Tumorigenesis and Neoplastic Progression

Matrix Metalloproteinase 3 Is Present in the Cell Nucleus and Is Involved in Apoptosis

Karim Si-Tayeb,^{*†¶} Arnaud Monvoisin,^{*†¶}
Claire Mazzocco,^{*†¶} Sébastien Lepreux,^{*†‡}
Marion Decossas,^{§†¶} Gaëlle Cubel,^{*†¶}
Danièle Taras,^{*†¶} Jean-Frédéric Blanc,^{*†¶}
Derrick R. Robinson,^{‡†¶} and Jean Rosenbaum^{*†¶}

From INSERM E362,^{*} Bordeaux; Université Victor Segalen Bordeaux 2,[¶] Bordeaux; and IFR 66,[†] Bordeaux; CHU de Bordeaux,[‡] Hôpital Pellegrin, Service d'Anatomie Pathologique, Bordeaux; and CNRS,[§] UMR 5162, Equipe ATIP: Interaction Mitochondrie-Cytosquelette chez les Trypanosomes Bordeaux, France

Matrix metalloproteinase (MMP)-3 is a protease involved in cancer progression and tissue remodeling. Using immunofluorescence and immunoelectron microscopy, we identified nuclear localization of MMP-3 in several cultured cell types and in human liver tissue sections. Western blot analysis of nuclear extracts revealed two immunoreactive forms of MMP-3 at 35 and 45 kd, with the 35-kd form exhibiting caseinolytic activity. By transient transfection, we expressed active MMP-3 fused to the enhanced green fluorescent protein (EGFP/aMMP-3) in Chinese hamster ovary cells. We showed that EGFP/aMMP-3 translocates into the nucleus. A functional nuclear localization signal was demonstrated by the loss of nuclear translocation after site-directed mutagenesis of a putative nuclear localization signal and by the ability of the MMP-3 nuclear localization signal to drive a heterologous protein into the nucleus. Finally, expression by Chinese hamster ovary cells of EGFP/aMMP-3 induced a twofold increase of apoptosis rate, compared with EGFP/pro-MMP-3, which does not translocate to the nucleus. Increased apoptosis was abolished by site-directed mutagenesis of the catalytic site of MMP-3 or by using the MMP inhibitor GM6001. This study elucidates for the first time the mechanisms of nuclear localization of a MMP and shows that nuclear MMP-3 can induce apoptosis via its catalytic activity. (Am J Pathol 2006; 169:1390–1401; DOI: 10.2353/ajpath.2006.060005)

Proteolysis is a major mechanism for the activation or inactivation of proteins. It is also frequently used as a

highly effective posttranslational regulatory mechanism and may dramatically change protein functional properties. Matrix metalloproteinases (MMPs) were characterized by their ability to degrade extracellular matrix components. The MMP family includes ~25 members that play roles in morphogenesis, angiogenesis, and tissue remodeling.^{1–4} MMP activity is mainly regulated at the transcriptional level and through the action of tissue inhibitors of metalloproteinases. Disruption of this regulation is involved in the pathogeny of arthritis, atherosclerosis, fibrosis, tumor growth, and metastasis.^{5–8}

MMPs are synthesized as zymogen forms that require cleavage of their prodomain for activation.^{9,10} Indeed, the prodomain contains a conserved cysteine residue that interacts with the zinc ion in the catalytic site, thus maintaining the protein in an inactive state. This cysteine switch must be opened for activation.¹¹ MMPs are best known as being either secreted or transmembrane proteins. However, recent publications have identified unusual localizations for several MMPs. Luo and colleagues¹² found a variant of MMP-11/stromelysin 3 that is expressed as an intracellular active form. Golubkov and colleagues¹³ identified MMP-14/MT1-MMP in the centrosome. Most recently, Kwan and colleagues¹⁴ found a nuclear localization of MMP-2 in cardiac myocytes and rat liver.

MMP-3, or stromelysin-1, can degrade a variety of extracellular matrix substrates, such as type III, IV, and V collagens; laminins; fibronectin; osteopontin; and proteoglycans.¹⁵ In addition, MMP-3 is involved in the shedding of protein ectodomains from the cell surface¹⁶ and can also proteolytically activate other MMPs. MMP-3 is secreted as a precursor form that is activated in the extracellular space by proteases, notably plasmin.¹⁷

Supported by the Comité de la Dordogne from the Ligue Nationale Contre le Cancer, the Association pour la Recherche sur le Cancer, and the Conseil Régional d'Aquitaine.

K.S.-T. and A.M. contributed equally to this work.

Accepted for publication July 7, 2006.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Jean Rosenbaum, INSERM E362, Université Victor Segalen Bordeaux 2, 33076 Bordeaux, France. E mail: jean.rosenbaum@gref.u-bordeaux2.fr.

We have shown that MMP-3 was used by liver cancer cells to invade the extracellular matrix in response to hepatocyte growth factor.¹⁸ In subsequent experiments, we undertook to study the expression of MMP-3 in liver tissue using immunohistochemistry. This led us to the unexpected discovery of a nuclear staining for MMP-3. The aim of this study was to examine the mechanisms of nuclear translocation of MMP-3 and the function of nuclear MMP-3.

Materials and Methods

Liver Samples and Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of nontumoral liver and hepatocellular carcinoma (HCC) were used. Samples were obtained after either hepatectomy or liver transplantation. In 11 cases, HCC was developed on cirrhotic liver. All patients were male, with a mean age of 57.5 years. The etiology of liver disease was alcoholism ($n = 3$), HCV infection ($n = 3$), HBV infection ($n = 1$), nonalcoholic steatohepatitis ($n = 1$), and unknown ($n = 2$). In eight cases, the nontumoral liver was noncirrhotic. Seven patients were male, and the mean age was 72.6 years. In addition, four cases of histologically subnormal liver surrounding liver metastasis were also studied.

For immunohistochemistry, samples were first heated for 5 minutes in pH 6 citrate buffer in a steam cooker. Endogenous peroxidases were inhibited with 3% H_2O_2 in methanol. Samples were incubated 30 minutes with a polyclonal anti-MMP-3 antibody (AB810, 1:3000; Chemicon International, Temecula, CA) diluted 1/1250 in Tris-buffered saline, then in Envision+ peroxidase rabbit K4002 (DAKO, Carpinteria CA). Staining was revealed using Dako Liquid Dab+ large volume substrate with chromogene solution K3468. Nuclei were lightly counterstained with hemalum.

Several negative controls were performed: omission of the primary antibody, use of a nonrelevant rabbit IgG, or adsorption of antibody on recombinant MMP-3. For this latter control, recombinant MMP-3 (R&D Systems, Minneapolis, MN) was coupled to AminoLink Coupling Gel (Pierce, Rockford, IL) with 1 mol/L sodium cyanoborohydride (AminoLink Reductant; Pierce) in 0.01 N NaOH, using 0.1 mol/L sodium phosphate buffer, pH 8, coupling buffer. After mixing overnight at 4°C, the gel was washed with a quenching buffer (1 mol/L Tris, pH 7.5) and mixed 30 minutes at room temperature with quenching buffer and sodium cyanoborohydride. Finally, the gel was washed several times with 1 mol/L NaCl. The antibody was incubated overnight with coupled recombinant MMP-3, and the supernatant was used for immunohistochemistry.

Immunoelectron Microscopy in Tissue Sections

MMP-3 was detected by the pre-embedding immunogold technique. Samples were prepared as described,¹⁹ with the following changes. Fresh pieces from two cases of histologically normal liver surrounding colon cancer me-

tastasis and two cases from HCC were fixed for 2 hours at 4°C in 4% paraformaldehyde/0.2% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, then stored overnight in 4% paraformaldehyde at 4°C. Sections were cut on a vibrating microtome, collected in phosphate-buffered saline (PBS), equilibrated in a cryoprotectant solution (0.05 mol/L phosphate buffer, pH 7.4, containing 25% saccharose and 10% glycerol), and freeze-thawed by freezing in isopentane cooled in liquid nitrogen and thawing in PBS. Sections were preincubated in 4% normal goat serum in PBS for 30 minutes at room temperature, and then for 15 hours with the anti-MMP-3 antibody (or a control rabbit IgG) diluted 1/50 in PBS supplemented with 1% normal goat serum. After two washes in PBS and two in PBS supplemented with 2% bovine serum albumin-c (BSA-c) (Aurion, Costerweg, Wageningen, The Netherlands) and 0.2% ice-cold fish gelatin (PBS-BSA-c-gelatin), sections were incubated for 2 hours with goat anti-rabbit IgG conjugated to ultrasmall gold particles (0.8 nm, 1/100; Aurion) diluted in PBS-BSA-c-gelatin. After silver enhancement (HQ Silver; Nanoprobes, Yaphank, NY) for 6 minutes, after fixation, and embedding, ultrathin sections were cut, collected on pioloform-coated single-slot copper grids, stained with 2.7% lead acetate, and examined with a Technai electron microscope (Philips, Eindhoven, The Netherlands).

Cell Culture

The HepG2 human HCC cell line was obtained from American Type Culture Collection (Rockville, MD) and was cultured in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum. Chinese hamster ovary (CHO)-K1 cells (American Type Culture Collection, Manassas, VA) were cultured in Iscove's modified Eagle's medium supplemented with 6.7% fetal calf serum. Human hepatic myofibroblasts were obtained from explants of nontumoral liver and characterized as previously described.^{20,21} Myofibroblasts were grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum, 5% human serum, and 5 ng/ml human epidermal growth factor. Cell lines were used between passages 2 to 10 after thawing, whereas primary myofibroblasts were used between passages 2 to 6.

Nuclear Extraction

Nuclear extraction was performed as described previously.²² Nuclear integrity was controlled as follows: after washing the nuclear pellet twice, an aliquot of the nuclear suspension was deposited on a glass slide by cytocentrifugation, stained with hemalum and examined under a microscope to determine nuclei integrity and whether nuclei were free of cytoplasmic debris. In addition, fractions were analyzed by Western blot as described below.

Table 1. Primer Sequences and Oligonucleotides Coding for MMP-3-NLS and SV40-Tag-NLS

	Primers	Resulting sequence
a		
Pro-MMP-3	5'-GC GAGCTC AATGGAGAGTCTTCCAATCC-3' 5'-GAACTTGAGCGTGAATCTGT GAATTC GC-3'	Amino acids 1 to 477
aMMP-3	5'-GC GAGCTC TTAGAACCCTTCCTGGCAT-3' 5'-GAACTTGAGCGTGAATCTGT GAATTC GC-3'	Amino acids 100 to 477
b		
NLS MMP-3	5'- CATCCCGAAGTGGAGGAAAACCCACCTTG -3' 3'- TCGAG TAGGGCTTCACCTCCTTTTGGGTGGAA CTTAA -5' I P K W R K T H L	
NLS TagSV40	5'- CATCCCGAAGAAGAAGAGGAAGGTCCTTG -3' 3'- TCGAG TAGGGCTTCTTCTCTCTCCAGGA CTTAA -5' I P K K K R K V L	

a: Primers used for PCR amplification of active MMP-3 cDNA. b: Oligonucleotides used for fusion of NLS sequences to EGFP. Bold characters denote restriction sites used for oriented cloning.

Plasmid Construction

A plasmid coding enhanced green fluorescent protein (EGFP)-tagged active MMP-3 (aMMP-3) and pro-MMP-3 were constructed on the basis of the pEGFP-C3 plasmid (Clontech, Mountain View, CA), which allows aMMP-3 fusion to the C terminus of EGFP. Primers (Table 1) corresponding to the N- and C-terminal regions of active MMP-3 were tagged with *SacI* and *EcoRI* sites, respectively, and used in PCR reactions with products from reverse-transcription of human hepatic myofibroblasts as template. The products were cloned into the pGEM T-easy vector (Promega, Madison, WI). The resulting plasmids were digested with *SacI* and *EcoRI* and the inserts were subcloned into the pEGFP-C3 vector to allow an in-frame fusion (EGFP/aMMP-3). For EGFP-nuclear localization signal (NLS) fusion, oligonucleotides corresponding to the MMP-3 NLS sequence or the SV40 Tag NLS sequence^{23,24} were tagged with *SacI* and *EcoRI* (Table 1) and cloned into the pEGFP-C3 vector.

Site-Directed Mutagenesis

Mutations of specific amino acids in MMP-3 were constructed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The function of the putative NLS was tested using deletion Arg¹¹⁰-Lys¹¹¹ ($\Delta R^{110}K^{111}$).²⁵ The sense-strand oligonucleotide used was 5'-CTGGCATCCCGAAGTGGACCCACCTTACATACAG-3', and anti-sense 5'-CTGTATGTAAGGTGGTCCACTTCGGGATGCCAG-3' (EGFP/aMMP-3 Δ NLS). We also substituted Arg¹¹⁰-Lys¹¹¹ with Asn and Gln (R110N/K111Q). The sense-strand oligonucleotide was 5'-CTGGCATCCCGAAGTGGGAATCAAACCCACCTTACATACAG-3', and anti-sense 5'-CTGTATGTAAGGTGGTTTGATTCCACTTCGGGATGCCAG-3'. To inhibit MMP-3 catalytic activity, we mutated Glu²¹⁹ as described.²⁶ For substitution of Glu²¹⁹ with Ala (E219A), the sense-strand oligonucleotide was 5'-GCTGCTCATGCAATTGGCCAC-TCCCTG-3', and the anti-sense-strand oligonucleotide was 5'-GTGGCCAATTGCATGAGCAGCAACG-3' (EGFP/aMMP-3-E219A).

Western Blot

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred to a polyvinylidene difluoride membrane by semi-dry transfer (Transblot-SD; Bio-Rad, Marnes-la-Coquette, France) as described.²⁷ The membrane was incubated with the polyclonal anti-MMP-3 antibody diluted 1:1000 followed by a peroxidase-conjugated anti-rabbit IgG antibody. Detection was achieved with the ECL reagent (Amersham Bioscience, Saclay, France). In some experiments, recombinant pro-MMP-3 was heat-activated by incubation at 55°C for 30 minutes as described²⁸ and was used as a positive control. For the control of the purity of nuclear and cytoplasmic extracts, aliquots (15 μ g) were analyzed by Western blot with antibodies to a nuclear marker (U1 small nuclear ribonucleoprotein, 1/200; Santa Cruz Biotechnology, Santa Cruz, CA) or a cytosolic marker (α -tubulin, 1/10,000; Sigma, Lyon, France).

Casein Zymography

Casein zymography was performed essentially as previously described.²⁹ In brief, β -casein (Sigma) was added to the gel mixture at a final concentration of 8 mg/ml. Samples were mixed with sample buffer (10% sodium dodecyl sulfate, 22% glycerol, 0.25 mol/L Tris-HCl, pH 6.8, and 0.1% bromophenol blue) and loaded without boiling. After electrophoresis, the gels were soaked in 2.5% Triton X-100, rinsed, and incubated for 48 hours at 37°C in substrate buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10 mmol/L CaCl₂, and 50 μ mol/L ZnSO₄). After incubation, gels were stained for 15 to 30 minutes in 0.25% Coomassie Blue R-250 in acetic acid/methanol/H₂O (1:4:5), destained, and photographed.

α -Casein Affinity Column

One hundred μ l of α -casein immobilized on cyanogen bromide-activated agarose (Sigma) were incubated with 150 μ l of nuclear extract (corresponding to 3.5×10^7

nuclei) overnight under slow shaking at 4°C. The beads were transferred to a column and interacting proteins were eluted with increasing NaCl concentrations (0.3, 0.4, 0.5, and 1 mol/L). Each fraction was concentrated 2.5-fold (Microcon column 10) and analyzed by Western blot with the anti-MMP-3 antibody.

Transfection

Transfections were performed using the Fugene 6 reagent according to instructions from the manufacturer (Roche, Basel, Switzerland). Briefly, CHO cells were seeded in 24-well plates or in 35-mm dishes at a density of $2 \times 10^4/\text{cm}^2$ on the day before transfection. Respectively, 2 or 6 μl of Fugene 6 were diluted in 100 or 300 μl of serum-free OptiMem medium (Invitrogen, Paisley, UK). DNA (0.5 or 2 μl) was added to the diluted Fugene 6 and incubated for 15 minutes at room temperature. The DNA/Fugene 6 mix was finally added to the cells.

Flow Cytometry

Nuclei were isolated from transfected cells at 3 days after transfection and were analyzed on a FACScalibur (Beckton Dickinson Bioscience, le Pont de Claix, France). To avoid leaking of nuclear proteins, nuclei were fixed immediately at the end of the isolation procedure. The nucleus population was gated on the basis of forward angle and side scatter, and a minimum of 5000 nuclei was analyzed. The mean fluorescence intensity (MFI) of EGFP was recorded and results were expressed as $\text{MFI}_{\text{NLS-EGFP}}/\text{MFI}_{\text{EGFP}}$ ratios.

Apoptotic Cell Quantification

CHO cells were transfected with plasmids coding for EGFP-aMMP-3 or EGFP-aMMP-3-E219A. In another set of experiments, cells were preincubated with a broad-spectrum MMP inhibitor (GM6001, 10 $\mu\text{mol/L}$) 2 hours before transfection. Apoptosis was detected by labeling cells with an antibody to activated caspase-3. Caspase-3-positive cells were counted under the fluorescence microscope (Zeiss Axioplan 2; objective $\times 10$), and the EGFP status of each cell was recorded. Approximately 2000 cells were counted per condition, and experiments were repeated three times for each condition.

Immunodetection of MMP-3 in Cultured Cells

Immunofluorescence

Cells on coverslips were fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.5% Triton X-100. After blocking in 4% BSA/PBS, coverslips were incubated with primary antibodies, washed, and incubated with the secondary antibody together with 4,6-diamidino-2-phenylindole (DAPI) (1 $\mu\text{g/ml}$). Antibodies used included the rabbit polyclonal anti-MMP-3 antibody, mouse monoclonal MMP-3 antibody (SC-21732, 1:30; Santa Cruz), rabbit anti-activate caspase-3 (AF835, 1:2500; R&D Systems,

Lille, France), and anti-rabbit Ig labeled with Alexa red 594 (1:300; Molecular Probes, Invitrogen). Fluorescent microscope Axioplan 2 and confocal microscope LSM Meta 510 from Zeiss were used for observation.

Immunoelectron Microscopy

Cells were incubated in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 15 minutes and then two times for 10 minutes in 100 mmol/L glycine to block unsaturated aldehyde sites. Cells were then treated with 0.1% Triton X-100. After washing in PBS, cells were incubated in 4% BSA and 0.2% Tween 20 in PBS for 30 minutes, then with the polyclonal MMP-3 antibody (1:3000) or preimmune IgG in 2% BSA and 0.2% Tween 20 in PBS for 1 hour at room temperature. Cells were washed in 2% BSA and 0.2% Tween 20 in PBS and then in PBS-BSAc-gelatin before incubation with goat anti-rabbit IgGs conjugated to ultrasmall gold particles (0.8 nm in diameter, 1:100 in PBS-BSAc-gelatin; Electron Microscopy Sciences, Harfield, PA) for 2 hours. Cells were then washed three times in PBS and postfixed in 2.5% glutaraldehyde for 10 minutes. After washing in sodium acetate buffer (0.1 mol/L, pH 7.0), the signal was enhanced with silver as above. The reaction was stopped by several washes in acetate buffer (0.1 mol/L, pH 7.0). Samples were postfixed and embedded as above.

Statistical Analysis

Data are presented as means $\pm$ SEM. A paired Student's *t*-test was used to compare the subcellular localization of MMP-3 after transfection of plasmids with or without mutations in the nuclear localization sequence, and the apoptosis rates.

Results

Immunolocalization of Endogenous MMP-3

We have previously shown that MMP-3 was overexpressed in a majority of HCCs.¹⁸ To define the distribution of tissue MMP-3 in the liver, we performed immunohistochemistry experiments. This was done with a rabbit polyclonal antibody directed against the human MMP-3 hinge region (Figure 4A). In immunoblots of conditioned medium samples, this antibody labels specifically the 57- to 60-kD pro-MMP-3 doublet and the processed 45-kD form of MMP-3 (not shown). The major finding was that MMP-3 was detected in the nuclei of tumor cells in most HCC samples (Figure 1, A and C). Although the overall staining in HCC was usually homogeneous, not all tumor cells were positively stained in a given area (especially obvious in Figure 1C). Altogether, 17 of 19 HCCs exhibited a MMP-3 nuclear staining (10 of 11 from the cirrhotic group, seven of eight from the noncirrhotic group). The matched peritumoral liver from the same patients also showed often a positive nuclear staining. However, in most cases, the staining was less homogeneous and/or intense than in the tumors (Figure 1B), and it was absent

Figure 2. MMP-3 is present in the nucleus of cultured cells. Immunodetection of MMP-3 in HepG2 cells and liver myofibroblasts. **A:** HepG2 nuclei were stained with DAPI. **B:** MMP-3 was detected using a polyclonal antibody. **C:** Merging of the signals confirms the mainly nuclear staining for MMP-3 in HepG2 cells. **D:** Myofibroblast nuclei were stained with DAPI. **E:** Immunofluorescent detection of MMP-3 in myofibroblasts with a monoclonal antibody directed against the proximal part of the hemopexin-like domain of MMP-3 (Santa Cruz 21732). **F:** Merging of the signals confirms the combined nuclear and cytoplasmic staining for MMP-3 in myofibroblasts. **G–I:** Double immunolabeling of myofibroblasts with the Santa Cruz 6839 antibody (**G**) showing only a cytoplasmic staining and with the polyclonal Chemicon antibody (**H**) showing a nuclear and cytoplasmic staining; **I** illustrates the nuclei stained with DAPI. **J:** Immunodetection of MMP-3 by confocal microscopy in myofibroblasts using the polyclonal antibody. **K:** Immunoelectron microscopy shows a strong nuclear labeling as well as some cytoplasmic staining for MMP-3 in a cultured myofibroblast.

in some cases (Figure 1D). Altogether in the peritumoral areas, the nuclear staining was seen in 6 of 11 cases in the cirrhotic group and in six of eight cases in the non-cirrhotic group (not different from HCC samples by χ^2 analysis). Immunoelectron microscopy confirmed the nuclear staining for MMP-3 in a case of HCC (Figure 1, E–H). Finally, positive staining was also seen in four of four cases of histologically normal liver surrounding metastasis; however, the staining was primarily heterogeneous with an enhancement in periportal areas with the remaining liver being mostly negative (not shown).

In addition to the nuclear staining, cytoplasmic staining was seen in scattered cells in portal tracts from noncirrhotic livers and within fibrous tracts of cirrhotic samples. These cells did not stain with markers of macrophages,

lymphocytes, mastocytes, or endothelial cells. However, they could be conclusively identified as plasmacytes based on a positive staining for syndecan-1, which confirms a previous report³⁰ (not shown). No staining was seen in the absence of primary antibody or when it was replaced by a nonrelevant IgG. Moreover, when the antibody was preadsorbed on recombinant MMP-3, the staining was abolished (Supplemental Figure 1 at <http://ajp.amjpathol.org>).

These findings were confirmed on cultured cells. We have previously shown that HepG2 human HCC cells express MMP-3 transcripts and secrete MMP-3 in their culture medium.¹⁸ We first used the rabbit polyclonal antibody directed against the human MMP-3 hinge region. Fluorescence microscopy analysis showed a pre-

Figure 1. Immunodetection of MMP-3 in the nuclei of hepatocytes in human liver. **A:** Immunostaining of a HCC section in a cirrhotic patient shows staining in a majority of tumor cells. **B:** Immunostaining of the peritumoral part of the same patient shows a much lighter staining than in the tumor. **C:** Immunostaining of a HCC section in a noncirrhotic patient shows staining in a majority of tumor cells. **D:** Immunostaining of the peritumoral part of the same patient does not show any nuclear staining. **E:** Immunoelectron microscopy: several tumoral hepatocytes show a nuclear labeling for MMP-3. **F:** Higher magnification of the zone delimited in **E**. **G:** Negative control for immunoelectron microscopy with a control rabbit IgG. **H:** Higher magnification of the zone delimited in **G** clearly shows the lack of any nuclear staining.

Figure 3. Biochemical characterization of nuclear MMP-3. **A:** Control for the purity of the nuclear and cytoplasmic fractions. Equal amounts (15 μ g) of nuclear (N) or cytoplasmic (C) proteins were analyzed by Western blotting with antibodies to U1 small nuclear ribonucleoprotein (top) or α -tubulin (bottom). **B:** Western blot analysis of HepG2 cytosolic (C) and nuclear (N) extracts using anti MMP-3 antibody. The apparent molecular mass of the proteins (in kd) is indicated by arrows. **C:** MMP-3 Western blot. **Lane 1:** Recombinant pro-MMP-3 partially activated by heat treatment showing the presence of the cleaved 45- and 35-kd forms. **Lanes 2 to 6:** Nuclear extracts from myofibroblasts, HT1080, Hep3B, HuH7, and HepG2 cells. Molecular weight markers are indicated on the left. The arrows on the right side point to the 45- and 35-kd forms of MMP-3, and the asterisk indicates pro-MMP-3. **D:** β -Casein substrate gel zymography. The control lane contains myofibroblast-conditioned medium showing the expected 57- to 60-kd doublet. Other lanes contain increasing amounts of HepG2 nuclear extract (50, 100, or 150 μ g). **E:** HepG2 nuclear extracts were chromatographed on an immobilized α -casein column and fractions analyzed by Western blot with the MMP-3 antibody. **Lane 1:** initial input; **lanes 2 and 3:** PBS washes; **lanes 4–7:** 0.3, 0.4, 0.5, and 1 mol/L NaCl eluted fractions, respectively.

dominant nuclear distribution of MMP-3 in HepG2 cells (Figure 2, A–C). Identical results were obtained in primary human liver myofibroblasts with the same antibody (not shown) and also when using a monoclonal antibody directed against the proximal part of the hemopexin-like domain of MMP-3 (Santa Cruz 21732; Figure 2, D–F). In contrast, no nuclear staining was seen with antibodies directed to amino- or carboxy-terminal extremities of MMP-3 (Sigma M-5032 and Santa Cruz 6839, respectively), although these antibodies correctly labeled cytoplasmic MMP-3 in myofibroblasts (Figure 2, G–I). The polyclonal antibody was preferred for further studies because it gave a more intense staining. No staining was seen when primary antibodies were omitted or replaced with a species-matched IgG. The nuclear localization of MMP-3 was confirmed in experiments that used confocal microscopy (Figure 2J) and also with immunoelectron microscopy. Thus, Figure 2K clearly shows nuclear staining of cultured human hepatic myofibroblasts with the MMP-3 antibody. In contrast, a β -tubulin antibody showed the expected cytoskeletal staining (data not shown).

Cell Fractionation Studies

HepG2 cells were fractionated into nuclear and cytosolic fractions. The absence of cross-contamination was checked by Western blotting with antibodies directed to specific markers, the U1 small nuclear ribonucleoprotein

for the nuclear compartment and α -tubulin for the cytosol (Figure 3A). In addition, examination of cyto-centrifuged aliquots confirmed the integrity of nuclei and that they were free of cytoplasmic debris (Supplemental Figure 2 at <http://ajp.amjpathol.org>). Western blot analysis of nuclear extracts showed two major immunoreactive bands of 45 kd and 35 kd (Figure 3B). MMP-3 is known to be processed into a 45-kd form resulting from the cleavage of the signal peptide and the prodomain^{28,31} (Figure 4A). A 35-kd processed form has also been described.³² In our experiments, the 45-kd form was detected in both nuclear and cytoplasmic extracts, whereas the 35-kd form was present only in nuclei and could thus be a specific nuclear form. Both these forms can be seen with recombinant pro-MMP-3 activated *in vitro* by heat treatment (Figure 3C, lane 1) and they co-migrate with the 45- and 35-kd forms found in nuclear extracts from a variety of cultured cells (Figure 3C, lanes 2 to 6).

Functionality of Nuclear MMP-3

The functionality of nuclear MMP-3 was evaluated with β -casein substrate gel zymography. Figure 3D shows that nuclear extracts contained a caseinase activity migrating at an approximate molecular weight of 35 kd. To confirm that this 35-kd band corresponded indeed to MMP-3, casein-binding proteins in nuclear extracts from the human HCC cell line HepG2 were enriched using an affinity column of immobilized α -casein. Western blot analysis detected a sin-

Figure 4. MMP-3 nuclear translocation depends on a functional NLS. **A:** Schematic representation of MMP-3 module organization. **B:** CHO cells were transiently transfected with a vector encoding EGFP/aMMP-3. Top: Transfected cells were observed by confocal microscopy. Arrows indicate fluorescent nuclei; bottom: the number of cells with the different subcellular fluorescence patterns was counted 24 hours after transfection ($n = 3$) (white bar, cytosolic; black bar, nuclear; gray bar, both). **C:** CHO cells were transiently transfected with a vector encoding EGFP/pro-MMP-3. Top: Transfected cells were observed by confocal microscopy. Arrows indicate fluorescent nuclei; bottom: the number of cells with the different subcellular fluorescence patterns was counted 24 hours after transfection ($n = 3$) (white bar, cytosolic; black bar, nuclear; gray bar, both). **D:** Alignment of a portion of the amino acid sequence of human, rat, and mouse MMP-3 illustrates the conservation of the putative NLS between species. **E:** Comparison of the subcellular localization of MMP-3 in cells transfected with either EGFP/a-MMP-3 (left) or EGFP/aMMP-3ΔNLS (right). The number of cells with the different fluorescence patterns was counted 24 hours after transfection ($n = 3$) (white bar, cytosolic; black bar, nuclear; gray bar, both). **F:** CHO cells were transfected with EGFP fused to MMP-3 or Tag SV40 NLS. Isolated nuclei were analyzed by flow cytometry, the MFI of EGFP was recorded and results were expressed as MFI.NLS-EGFP/MFI.EGFP ratios ($n = 3$).

gle immunoreactive 35-kD band in the eluted fractions with the MMP-3 antibody (Figure 3E). Taken together, these experiments demonstrate that nuclear extracts contain two immunoreactive MMP-3 fragments and that only the 35-kD protein seems to retain a proteolytic activity.

Mechanisms of the Nuclear Localization of MMP-3

MMP-3 is synthesized as a preproenzyme (Figure 4A) containing a signal peptide, and the final destination of this molecule would thus be the extracellular space. The apparent molecular mass of nuclear MMP-3, the fact that it has enzymatic activity, together with the indications obtained from the use of antibodies directed against different epitopes, indicate that nuclear MMP-3 contains the catalytic domain and at least a part of the hemopexin domain. To gain insight into the mechanisms governing the nuclear translocation of MMP-3, we constructed a chimera in which EGFP is fused to the N terminus of the processed, 45-kD active fragment of MMP-3 (EGFP/

aMMP-3) (Figure 4B) and examined the subcellular localization of the protein by fluorescence microscopy 24 hours after transfection of CHO cells. We observed a nuclear localization of the chimeric construct in most cells, either strictly nuclear in $61.8 \pm 5.9\%$ of the transfected cells or mixed nuclear and cytosolic in $21.1 \pm 6.4\%$ of cells (Figure 4B). On the other hand, when EGFP was fused to the N-terminal region of the prepro-MMP-3 (EGFP/pro-MMP-3), the localization of the chimeric protein was exclusively cytoplasmic in $53.4 \pm 3.1\%$ of transfected cells (Figure 4C), mixed nuclear and cytosolic in $36.0 \pm 5.4\%$ of cells, and strictly nuclear in only $9.9 \pm 1.6\%$ of cells.

In most cases, proteins enter the nucleus through the nuclear pore via a mechanism involving recognition of NLSs by transporter proteins.³³ The bioinformatics software PSORT (<http://psort.hgc.jp/>)³⁴ detected the putative NLS (PKWRKTH) at position 107 to 113. Alignment of the human sequence with rat and mouse sequences showed a very good conservation of this motif, suggesting its biological relevance (Figure 4D). As shown in Figure 4E,

Figure 5. Transfection of EGFP/aMMP-3 increases the apoptotic index. The apoptotic index was measured by counting caspase 3-positive cells in transfected (GFP+) or untransfected cells (GFP-) in the same microscopic fields. At least 2000 cells were counted for each setting, and the results are the mean $\pm$ SEM of $n = 3$ experiments.

deletion of two amino acids of the putative NLS led to a large decrease in the nuclear localization of the chimeric proteins because only $12.4 \pm 2.4\%$ of the transfected cells showed a nuclear fluorescence ($P = 0.01$ as compared with the wild-type construct, $n = 3$). Similar results were observed after substitution of the same amino acids with Asn and Gln (R110N/K111Q) (data not shown).

The functionality of the MMP-3 NLS was further tested in experiments in which we evaluated its ability to drive a nonrelated protein to the nucleus. Thus, we fused the sequence of the putative NLS of MMP-3 downstream of the sequence of EGFP. Because some EGFP is able to enter the nucleus without an NLS because of its small mass, we used flow cytometry, which gives more quantitative results than fluorescence microscopy, and we compared the fluorescence of isolated nuclei from cells transfected with the different plasmids. We observed approximately a sixfold increase of nuclear mean fluorescence intensity from cells expressing EGFP-NLS-MMP3 fusion protein as compared with EGFP alone (Figure 4F; $P = 0.025$, $n = 3$). In comparison, the SV40 Tag NLS used as a control increased this figure by threefold. These results show that the MMP-3 NLS is functional and suggest that the nuclear translocation of MMP-3 is operated by an NLS-dependent mechanism.

Correlation between Nuclear Localization of MMP-3 and Apoptosis

In the course of our experiments, we noticed that many cells transfected with EGFP/aMMP-3 had fragmented nuclei characteristic of apoptosis. To quantify this observation, we labeled cells with an antibody directed against activated caspase-3. As a control for the effects of transfection, we used cells that were transfected with a plasmid coding a fusion of EGFP to the N-terminus of pro-MMP-3 (EGFP/pro-MMP-3). Western blot showed that the

Figure 6. The proapoptotic effect of MMP-3 is dependent on its protease activity. **A:** Cells were transfected with either EGFP/aMMP-3 or EGFP/aMMP-3-E219A. The apoptotic index was measured as in Figure 5. **B:** Cells were transfected with EGFP/aMMP-3 in the absence or in the presence of the broad spectrum MMP inhibitor GM6001 (10 μ mol/L).

resulting protein does not undergo signal peptide cleavage, likely as a result of conformational changes imposed by the presence of EGFP, and thus remains cytosolic in most cells as shown by fluorescence microscopy (data not shown). As shown on Figure 5, untransfected (GFP-) cells had a low rate of basal apoptosis. Although transfection efficiency was similar with both plasmids, cells transfected with EGFP/aMMP-3 had a more than 20% apoptotic rate, which was twice as high as that of EGFP/pro-MMP-3 cells ($P = 0.008$). These data suggest that the nuclear localization of MMP-3 is associated with an increased rate of apoptosis.

We next questioned the role of the proteolytic activity of nuclear MMP-3 in apoptosis induction. First, we constructed the E219A catalytic domain mutant that lacks catalytic activity.²⁶ This mutation did not modify the sub-cellular localization of the encoded protein that remained predominantly nuclear, and transfection efficiency was similar to that of the native plasmid (not shown). As shown in Figure 6A, transfection of the mutant significantly reduced the apoptotic index ($P = 0.013$, $n = 4$). In a second series of experiments, cells were treated with the broad-spectrum MMP inhibitor GM6001 for 2 hours before transfection with the EGFP/aMMP-3 construct. As compared with untreated cells, treatment with GM6001 significantly reduced the apoptotic index of cells ($P = 0.03$, $n = 3$) (Figure 6B). Actually, the apoptosis rate after inhibition of MMP-3 activity was close to that of cells transfected with EGFP/pro-MMP-3 (compare Figure 6 with Figure 5). These results underlie a link between the presence of active MMP-3 in the cell nucleus and its pro-apoptotic potential and suggest that this property is attributable to MMP-3 catalytic activity.

Discussion

This is the second study that reports the presence of an MMP in the cell nucleus and the first to elucidate the mechanisms of its nuclear translocation and give a hint about its function. Nuclear localization of MMP-3 was demonstrated by immunofluorescence, laser confocal

microscopy, and immunoelectron microscopy not only in cultured cells but also in human liver tissue. These results were obtained with two different antibodies that target the same part of the molecule (hinge region and proximal part of the hemopexin-like domain). Two nuclear immunoreactive bands of 45 and 35 kd were characterized. Previous publications have identified MMP-3 maturation products corresponding to both these bands.^{28,31} The 45-kd form corresponds to the catalytic domain, the hinge region, and the C-terminal domain of MMP-3.^{28,31} A protein with an estimated molecular mass of 35 kd on sodium dodecyl sulfate gels was also found to degrade casein in zymography assays.^{32,35} Its domain composition is unknown, but it may be identical to the 28-kd (as estimated by size exclusion chromatography) form purified by Okada and colleagues.⁵ This 28-kd form has the same N terminus as the 45-kd form, contains the catalytic domain but lacks the hemopexin domain.^{28,36} Our data also indicate that the 35-kd form contains the catalytic domain of MMP-3 because it is able to degrade casein. Surprisingly, we did not detect an enzymatic activity associated with the 45-kd form. Moreover, using a casein affinity column, we purified the 35-kd form but not the 45-kd form. We hypothesize that the mild denaturing conditions used for nuclear extract preparation and for zymography are not sufficient to prevent interaction of this protein with a partner that can prevent the 45-kd form from interacting with its substrate or degrade it.

We next confirmed that the active form of MMP-3 is efficiently transported into the nucleus. This was shown in experiments in which EGFP was fused to the N terminus of the active 45-kd form of MMP-3. Transfection and fluorescence microscopy examination showed clearly that EGFP/aMMP-3 was highly enriched in the nucleus. Most proteins larger than 20 to 40 kd³⁷ enter the nucleus through a carrier-mediated mechanism involving the recognition of NLSs by specialized proteins.³³ Bioinformatic analysis of the human MMP-3 sequence showed a putative NLS that was well conserved between human, mouse, and rat, suggesting its functionality. Point mutation or deletion of this NLS dramatically disturbed the nuclear localization of MMP-3. In addition, fusion of the MMP-3 NLS to the unrelated protein EGFP increased the transport of the EGFP chimera to the nucleus and greatly increased the mean fluorescence of nuclei, indicating the functionality of this NLS. In our hands, very little if any, pro-MMP-3 was found in the nucleus. We hypothesize that the NLS, like the catalytic cleft, is shielded by the prodomain, preventing its interaction with the nuclear pore complex. Recently, Kwan and colleagues¹⁴ found full-length pro-MMP-2 in the nucleus of cardiomyocytes and in rat liver extracts. Pro-MMP-2 carries a putative NLS on its C terminus that should be accessible without the need of propeptide cleavage, thus allowing transfer of the full-length protein to the nucleus. The ability to translocate to the nucleus may be a feature of many MMPs. Indeed, we have analyzed the sequence of human MMPs and found that, besides MMP-2 and MMP-3, MMPs 1, 8, 10, 13, 14, 16, 17, 19, 20, 23A, and 24 carry some type of putative NLS.

Several explanations can be proposed to understand why a short MMP-3 form is found in the nucleus. First, it could be the result of alternative splicing of the primary pro-MMP-3 transcript or of alternative promoter usage. Such a mechanism was recently shown to account for the synthesis of an active intracellular form of MMP-11/stromelysin 3 (ST3).¹² Second, pro-MMP-3 could be cleaved intracellularly. We favor this hypothesis because it was recently shown that MMP-3 was a substrate for the intracellular proprotein convertase furin that cleaved pro-MMP-3 into its active 45-kd form.³⁸ Further experiments need to address this point in more detail and also to explain the generation of the 35-kd form.

Finally, our data suggest that nuclear MMP-3 is associated with the onset of apoptosis. Indeed, cells expressing a nuclear form of MMP-3 contained a higher fraction of apoptotic cells than control cells. This was shown with immunostaining for activated caspase 3 that allowed precise definition of the apoptotic status of a cell while knowing at the same time whether it was transfected or not; biochemical assessment of apoptosis on the whole cell population proved unfeasible because of insufficient transfection efficiency with the MMP-3 plasmids. Moreover, evidence directly incriminates the metalloproteinase activity of MMP-3 in apoptosis induction. First, transfection of a mutated form of MMP-3, devoid of catalytic activity, greatly reduced the apoptotic rate. Secondly, the MMP inhibitor GM6001 also reduced the proapoptotic effect of MMP-3. Although GM6001 could have protective effects by other mechanisms, these combined arguments suggest that nuclear MMP-3 induces apoptosis via its MMP activity. In addition, these two experiments discount the hypothesis that the increased apoptotic rate was attributable only to the overexpression of a foreign protein in the nucleus, because in these experiments, the amount of nuclear proteins was the same in all conditions. As mentioned, MMP-2 also accumulates in the nucleus, although its translocation mechanism was not demonstrated.¹⁴ Purified MMP-2 was able to cleave PARP *in vitro*, thus suggesting a possible contribution of MMP-2 to apoptosis induction. Whether MMP-2 cleaves PARP *in vivo*, however, remains to be demonstrated. On the other hand, Limb and colleagues³⁹ have found very recently that intracellular MMP-1 was actually protective against apoptosis. A new concept is therefore emerging whereby intracellular MMPs may regulate apoptosis. Relationships between MMPs and apoptosis have been primarily shown but have been so far ascribed to extracellular proteolysis. Our data underscore the new concept that proteolysis of nuclear substrates by MMPs could result in apoptosis. It has been shown that overexpressing MMP-3 in mouse mammary gland leads to spontaneous breast cancers associated with stereotypical genetic changes.⁴⁰ Recently, this has been ascribed to enhanced intracellular levels of H₂O₂ through increased Rac1b expression.⁴¹ We hypothesize that in addition, nuclear MMP-3 could also damage the genomic environment, causing apoptosis of some cells, whereas damaged cells defective for apoptosis will be selected for tumorigenesis. This is consistent with the frequent finding of nuclear MMP-3 in samples of cirrhosis, a well-

known precancerous lesion. Persistent expression of nuclear MMP-3 in tumor cells may further damage the nuclear matrix and contribute to the accumulation of genetic and epigenetic abnormalities. The identification of the nuclear substrates of MMP-3 should shed light on these important issues.

Acknowledgments

We thank Vincent Pitard and Sophie Daburon for help in flow cytometry experiments, Evelyne Doudnikoff for immunoelectron microscopy, Véronique Neaud for immunofluorescence, and Nathalie Dugot-Senant for confocal microscopy.

References

- Massova I, Kotra LP, Fridman R, Mobashery S: Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J* 1998, 12:1075–1095
- Nagase H, Woessner JR Jr: Matrix metalloproteinases. *J Biol Chem* 1999, 274:21491–21494
- Sternlicht MD, Werb Z: How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001, 17:463–516
- Chang C, Werb Z: The many faces of metalloproteinases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol* 2001, 11:S37–S43
- Okada Y, Nagase H, Harris ED: A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. *J Biol Chem* 1986, 261:14245–14255
- Jones CB, Sane DC, Herrington DM: Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. *Cardiovasc Res* 2003, 59:812–823
- Noel A, Gilles C, Bajou K, Devy L, Kebers F, Lewalle JM, Maquoi E, Munaut C, Remacle A, Foidart JM: Emerging roles for proteinases in cancer. *Invasion Metastasis* 1997, 17:221–239
- Vaillant B, Chiaramonte MG, Cheever AW, Soloway PD, Wynn TA: Regulation of hepatic fibrosis and extracellular matrix genes by the th response: new insight into the role of tissue inhibitors of matrix metalloproteinases. *J Immunol* 2001, 167:7017–7026
- Bode W: A helping hand for collagenases: the haemopexin-like domain. *Structure* 1995, 3:527–530
- Ottl J, Gabriel D, Murphy G, Knauper V, Tominaga Y, Nagase H, Kroger M, Tschesche H, Bode W, Moroder L: Recognition and catabolism of synthetic heterotrimeric collagen peptides by matrix metalloproteinases. *Chem Biol* 2000, 7:119–132
- Van Wart HE, Birkedal-Hansen H: The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* 1990, 87:5578–5582
- Luo D, Mari B, Stoll I, Anglard P: Alternative splicing and promoter usage generates an intracellular stromelysin 3 isoform directly translated as an active matrix metalloproteinase. *J Biol Chem* 2002, 277:25527–25536
- Golubkov VS, Boyd S, Savinov AY, Chekanov AV, Osterman AL, Remacle A, Rozanov DV, Doxsey SJ, Strongin AY: Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits an important intracellular cleavage function and causes chromosome instability. *J Biol Chem* 2005, 280:25079–25086
- Kwan JA, Schulze CJ, Wang W, Leon H, Sariahmetoglu M, Sung M, Sawicka J, Sims DE, Sawicki G, Schulz R: Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly (ADP-ribose) polymerase (PARP) in vitro. *FASEB J* 2004, 18:690–692
- Sternlicht MD, Lochter A, Simpson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z: The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999, 98:137–146
- Bergers G, Coussens LM: Extrinsic regulators of epithelial tumor progression: metalloproteinases. *Curr Opin Genet Dev* 2000, 10:120–127
- Okada Y, Harris ED, Nagase H: The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts. Purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate. *Biochem J* 1988, 254:731–741
- Monvoisin A, Bisson C, Si-Tayeb K, Balabaud C, Desmouliere A, Rosenbaum J: Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells. *Int J Cancer* 2002, 97:157–162
- Dumartin B, Jaber M, Gonon F, Caron MG, Giros B, Bloch B: Dopamine tone regulates D1 receptor trafficking and delivery in striatal neurons in dopamine transporter-deficient mice. *Proc Natl Acad Sci USA* 2000, 97:1879–1884
- Win KM, Charlotte F, Mallat A, Cherqui D, Martin N, Mavie P, Preaux AM, Dhumeaux D, Rosenbaum J: Mitogenic effect of transforming growth factor-beta 1 on human Ito cells in culture: evidence for mediation by endogenous platelet-derived growth factor. *Hepatology* 1993, 18:137–145
- Blazewski S, Preaux AM, Mallat A, Brocheriou I, Mavie P, Dhumeaux D, Hartmann D, Schuppan D, Rosenbaum J: Human myofibroblastlike cells obtained by outgrowth are representative of the fibrogenic cells in the liver. *Hepatology* 1995, 22:788–797
- Schreiber E, Matthias P, Muller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989, 17:6419
- Murai N, Murakami Y, Matsufuji S: Identification of nuclear export signals in antizyme-1. *J Biol Chem* 2003, 278:44791–44798
- Kalderon D, Roberts BL, Richardson WD, Smith AE: A short amino acid sequence able to specify nuclear location. *Cell* 1984, 39:499–509
- Xiao Z, Liu X, Henis YI, Lodish HF: A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc Natl Acad Sci USA* 2000, 97:7853–7858
- Arza B, De Maeyer M, Felez J, Collen D, Lijnen HR: Critical role of glutamic acid 202 in the enzymatic activity of stromelysin-1 (MMP-3). *Eur J Biochem* 2001, 268:826–831
- Blanc JF, Lalanne C, Plomion C, Schmitter JM, Bathany K, Gion JM, Bioulac-Sage P, Balabaud C, Bonneau M, Rosenbaum J: Proteomic analysis of differentially expressed proteins in hepatocellular carcinoma developed in patients with chronic viral hepatitis C. *Proteomics* 2005, 5:3778–3789
- Koklit PA, Murphy G, Sutton C, Angal S: Purification of recombinant human prostromelysin. Studies on heat activation to give high-Mr and low-Mr active forms, and a comparison of recombinant with natural stromelysin activities. *Biochem J* 1991, 276:217–221
- Suzuki M, Raab G, Moses MA, Fernandez CA, Klagsbrun M: Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. *J Biol Chem* 1997, 272:31730–31737
- Di Girolamo N, Tedla N, Lloyd A, Wakefield D: Expression of matrix metalloproteinases by human plasma cells and B lymphocytes. *Eur J Immunol* 1998, 28:1773–1784
- Nagase H, Enghild JJ, Suzuki K, Salvesen G: Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. *Biochemistry* 1990, 29:5783–5789
- Chin JR, Murphy G, Werb Z: Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization, and substrates. *J Biol Chem* 1985, 260:12367–12376
- Mattaj JW, Englmeier L: Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* 1998, 67:265–306
- Nakai K, Kanehisa M: A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 1992, 14:897–911
- Newman KM, Ogata Y, Malon AM, Irizarry E, Gandhi RH, Nagase H, Tilson MD: Identification of matrix metalloproteinases 3 (stromelysin-1) and 9 (gelatinase B) in abdominal aortic aneurysm. *Arterioscler Thromb* 1994, 14:1315–1320
- Nagase H: Human stromelysins 1 and 2. *Methods Enzymol* 1995, 248:449–470
- Peters R: Fluorescence microphotolysis to measure nucleocytoplasmic transport. *Methods Enzymol* 1995, 248:449–470

- mic transport and intracellular mobility. *Biochim Biophys Acta* 1986, 864:305–359
38. Cao J, Rehemtulla A, Pavlaki M, Kozarekar P, Chiarelli C: Furin directly cleaves proMMP-2 in the trans-Golgi network resulting in a nonfunctioning proteinase. *J Biol Chem* 2005, 280:10974–10980
 39. Limb GA, Matter K, Murphy G, Cambrey AD, Bishop PN, Morris GE, Khaw PT: Matrix metalloproteinase-1 associates with intracellular organelles and confers resistance to lamin A/C degradation during apoptosis. *Am J Pathol* 2005, 166:1555–1563
 40. Sternlicht MD, Lochter A, Simpson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z: The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999, 98:137–146
 41. Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ: Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 2005, 436:123–127