumors *in vivo*, total RNA was extracted from subcutaneous tumors on the day 10 after
31 subcutaneous injection. Lung metastasis assay and IHC were performed on the day 20 after
32 subcutaneous injection. (B) Representative photomicrographs of LuM1 cells transfected with
33 siRNAs. Scale bars: 100 μm . (C) Gelatin zymography to examine sustainability of RNAi effect.
34 LuM1 cells were transfected with siRNA targeting *Mmp9* or non-silencing control dsRNA. Cell
35 culture supernatants (5 ml) were collected at 48, 72, and 96 hours after transfection, then
36 concentrated to a 1/20 volume. Protein samples (13.2 μg) were subjected to SDS-PAGE for
37 gelatin zymography (upper panel). The #1 and #2 indicate biological duplicates. The intensities
38 of the bands were quantified by using ImageJ and shown in the graph (lower panel). (D) *In vivo*
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5 gene expression levels of *Mmp3* in the MMP3-knockdown tumor (upper) and the levels of
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7 *Mmp9* in the MMP9-knockdown tumor (lower). Total RNA was prepared from the tumor on the
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9 day 10 after the subcutaneous injection of siRNA-transfected LuM1 cells and qRT-PCR was
10
11 performed. Relative mRNA levels normalized to *Actb* mRNA levels were shown.
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16 **Figure S3. Full images of Western blot and zymography shown in Fig 6.** (A) Western
17 blotting analysis of intracellular isoforms of MMP3 in the LuM1 cells and those knockdown.
18 (B) Casein zymography of extracellular isoforms of MMP3 and those knockdown in the LuM1
19 cells. (C) Intracellular gelatinase activities in the LuM1 cells. (D) Extracellular gelatinase
20 activities in the culture supernatant of the LuM1 cells. (E) Western blotting analysis of
21 intracellular isoforms of MMP9 in the lysate of LuM1 cells and those knockdown. (F) Gelatin
22 zymography of extracellular MMP9 and those knockdown in the LuM1 cells.
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Table 1. Functional annotation clustering.

Annotation Cluster 1	Enrichment Score: 2.49	P-value
GO:0044421	extracellular region part	0.0010483
GO:0005576	extracellular region	0.0050501
GO:0005615	extracellular space	0.0064459
Annotation Cluster 2	Enrichment Score: 2.11	P-value
GO:0007155	cell adhesion	0.0046972
GO:0022610	biological adhesion	0.0047962
GO:0016337	cell-cell adhesion	0.0213548
Annotation Cluster 3	Enrichment Score: 1.90	P-value
GO:0006954	inflammatory response	0.00592
GO:0006952	defense response	0.0092881
GO:0009611	response to wounding	0.0107201
GO:0002252	immune effector process	0.0416591
Annotation Cluster 4	Enrichment Score: 1.83	P-value
GO:0030246	carbohydrate binding	4.37E-04
GO:0030247	polysaccharide binding	0.0119368
GO:0001871	pattern binding	0.0119368
GO:0005539	glycosaminoglycan binding	0.0228992
GO:0008201	heparin binding	0.0477234

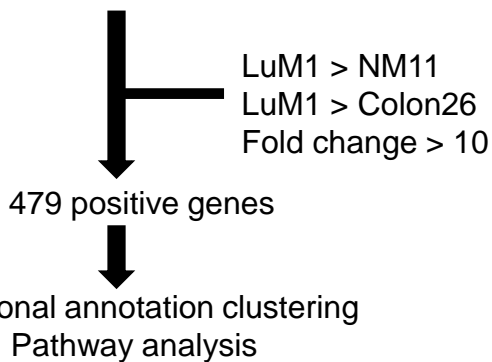
Genes expressed at a 10-fold greater level in LuM1 as compared to those in NM11 and Colon26 cells were analyzed.

Table 2. Ranking of altered pathways in LuM1 as compared to NM11 and Colon26 cells.

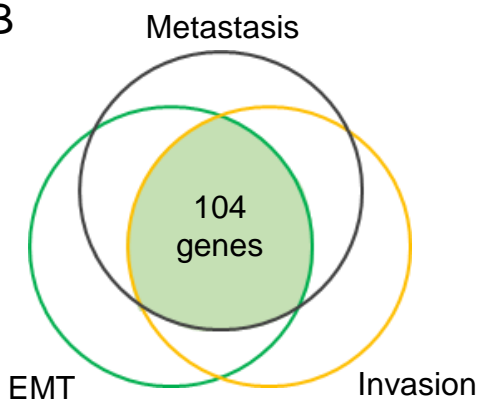
Category	Term	P-value
KEGG_PATHWAY	mmu04060:Cytokine-cytokine receptor interaction	0.0202
KEGG_PATHWAY	mmu04062:Chemokine signaling pathway	0.0253
KEGG_PATHWAY	mmu04142:Lysosome	0.0283
KEGG_PATHWAY	mmu04020:Calcium signaling pathway	0.0325

For Peer Review

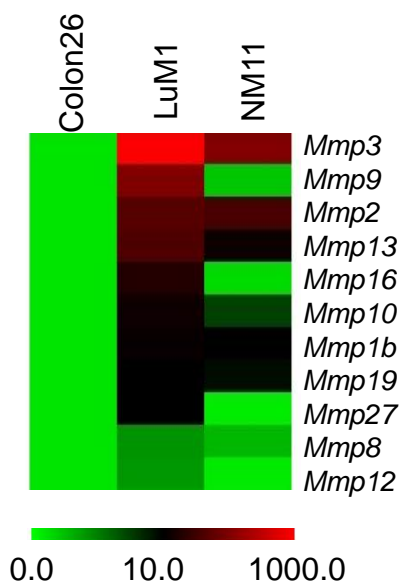
A Screening of 62976 genes by microarray



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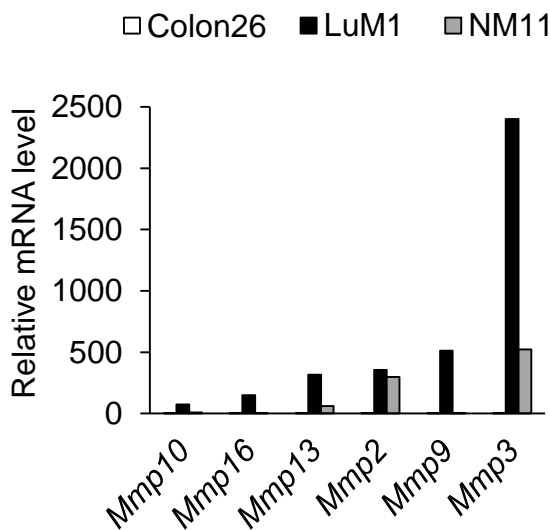
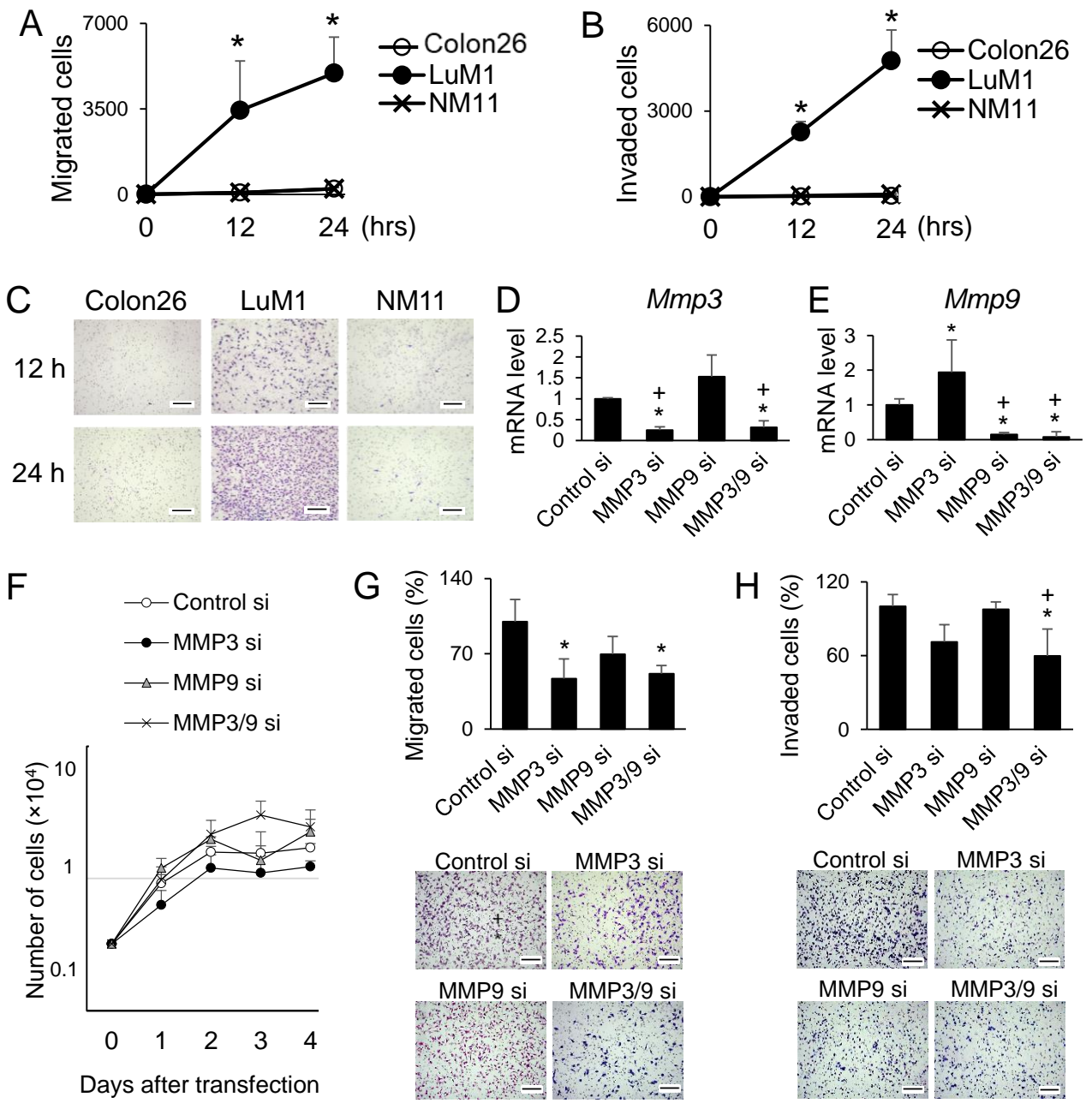


Fig. 1



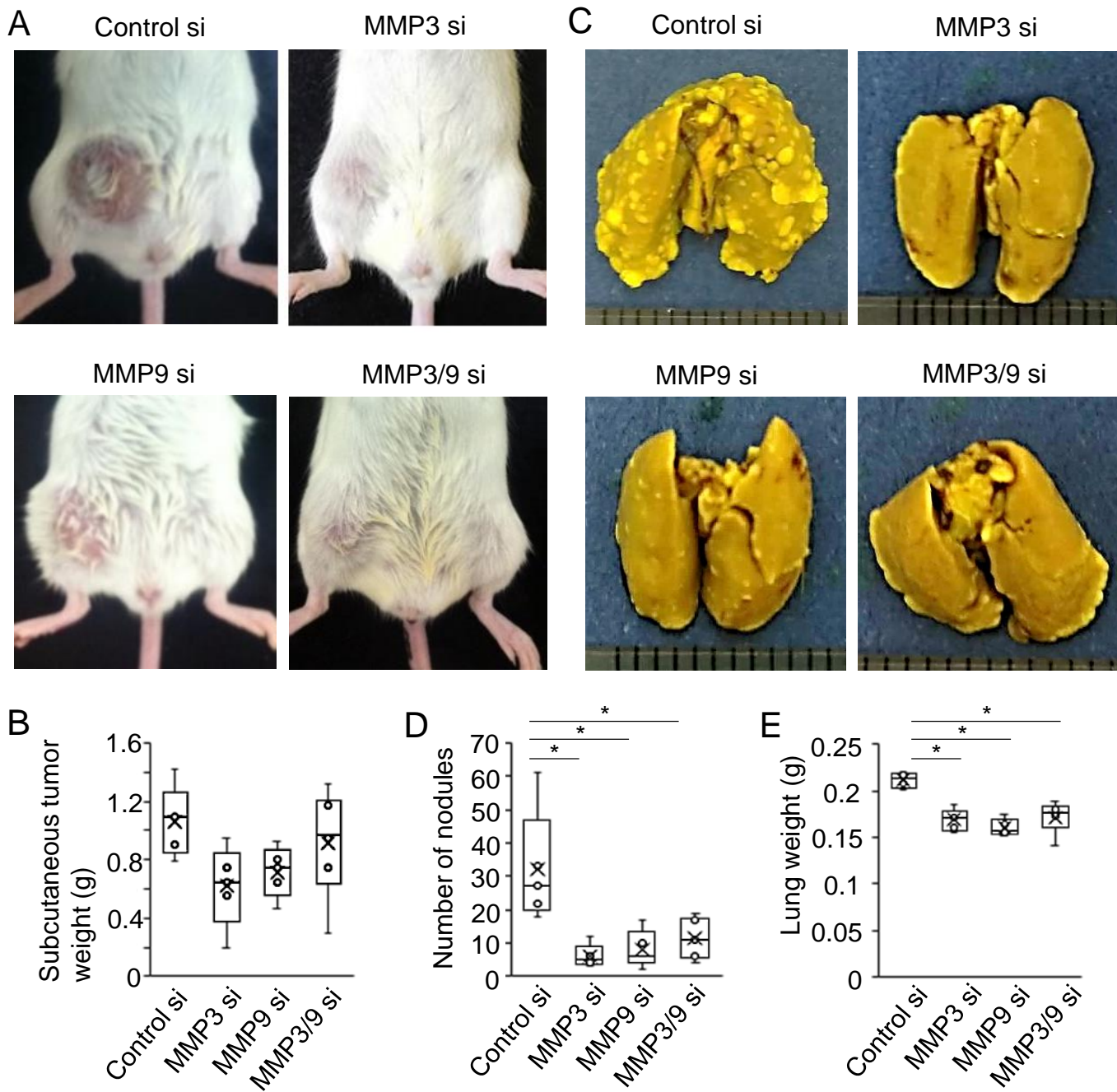


Fig. 3

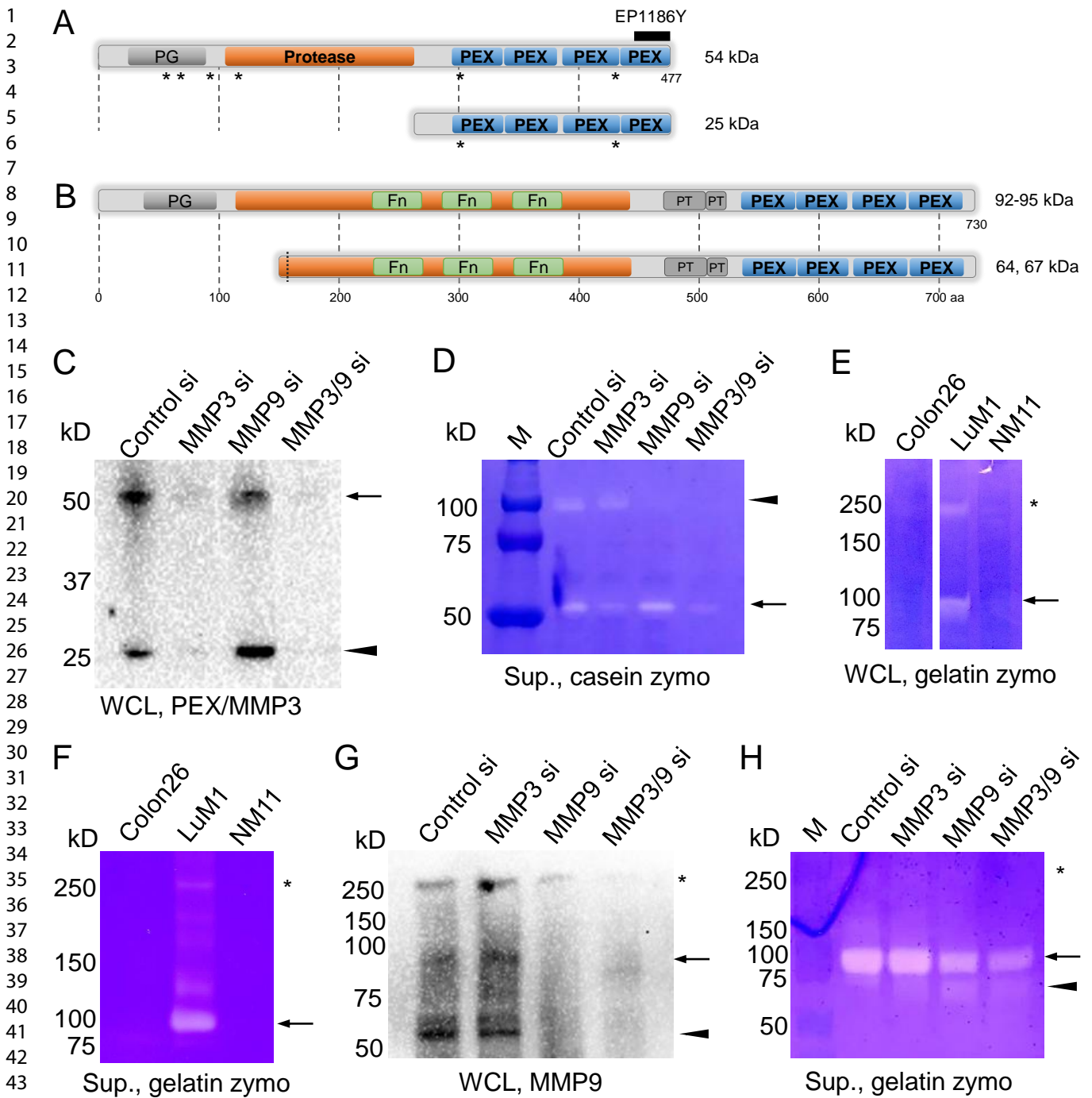


Fig. 4

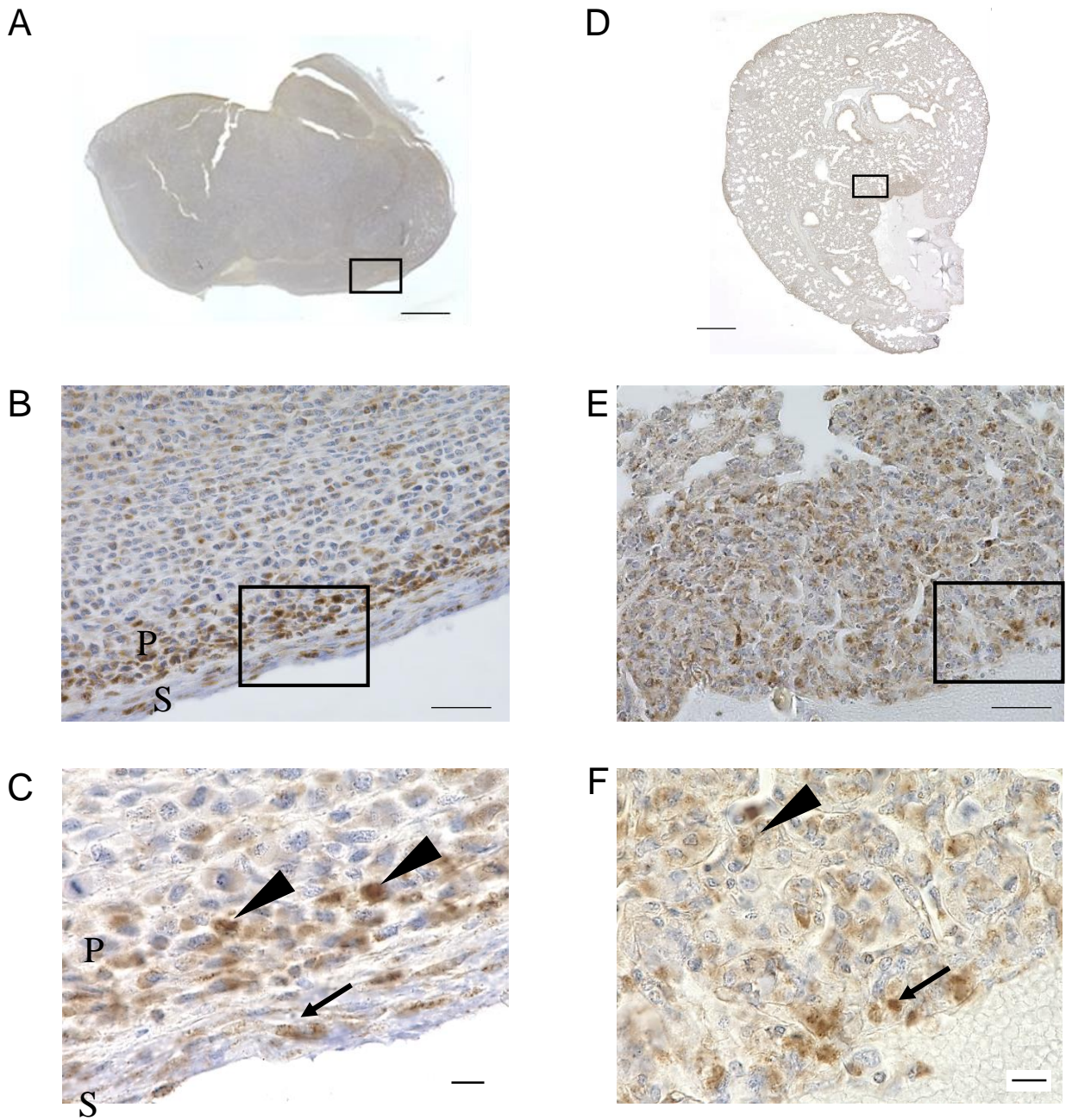


Fig. 5

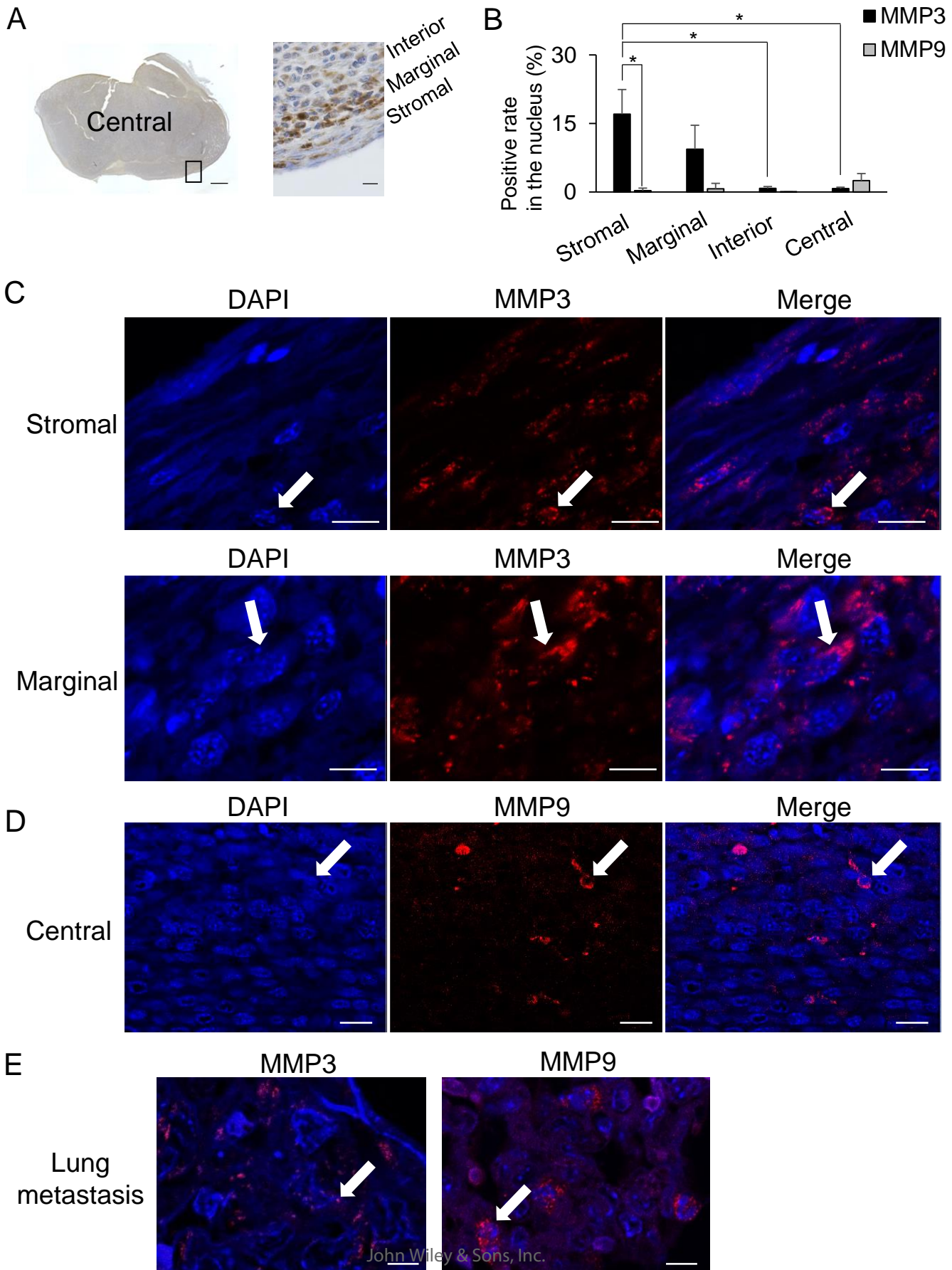


Fig. 6

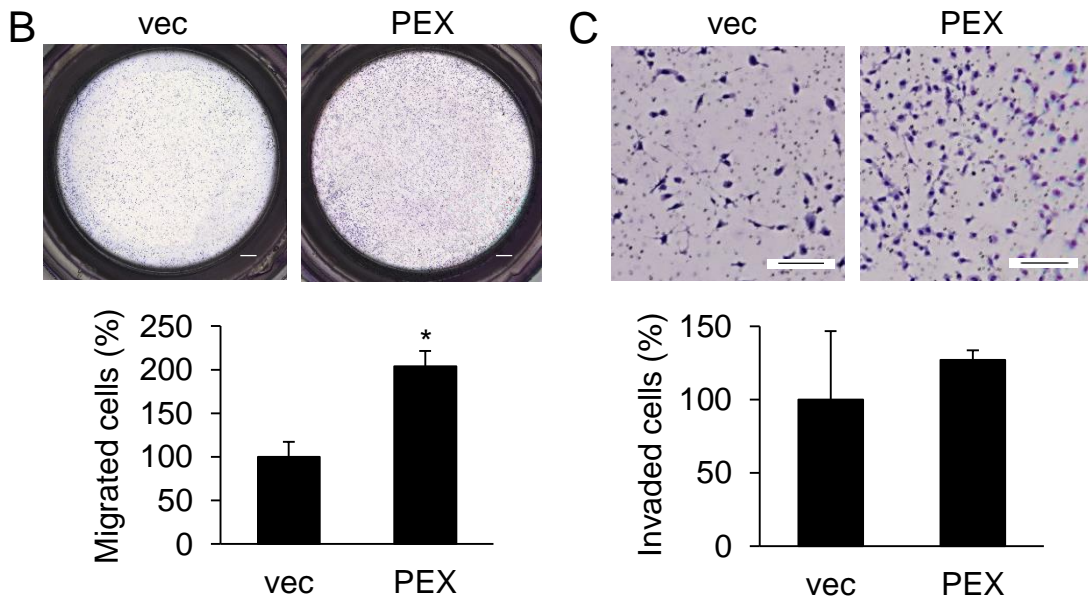
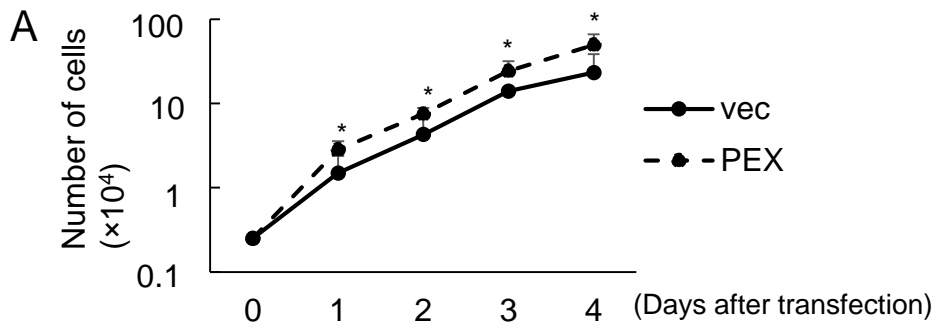
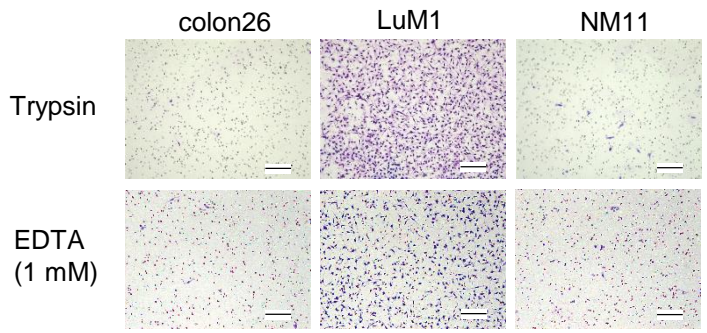


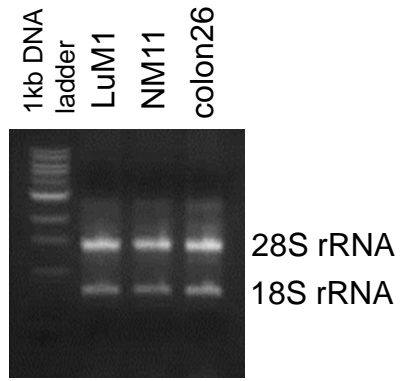
Fig. 7

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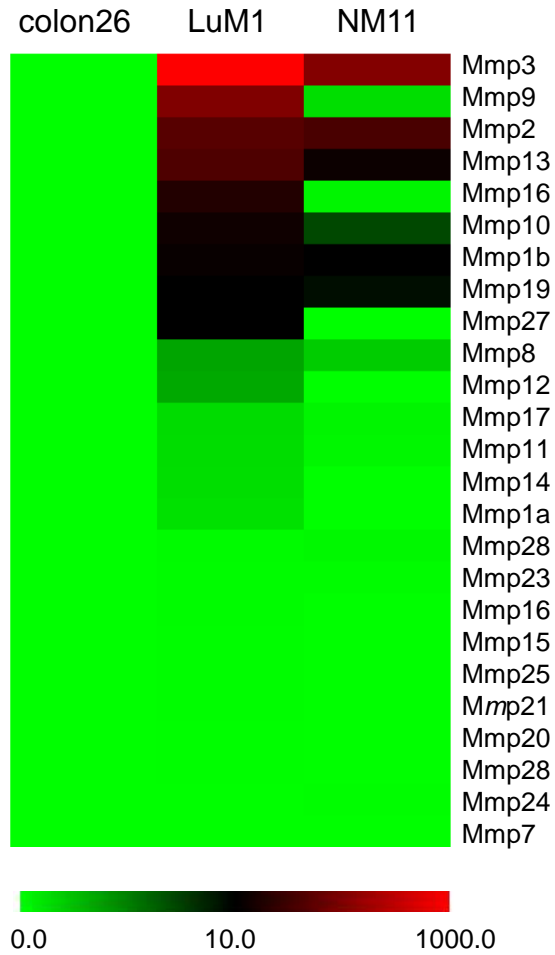


Fig. S1

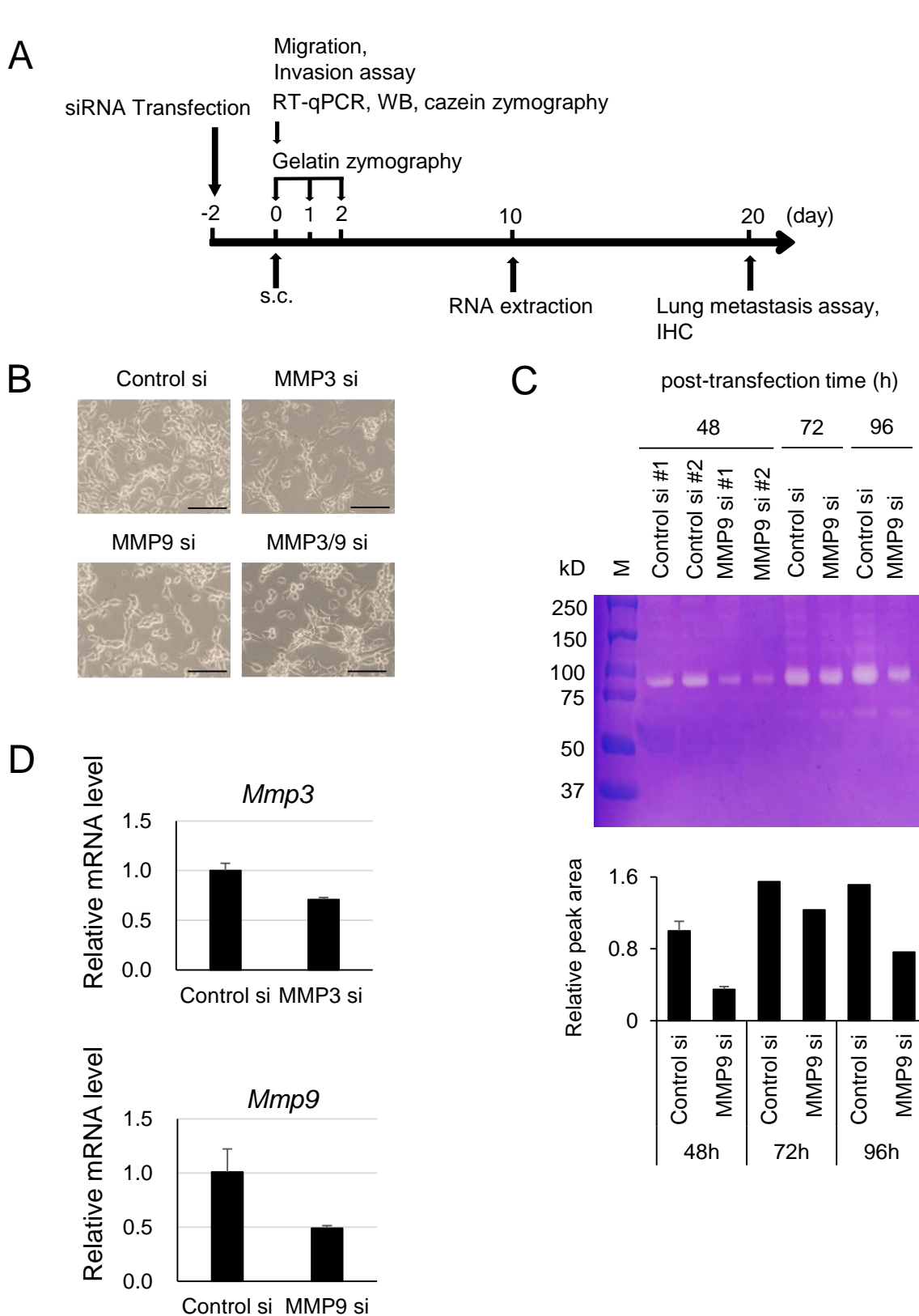
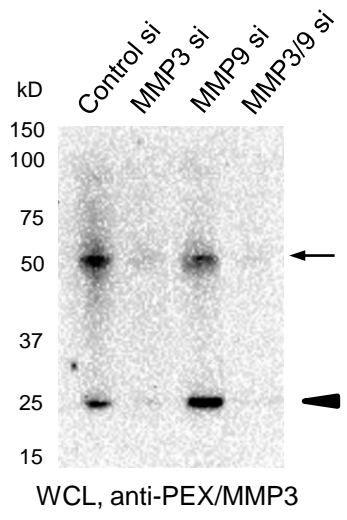


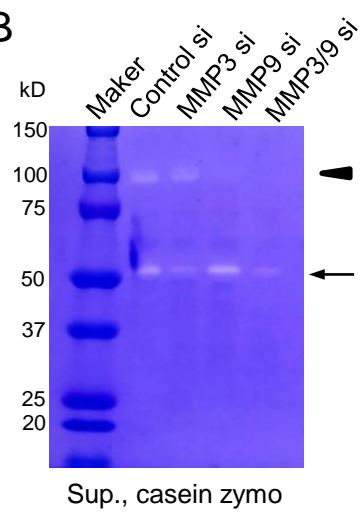
Fig. S2

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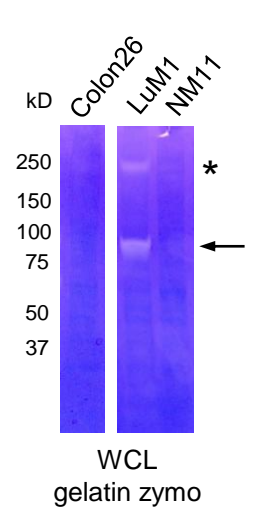
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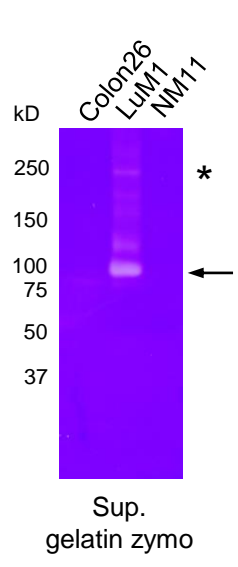
B



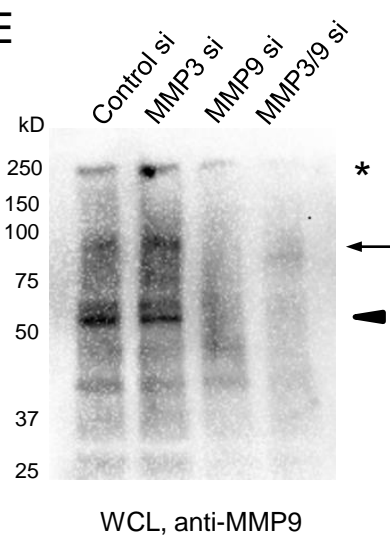
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