The structure and regulation of the human and mouse matrix metalloproteinase-21 gene and protein

George N. MARCHENKO, Natalia D. MARCHENKO and Alex Y. STRONGIN[1]

Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Matrix metalloproteinases (MMPs) play key roles in tissue remodelling under normal development and, especially, in diseases ranging from malignancies to stroke. We cloned and thoroughly characterized the novel human and mouse MMP gene encoding MMP-21. MMP-21 is the last uncharacterized MMP coded by the human genome. Human and mouse MMP-21 is the orthologue of *Xenopus laevis* X-MMP. The latent proenzyme of MMP-21 (569 amino acid residues) consists of the prodomain, the catalytic domain and the haemopexin-like domain, and is potentially capable of being activated in its secretory pathway to the extracellular milieu by furin-like proprotein convertases. Human MMP-21 is the probable target gene of the Wnt pathway. In addition, the expression of MMP-21 is controlled uniquely by Pax and Notch transcription factors known to be critical for organogenesis. MMP-21 is expressed transiently in mouse embryogenesis and increased in embryonic neuronal tissues. Our observations clearly indicate that there is an important specific function for MMP-21 in embryogenesis, especially in neuronal cells.

Key words: embryogenesis, extracellular matrix, glial tissue, human genome, matrix metalloproteinase-21, neuronal tissue.

INTRODUCTION

The matrixins or matrix metalloproteinases (MMPs) are known to degrade extracellular matrix proteins and to perform key functions in tumour progression, and in other diseases ranging from arthritis to stroke [1–4]. Certain MMPs have demonstrated the ability to modulate the activity of growth factors or their receptors, cytokines and adhesion integrins, and CD44 receptors [5–7]. To date, 23 individual human MMPs have been cloned and characterized partially. MMPs share a modular domain structure and consist of at least two essential domains, the prodomain and the catalytic domain. The catalytic domain of all known MMPs contains an active-site zinc that binds to three conserved histidine residues in the HE*XX*H*XX*G*XX*HS/T$(X)_5$M zinc-binding motif (where *X* is any amino acid). With the exception of MMP-23 [8] and MMP-26 [9], the prodomain of all known MMPs has a functional, highly conserved PRCXXPD sequence motif. This motif, called a cysteine switch, prevents latent proenzymes from becoming activated by binding to the catalytic zinc atom. The cysteine residue of the PRCXXPD motif can interact with the zinc ion of the catalytic domain and provide the fourth co-ordination site for the catalytic zinc ion. The cysteine residue may act in concert with other residues to maintain the conformation of the prodomain and shield the active site of the enzyme from substrates. All MMPs are synthesized as latent zymogens, which require proteolytic activation to remove the N-terminal prodomain. The release of the prodomain exposes the active site of the enzyme and generates the respective enzyme forms with complete functional activity. The prodomain of MMP-26 exhibits the dysfunctional PHCXXPD peptide sequence, which has no role in the proenzyme activation [10,11].

With the exception of matrilysins-1 and -2 (MMP-7 and MMP-26 respectively), MMPs have a haemopexin-like C-terminal domain which is essential for the regulation of activity and specificity of the enzyme's function. In the structure of certain MMPs, the haemopexin-like domain is separated from the catalytic domain by a proline-rich hinge region [12]. Membrane-tethered MMPs are distinguished by an additional transmembrane domain and a cytoplasmic domain (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP; where MT stands for membrane type), or a C-terminal hydrophobic segment that acts as a glycosylphosphatidylinositol anchor to link the protein molecule to the plasma membrane (MT4-MMP and MT6-MMP) [1]. The gelatinases (MMP-2 and MMP-9) have fibronectin-like gelatin-binding domains inserted into their catalytic domains. MMPs demonstrate a similar genomic arrangement, suggesting that they have evolved from the same ancestral gene. At least eight of the known human MMP genes are clustered in chromosomes 11q21–q23 (MMPs 1, 3, 7, 8, 10, 12, 13 and 20). Other known MMP genes are scattered among chromosomes 1, 8, 10, 12, 14, 16, 17, 20 and 22.

The purpose of the present study is to characterize the structure–function relationships and the regulation of the final member of the human MMP family, MMP-21. Human and mouse MMP-21 is the orthologue of X-MMP [13] and Cy-MMP [14], cloned previously in *Xenopus laevis* and *Cynops pyrrhogaster* (Japanese fire-bellied newt) respectively. Our results lead us to believe that MMP-21 has an important specific function in tumour progression and embryogenesis. These results confirm and extend the very recent findings of other authors [15].

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and all other reagents for molecular cloning were from

Abbreviations used: APMA, 4-aminophenylmercuric acetate; EMSA, electrophoretic mobility-shift assay; GSK, glycogen synthase kinase; HRP, horseradish peroxidase; LEF-1, lymphoid enhancer factor-1; MMP, matrix metalloproteinase; MT, membrane type; RACE, rapid amplification of cDNA ends; RBP-Jκ, repressor-binding protein Jκ; Tcf, T-cell factor.

[1] To whom correspondence should be addressed (e-mail strongin@burnham.org).

The nucleotide sequence data reported in this paper will appear in GenBank® Nucleotide Sequence Database under accession numbers AF520613, AY121358 and AY124569.

New England Biolabs (Beverly, MA, U.S.A.). Most chemicals were purchased from Sigma. Thermostable AdvanTaq™ Plus DNA polymerase was from ClonTech Laboratories (Palo Alto, CA, U.S.A.). Proof-reading Pfx DNA polymerase and cell culture media were from Invitrogen (Carlsbad, CA, U.S.A.). Radiochemicals, such as [γ-^{32}P]ATP and [α-^{32}P]ATP, were purchased from PerkinElmer (Boston, MA, U.S.A.). HeLa-cell nuclear extracts were from Promega (Madison, WI, U.S.A.). Synthetic oligonucleotides were from MWG-Biotech (High Point, NC, U.S.A.). A rabbit antibody RP3MMP-21 against the C-terminal region of the catalytic domain of human MMP-21 was a gift from Dr Preston Alexander (Triple Point Biologics, Portland, OR, U.S.A.). The specificity of the antibody was demonstrated by Western blotting.

Computer analysis

The gene and protein structures were analysed using the DNAstar software package (DNASTAR, Madison, WI, U.S.A.). MMP-21 signal peptide was identified, and its cleavage site was predicted using the SignalP server. MatInspector (http://www.genomatix.de) software package was employed for the identification of the putative transcription-factor-binding sites in the promoter regions of MMPs. A SSAHA (Sequence Search and Alignment by Hashing Algorithm; http://www.sanger.ac.uk/Software/analysis/SSAHA) search of the mouse genome database (http://www.ensembl.org/Mus_musculus) was performed with the exon coding sequences of the human MMP-21 gene. The highest alignment score was observed for the sequence region between 123 622 399 and 123 722 398 bp of the mouse chromosome 7. Our work has confirmed that this region includes the mouse MMP-21 gene.

Rapid amplication of cDNA ends (RACE)

The full-length human and mouse MMP-21 cDNA was obtained using a human Foetal Brain Marathon-Ready cDNA and mouse 11-day Embryo Marathon-Ready cDNA (ClonTech Laboratories) respectively. The 216 bp initial fragment of the human MMP-21 was extended in the RACE experiments. For the cloning of the mouse MMP-21 cDNA gene, we used the forward and reverse primers (M-mmp21-6-f and M-mmp21-end-r respectively), complementary to the predicted exon no. 6 and to the 3′-end of the gene. Following PCR amplification, the fragment was extended in the successive RACE experiments. Each successive cycle of RACE allowed for a 100–300 bp extension. After cloning and sequencing of the amplified products, new respective specific primers were synthesized and used for the next RACE experiment. The full-length cDNA from several independent individual clones was sequenced to exclude any PCR errors. All nucleotides were read in both strands using the BigDye Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). The MMP-21 sequence has been submitted to GenBank® under the accession numbers AF520613, AY121358 and AY124569 for the complete coding sequence of human MMP-21 mRNA, human genomic MMP-21 gene and mouse MMP-21 mRNA respectively.

Primer-extension analysis

The transcription start of the human MMP-21 gene was identified by primer-extension analysis. For this purpose, breast carcinoma MCF7 cells were transfected transiently with the pLUC21-{−1004} vector. The total cellular RNA was isolated, 24 h after transfection, from the transfected cells using the RNAqueous-4PCR kit (Ambion, Austin, TX, U.S.A.). Each of the mmp21-15-r and mmp21-17-r oligonucleotide primers was 5′-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. An aliquot (10 μg) of total RNA was mixed with the labelled primer (10^5 c.p.m.) and incubated for 3 min at 70 °C. Then the temperature was decreased slowly to 42 °C to allow the primer to hybridize with the target mRNA. The mRNA was reverse-transcribed for 1 h with avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN, U.S.A.). Next, the RNA was degraded by incubating the samples at 37 °C for 20 min with 1 μg/ml RNase (Ambion). The resulting DNA products generated by primer-extension reaction were analysed by electrophoresis on denaturing 6 % polyacrylamide gel. The stepwise sequencing ladder was prepared by using the fmol DNA Cycle Sequencing System (Promega) with M13 forward primer and the pGEM-3Zf(+) plasmid as template.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described previously [16] but with minor modifications. The [γ-^{32}P]ATP double-stranded specific (mmp21{−212-f}/mmp21{−212-r}) and mutant (mmp21-{−212m-f}/mmp21{−212m-r}) probes, purified from excess label with a DNAce Dye-Strip kit (Bioline, Randolph, MA, U.S.A.), were co-incubated with 2 μg of HeLa nuclear extract (Promega). Where indicated, a 100–150-fold molar excess of unlabelled specific or mutant double-stranded competitor oligonucleotides were added to the respective reactions. Finally, the samples were processed according to the previously published procedure [16].

Construction of the chimaeric reporter plasmids

The promoterless pLUC-null vector [17] was used to subclone the PCR-generated human MMP-21 promoter fragments upstream of the *Renilla* luciferase-reporter gene. The 1066, 374 and 315 bp fragments were amplified by PCR using 100 ng of human genomic DNA (Oncomatrix, San Diego, CA, U.S.A.) as template, and the mmp21-16-r reverse and forward primers mmp21-{−1004-f}, mmp21{−212-f} and mmp21{−153-f} respectively. The mmp21{−212-m-f} and mmp21{−153-m-f} primers bearing the respective nucleotide substitutions were employed to generate the mutant 374 and 315 bp PCR products with the functionally inert T-cell factor (Tcf) and repressor-binding protein Jκ (RBP-Jκ) transcription-factor-binding sites.The generated PCR products were cloned directly into pCR-Blund II-TOPO vector (Invitrogen) and further recloned into the *Xho*I–*Hin*dIII sites of the pLUC-null vector to yield the pLUC21{−1004}, −212, −212 m, −157 and −157 m reporter plasmids. The *Bgl*II and *Eco*72I fragments were deleted from the sequence of the pLUC21{−1004} plasmid to generate the shorter pLUC21-{−419} and −167 reporter plasmids respectively.

Transient transfection and luciferase assay

Breast carcinoma MCF7 cells (A.T.C.C., Manassas, VA, U.S.A.) were used in our transfection experiments. Cells were grown routinely in Dulbecco's modified Eagle's medium, supplemented with 10 % foetal bovine serum at 37 °C in a humidified 5 % CO_2 incubator. Cells (0.5×10^4 cells/well) were then placed in wells of the 48-multiwell plastic plate (Costar, Corning, NY, U.S.A.) in fresh Dulbecco's modified Eagle's medium with 10 % foetal bovine serum and grown for 16–24 h to 90 % confluence. Next, cells were co-transfected transiently

with a mixture containing 300 ng of the individual luciferase-reporter plasmid and 50 ng of pβgal-control plasmid (an internal control; ClonTech Laboratories) by using LIPOFECTAMINE 2000 (Invitrogen). Cells transfected with the promoterless pLUC-null vector were used as a negative control. Cells transfected with 50 ng of pRL-CMV plasmid, where the *Renilla* luciferase gene was placed under the control of the cytomegalovirus promoter (Promega), were used as a positive control in our transfection studies. The pCS2+-axin plasmid bearing the full-length gene of axin, the pcDNA3-GSK3β plasmid expressing the full-length gene of glycogen synthase kinase 3β (GSK3β), the pBZ13-LEF-1 plasmid expressing the lymphoid enhancer factor (LEF)-1 transcription factor, the pβcatS33Y plasmid encoding the degradation-resistant β-catenin S33Y mutant (βcatS33Y) and the pE-cadCytoΔ bearing an E-cadherin cytoplasmic domain with the β-catenin interaction region deleted (E-cadCytoΔ) were co-transfected (0.3 μg each) with the *Renilla* luciferase reporter plasmids. The axin, GSK3β, LEF-1 and mutant β-catenin plasmids were kindly provided by Dr Zhuohua Zhang (The Burnham Institute, La Jolla, CA, U.S.A.). The E-cadCytoΔ plasmid was a gift from Dr H. Crawford (Vanderbilt University, Nashville, TN, U.S.A.). After 20–24 h transfection, the luciferase and β-galactosidase activities were measured in the cell lysates as described previously [17]. The mean of three measurements was taken to obtain the relative luciferase activity expressed in relative units defined as the ratio of *Renilla* luciferase to β-galactosidase activity. Results are represented as means ± S.E.M. for at least three independent transfection experiments performed in quadruplicate.

Tissue specificity of the MMP-21 gene

The expression of MMP-21 in various tissues was analysed by PCR using the PCR-ready human and mouse multiple tissue DNA, complementary to RNA (cDNA) Multiple Tissue and Cell Line cDNA Panels (ClonTech Laboratories). The mmp21-20-f forward and mmp21-5-r reverse primers were used to generate a 517 bp fragment of human MMP-21. The M-mmp21-6-f forward and M-mmp21-end-r reverse primers were employed to generate a 472 bp fragment of mouse MMP-21. The following PCR conditions were used: 95 °C for 3 min, 38 cycles of 95 °C for 30 s and 68 °C for 30 s followed by 72 °C for 5 min.

Single-nucleotide polymorphism

The genomic DNA (Oncomatrix) from 49 healthy individuals was screened for 572C/T polymorphism existing in the human MMP-21 gene sequence. For these purposes, the 124 bp fragment of the MMP-21 gene was amplified in PCR, employing the individual DNA samples (40 ng) as template, and the mmp21-13-f and mmp21-12-r oligonucleotides as the forward and reverse primers respectively. The amplified fragment was digested for 3 h at 37 °C with *Sac*II endonuclease. The digested fragments were separated by electrophoresis in 3 % agarose to discriminate the *Sac*II-sensitive ccgC^{572}gg samples from the *Sac*II-resistant ccgT^{572}gg DNA samples.

Expression of MMP-21 in *Escherichia coli*

The 912 bp fragment encoding for Glu^{25}-Gly^{327} sequence of the prodomain–catalytic domain construct of human MMP-21 was amplified using the mmp21pro-f and mmp21cat-r oligonucleotides as the forward and reverse primers respectively. The 909 bp fragment coding for the Glu^{25}-Gly^{326} sequence of the mouse prodomain–catalytic domain construct was amplified with M-mmp21pro-f forward and M-mmp21cat-r reverse primers. The amplified fragments were cloned into the pCRII plasmid. The fragments were then subcloned with *Nde*I and *Eco*RI into the pET21(+) expression vector under the control of regulatory elements of the T7 promoter. Each of the constructs was transformed into *E. coli* BL21(DE3) competent cells and grown in 2YT medium (16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl), supplemented with 50 μg/ml ampicillin. The expression of MMP-21 was induced with 1 mM isopropyl β-D-thiogalactoside. After incubation for 2–4 h at 37 °C, *E. coli* cells were collected by centrifugation and lysed. Inclusion bodies containing the denatured MMP-21 forms were isolated from cell lysates by centrifugation and purified using the BugBuster reagent (Novagen, Madison, WI, U.S.A.). Purified samples were solubilized in 20 mM Tris buffer (pH 7.5), containing 8 M urea and 5 mM dithiothreitol, and subjected to ion-exchange chromatography on a Mono Q column (1 cm × 5 cm; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Bound material was eluted with a linear NaCl gradient (0–500 mM). The eluted fractions were analysed for the presence of MMP-21 by SDS/PAGE. To refold MMP-21, the combined fractions containing the purified material were concentrated on an Apollo-10 k Centrifugal Concentrator (Orbital Biosciences, Topsfield, MA, U.S.A.) to reach a 2 mg/ml protein concentration, and then stepwise-diluted 2-fold in 20 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.01 % Brij 35 to reach the final concentration of 250 mM urea. The refolded material was used immediately in enzyme assays.

Activation and enzyme assay of MMP-21

Refolded human proMMP-21 (200 ng) was activated by co-incubation for 2 h at ambient temperature (20 °C) with 2 mM 4-aminophenylmercuric acetate (APMA) in 20 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.01 % Brij 35. Activated MMP-21 was incubated for 2 h with 0.5 μg of α1-anti-trypsin. The samples were analysed by SDS/PAGE (12 % gel) to estimate the efficiency of the α1-anti-trypsin proteolysis by MMP-21.

Western blotting

The purified samples of MMP-21 were separated by SDS/PAGE (12 % gel). The proteins were transferred from gel to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The RP3MMP-21 anti-MMP-21 rabbit antibody and goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP; Chemicon International, Temecula, CA, U.S.A.) were used as primary and secondary antibodies respectively. The protein bands were visualized by chemiluminescence employing a SuperSignal West Pico chemiluminescence system (Pierce, Rockford, IL, U.S.A.).

Immunohistochemistry

Formalin-fixed paraffin-embedded specimens of mouse embryo were used for immunohistochemical analyses. The sections were incubated for 30 min at room temperature (20 °C) with 0.3 % H_2O_2 to inactivate endogenous peroxidase and blocked for 1 h with 2 % normal goat serum in PBS/0.1 % Tween 20. The samples were incubated overnight at 4 °C with 1 μg/ml RP3MMP-21 anti-human MMP-21 rabbit antibody. The samples were then incubated for 1 h at room temperature with a biotin-labelled goat

anti-rabbit secondary antibody (Chemicon International) and, next, with streptavidin–HRP and for 5 min with diaminobenzidine (both from Vector Laboratories, Burlingame, CA, U.S.A.). For negative controls, we processed parallel sections with pre-immune serum. The slides were counterstained with haematoxylin. The sections were examined using a Nikon Eclipse TE300 microscope equipped with a RT slider Spot camera SP402–115 (Diagnostic Instruments, Sterling Heights, MI, U.S.A.). The images were acquired using the SpotCam 32 software, version 3.04 (Diagnostic Instruments).

RESULTS

Cloning of the full-length human and mouse MMP-21 cDNA genes

Recently, we reported the identification of the human 1038 bp sequence of MMP-21 encoding a partial sequence of the putative catalytic domain followed by the complete sequence of the putative haemopexin-like domains [17,18]. The known amino acid sequence of human MMP-21 was 73 % identical with the corresponding region of *X. laevis* X-MMP/MMP-21 [13]. The computer search in the mouse chromosomal DNA Ensembl database (www.ensembl.org) found the presence of the nucleotide sequence potentially encoding MMP-21 in chromosome 7 of the mouse genome. These findings suggest that MMP-21 found in *Xenopus* is also expressed in human and mouse tissues.

We subsequently used human foetal brain and mouse 11-day-old embryo cDNA in RACE experiments to extend the length of the novel human and mouse MMP-21 genes respectively. Successive 3′- and 5′-RACE experiments allowed us to obtain fragments that were long enough to contain the entire coding information for the 1710 bp human MMP-21 gene and the 1707 bp mouse MMP-21 gene.

MMP-21 peptide sequence

Computer analysis of the DNA sequence of the MMP-21 gene revealed an open reading frame that coded for a 569 (human) and 568 (mouse) amino acid preprometalloproteinase, with a predicted molecular mass of 62 and 49 kDa for the latent proenzyme and the active enzyme respectively (Figure 1). In the structure of human MMP-21, a hydrophobic signal peptide containing 24 residues follows the initiation methionine residue, indicating that this proteinase is secreted. Downstream of the signal peptide, a prodomain containing a putative unique unpaired Cys^{117} of the $PRC^{117}GVPD$ cysteine-switch motif is followed by the $RSRR^{144}$ furin cleavage motif, suggesting that furin and related proprotein convertases may activate proMMP-21 in its secretory pathway to the extracellular milieu [19]. There is a 20-residue-long proline-rich insert between the cysteine switch and the furin cleavage motifs in the peptide sequence of MMP-21. A similar insert exists in MMP-21 of *X. laevis* and *C. pyrrhogaster* [13,14]. The furin cleavage motif is followed by the catalytic domain representing the active site of the enzyme and exhibiting the highly conserved zinc-binding HEIGHVLGLPH sequence, a hallmark of MMPs [2,3]. There is a haemopexin-like C-terminal domain in human and mouse MMP-21. Neither the hinge regions nor the transmembrane and cytoplasmic domains are present in the peptide sequence of MMP-21.

Both human and mouse MMP-21 show high overall similarity to X-MMP [13] and Cy-MMP [14] (Table 1). The sequence of human MMP-21 was found to be 80.4, 61.5 and 63.5 % identical with that of mouse MMP-21, X-MMP from *Xenopus* and Cy-MMP of *C. pyrrhogaster* respectively. The catalytic and haemopexin domains of these enzymes are highly conserved, whereas similarity between MMP-21 from different species in the prodomain and signal sequences is relatively low. X-MMP and Cy-MMP both have 37–44-residue-long vitronectin-like inserts between the cysteine-switch sequence and the furin cleavage motif. No similarity to vitronectin was found in the sequence of this insert of human or mouse MMP-21.

Pairwise comparisons between MMP-21 and other MMPs showed that amino acid identities ranged from 25 to 43 %. Thus the protein sequence of human MMP-21 is similar to MMP-11, MMP-12 and MMP-23 (43.0, 39.2 and 38.7 % similar respectively).

According to the predicted peptide sequence, MMP-21 cannot be classified as a gelatinase, collagenase, matrilysin, stromelysin or membrane-type MMP as it does not share the specific motifs of these groups. Apparently, MMP-21 should be included, at least temporarily, in a group of ‘unclassified’ MMPs such as MMP-23, MMP-19 and MMP-28. Remarkably, each of MMP-23 [8,20,21], MMP-19 [22–24] and MMP-28 [25,26] functions unconventionally in cells and tissues, suggesting a similar unconventional role for MMP-21.

Chromosomal localization and genomic structure

Recently, a nucleotide sequence (accession no. AL360176) has been released by GenBank®. This sequence represents a draft of human chromosome 10 clone RP11-124H7 and includes the genomic sequence of MMP-21. Similarly, the genomic sequence of mouse gene was found on chromosome 7 by searching the Ensembl sequence database (www.ensembl.org). We mapped the exon–intron junctions and determined an exon–intron map of human and mouse MMP-21 by comparing the respective cDNA and genomic sequences. Whereas most MMP genes have ten exons, the MMP-21 chromosomal gene has only seven exons, and most of the splice sites are not conserved relative to other MMPs [15]. Analysis of the genomic sequences allowed us to identify the exon–intron structure of the novel human and mouse MMP-21 gene. The size of the human and mouse genomic sequences of MMP-21 gene are 9160 and 5637 bp respectively. Exon–intron boundaries and the sizes of introns and exons are shown in Table 2. The exon–intron boundaries conform to the GT/AT rule for splice sites. Exon 1 includes a signal sequence and a part of the prodomain. Exon 2 encodes another part of the prodomain and includes the N-terminal part of the catalytic domain. Exons 3 and 4 cover another part of the catalytic domain, whereas exons 5–7 code for the haemopexin-like domain. The exon structure of mouse MMP-21 is highly similar. The association of MMP-21 with human chromosome 10 as well as the location of splice sites in the MMP-21 gene differ from those of classical MMPs clustered in chromosome 11.

Mapping of the transcription start site

The low levels of expression greatly complicate the analysis of the transcriptional regulation of MMP-21 in cells and tissues. Thus expression of the MMP-21 gene in human embryonic kidney HEK-3, breast carcinoma MCF7 and lung carcinoma A549 cell lines was barely detectable, providing insufficient amounts of the MMP-21 mRNA for a subsequent analysis (results not shown). To define the site of transcription-initiation of the human MMP-21 gene, we used primer-extension analysis with polyadenylated-rich mRNA isolated from breast carcinoma MCF7 cells transfected transiently with the pLUC21{−1004} plasmid bearing the cloned 1 kb 5′-fragment of the MMP-21

Figure 1 The amino acid sequence alignment of human, mouse, *X. laevis* and *C. pyrrhogaster* MMP-21

Peptide sequences were retrieved from the GenBank® for *Xenopus* X-MMP/MMP-21 (accession no. U82541) and *Cynops* Cy-MMP/MMP-21 (accession no. AB054185), and aligned with MMP-21 peptide sequence. The deduced peptide sequence of human and mouse MMP-21 was submitted to GenBank® (accession nos. AF520613 and AY124569 respectively). Stars depict identical amino acid residue positions. Cysteine switch, furin cleavage and zinc-binding sites are indicated by an open box.

gene. This procedure significantly increased the levels of the mRNA transcribed by the MMP-21 gene promoter in the cells. Following transfection, the [γ-^{32}P]mmp21-15-r and mmp21-17-r oligonucleotides complementary to the first exon of the human gene were each used as a primer. The primer extension resulted in a single 164 and 156 bp band with the mmp21-15-r and mmp21-17-r primers respectively (Figure 2). These findings indicate the existence of a single transcription-initiation start of the

Table 1 Sequences of the oligonucleotides used in PCR

Mutant nucleotide positions are shown in lower case.

Primers	Nucleotide sequence	Orientation
Human MMP-21		
mmp21-51–5-r	5′-CTGTGAGGGCCTGATCCTTGTCACTG-3′	Reverse
mmp21-12-r	5′-CACCTCGCTCCACATCCTGAAGGC-3′	Reverse
mmp21-13-f	5′-CTGCCCAGGCCTTCTCCAAGAGGAC-3′	Forward
mmp21-15-r	5′-CCGAGCGGTCCCGGCTGTGG-3′	Reverse
mmp21-16-r	5′-CTGAGCAGCGTGGAGGTCGGCAATG-3′	Reverse
mmp21-17-r	5′-TCCCGGCTGTGGAAGAGACTCTCG-3′	Reverse
mmp21-20-f	5′-GACATTCACTTTGACGACGACG-3′	Forward
mmp21cat-f	5′-ACATATGGCGCCGCTGTCCTTGTCC-3′	Forward
mmp21cat-r	5′-TGAATTCTTAACCATACAGCTTTTGAATTGC-3′	Reverse
mmp21pro-f	5′-AACATATGGAGAGTCTCTTCCACAGCC-3′	Forward
mmp21{−1004-f}	5′-CTCAATATTCCCATGGACGGCTGTG-3′	Forward
mmp21{−212-f}	5′-GGTGAGCCTGGGGTCTTCAAAGGCAGCACGCCCA-3′	Forward
mmp21{−212-r}	5′-CTGGGCGTGCTGCCTTTGAAGACCCCAGGCTCACC-3′	Reverse
mmp21{−212-m-f}	5′-GGTGAGCCTGGGGTCgaCtAAGGCAGCACGCCCAG-3′	Forward
mmp21{−212-m-r}	5′-CTGGGCGTGCTGCCTTaGtcGACCCCAGGCTCACC-3′	Reverse
mmp21{−153-f}	5′-GGCACACTTCAGGGCTGGG-3′	Forward
mmp21{−153-m-f}	5′-GGCACACTTCAGGGCTGGGaGAGgcATGCTTGGAAACC-3′	Forward
Mouse MMP-21		
M-mmp21-6-f	5′-GAAGCCAATACTGGAGATATGACAG-3′	Forward
M-mmp21-end-r	5′-TTACATGTTCAGGGTAGAGGTGTG-3′	Reverse
M-mmp21pro-f	5′-aacatatgGAGAGGCTCTTCCACAGCC-3′	Forward
M-mmp21cat-r	5′-tgaattcttaACCATATAGTCTTTGAATTGC-3′	Reverse
M-mmp21tcf-f	5′-GTGGCCCTGGGGTCTTCAAAGGCAGCTTGCTTG-3′	Forward
M-mmp21tcf-r	5′-CAAGCAAGCTGCCTTTGAAGACCCCAGGGCCAC-3′	Reverse
M-mmp21tcf-m-f	5′-GTGGCCCTGGGGTCgaCtAAGGCAGCTTGCTTG-3′	Forward
M-mmp21tcf-m-r	5′-CAAGCAAGCTGCCTTaGtcGACCCCAGGGCCAC-3′	Reverse

Table 2 Exon–intron structure of the genomic sequence of human and mouse MMP-21 gene

The sequence of clone RP11-124H7 (a draft of *Homo sapiens* chromosome 10) from GenBank® with accession no. AL360176 was used to identify introns and exons of the genomic sequence of MMP-21 gene. The sequence of mouse chromosome 7 (the Ensembl database at http://www.ensembl.org) was employed to identify introns and exons of the mouse genomic sequence. Intron and exon sequences are in lower and upper case respectively.

Exon position	Splice donor	Splice acceptor	Intron size
Human			
1–223	*ggagaggtgacggggacgg	CGCTGCTCAG**gt**aggaggaa	1294
1518–2052	tctcccac**ag**CGGTTCCTGT	TTTGGGAGAG**gt**aagagcac	1080
3133–3272	ccctcgcc**ag**GCCGGCACCT	CCTTCTCAAG**gt**cagtggtc	251
3524–3665	ccccttct**ag**GTGGCCGTCC	AAGCTGTATG**gt**aagactgc	1626
5292–5549	ttttttta**ag**GCTCCTGTGA	TTTTTTCAAG**gt**acagattc	2629
8179–8351	tggctctc**ag**GAAATCAATA	GGAGTCCCTT**gt**aagtagaa	570
8922–9221	†tgctttgc**ag**GTATTTGCAT	TCTCCACACTGAACATGtaa†	
Mouse			
1–210	ggagacagggacgcagtaa	TGATGCTCAG**gt**aagaggag	807
1018–1549	tttcccac**ag**AGCTTCCTAT	TTTGGGAGAG**gt**aagagggg	886
2436–2575	ctcacacc**ag**GCCGGCACCT	CCTTCTCAAA**gt**aagaggcc	234
2810–2951	cccctcct**ag**GTAGCTGTCC	AGACTATATG**gt**aagatggt	1024
3976–4233	ctctctcc**ag**GTTCGTGCAA	TTTTTCAAAG**gt**aacactgc	823
5057–5229	tgactctc**ag**GAAGCCAATA	GGAGTCTCTA**gt**aagtgtca	160
5390–5690	tactttac**ag**GTATTTGCAT	CCTCTACCCTGAACATGtaa†	

* The transcription-initiation start (underlined) of the human MMP-21 gene. The transcription-initiation start of the mouse gene was predicted by sequence similarity to the human gene.
† The translation termination codon (underlined) of the MMP-21 gene. Donor and acceptor sites are in boldface.

MMP-21 gene located 61 nucleotides upstream of the ATG translation initiation codon.

Promoter region of the MMP-21 gene

The sequence of the promoter region of the MMP-21 gene is distinct from all known MMPs and demonstrates a putative recognition site for several transcription regulatory proteins [27]. The sequence of human MMP-21 exhibits an evident TATA box located 92 bp upstream of the transcription-initiation ATG codon (Figure 3). The putative binding sites of the Tcf-4 (5′-TTCAAAG-3′) [28], RBP-Jκ (5′-GTGAGAA-3′) [29,30] and Pax-2/5/8 (5′-GGCACACTTCAGGG-3′) [31] were found at positions −197/−191, −135/−129 and −153/−140 upstream of the transcription-initiation site respectively. In contrast with many other MMPs, there were no consensus activator protein 1, transcription factor

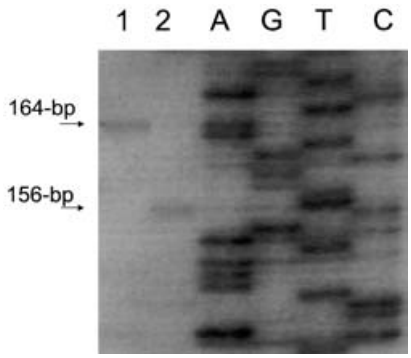

Figure 2 Determination of the transcription-initiation site of the human MMP-21 gene

Lanes 1 and 2, primer-extension reactions with the mmp21-15-r and mmp21-17-r oligonucleotides respectively of the mRNA isolated from MCF7 cells transfected transiently with the pLUC21{−1004} plasmid bearing the cloned 1 kb 5′-fragment of the MMP-21 gene. Lanes A, G, T and C, sequencing reactions of the pGEM-3Zf(+) plasmid (Promega) with the standard M13 forward primer. They are used to determine the exact position of the 164 and 156 bp extended products (arrows).

Sp-1 or polyoma virus enhancer A-binding protein-3 sites in the promoter of the MMP-21 gene. Remarkably, a few of the known MMPs exhibit either Pax or RBP-Jκ transcription-factor-binding sites (Figure 4). On the other hand, the structure of the 5′-flanking region of MMP-21 as well as the presence of the highly unusual Pax and RBP-Jκ/Notch motifs in the MMP-21 gene promoter suggest unconventional regulation and tissue expression pattern of this proteinase. The presence of the Tcf-4 element in the MMP-21 promoter is an indication that MMP-21 is a probable target of the Wnt pathway [28].

EMSA

To assess further the role of the Tcf-4 motif in the regulation of the MMP-21 gene, we used the respective wild-type and mutant oligonucleotides by EMSA (Figure 5). For these purposes, [γ-^{32}P]ATP double-stranded oligonucleotides mmp21{−212-f}/mmp21{−212-r} (human) and M-mmp21tcf-f/M-mmp21tcf-r (mouse), containing the potential Tcf-4-binding site of the human and mouse MMP-21 gene, were each incubated with HeLa nuclear extract. Specific or non-specific competition of TCF-4 was performed by the addition of 100–150-fold molar excess of the unlabelled competitor. One specific protein–DNA complex was observed in the Tcf-4 ^{32}P-labelled oligonucleotides incubated with the nuclear extract. The excess of non-labelled specific double-stranded competitor inhibited this complex. In turn, the functionally inert mutant Tcf-4 motif, when used as a competitor, failed to repress the protein–DNA complex. These results provided the foundation for further characterization of the regulation of the MMP-21 gene in cells and tissues.

Luciferase reporter assays

To perform a functional characterization of the regulatory structural elements found in the 5′-flanking region of the MMP-21 gene, DNA constructs containing various lengths of the promoter inserted in front of the *Renilla* luciferase reporter gene were expressed transiently in breast carcinoma MCF7 cells (Figure 6). The transcriptional activity corresponding to each promoter fragment was assessed in a dual luciferase/β-galactosidase assay. We examined five fragments with 5′-flanking sequences of the human MMP-21 gene that ranged from 153 to 1004 nucleotides (Figure 6). The longer pLUC21{−1004}, pLUC21{−419} and pLUC21{−212} constructs demonstrated

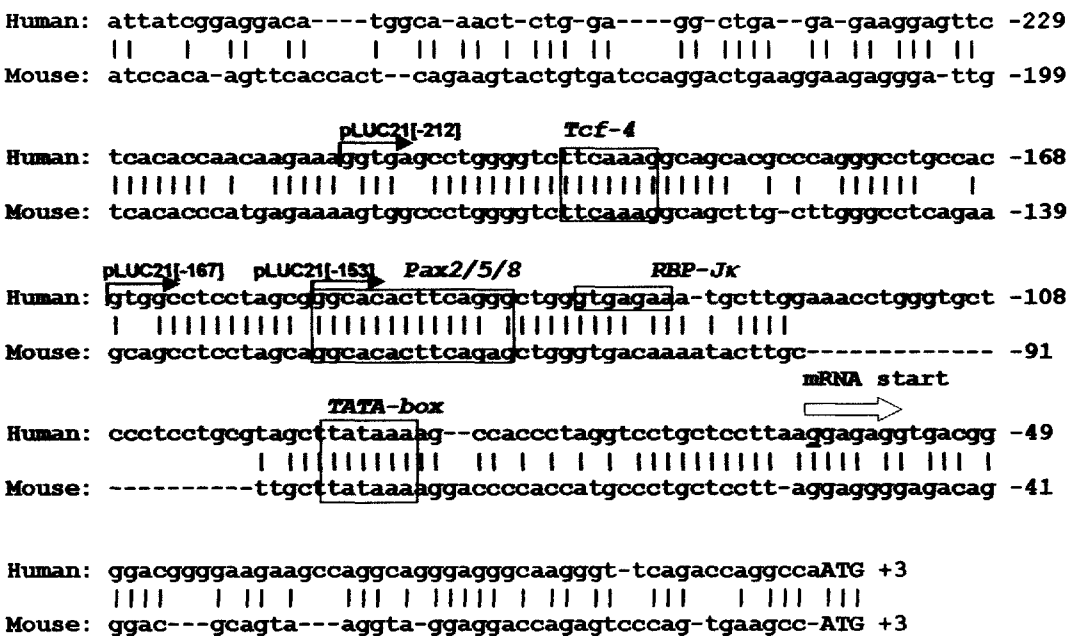

Figure 3 Sequence of the 5′-flanking region of the human and mouse MMP-21 gene

Numbering starts from the translation start codon ATG. The transcription–initiation site of the human gene is indicated by an open arrow. The first G^{61} nucleotide of the transcription-initiation site is underlined. MatInspector (http://www.genomatix.de) software package was used for the identification of the putative transcription-factor-binding sites in the promoter region of MMPs including MMP-21. The sites with the matrix similarity score > 0.9 were selected. The putative TATA box, Tcf-4, Pax2/5/8 and RBP-Jκ/Notch recognition motifs are boxed. The 5′-end of the DNA fragments inserted into the chimaeric luciferase reporter plasmids pLUC{−212}, pLUC{−167} and pLUC{−153} used in luciferase assay experiments are indicated by a bent arrow. The Tcf-4, Pax and RBP-Jκ/Notch site consensus sequences are A(A/T)CAAAG, C(A/T)NT(G/C)AAG(C/T)NTN(A/C) and GTG(G/A)GAA(A/C)NT, where N stands for A/G/C/T.

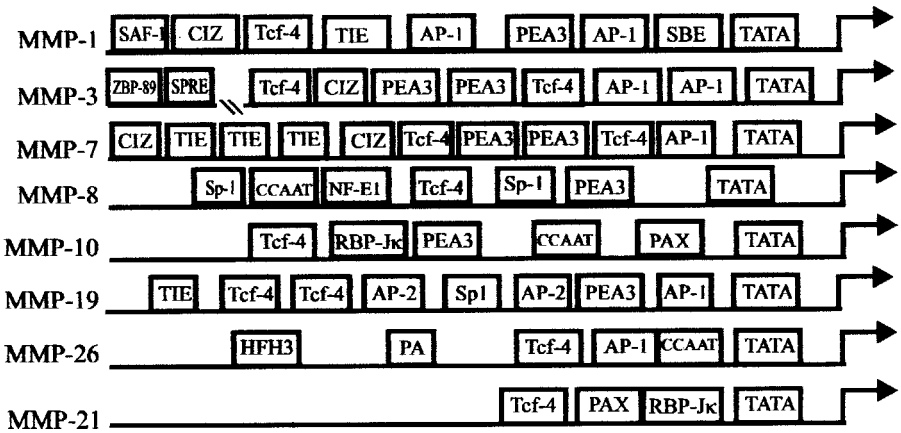

Figure 4 Regulatory elements of promoter regions of MMPs

Putative transcription-factor-binding sites are indicated within boxes. All individual MMPs exhibiting the Tcf-4 transcription-factor-binding site are shown. Transcription start is indicated by a bent arrow. Relative positions of the binding sites were identified using the available data from the literature. In addition, MatInspector (http://www.genomatix.de) software package was used for the identification of the putative transcription-factor-binding sites in the promoter region of MMPs. TATA, TATA box; AP-1, activator protein 1; AP-2, activator protein 2, HFH3, hepatocyte nuclear factor-3; PEA3, polyoma virus enhancer A-binding protein-3; PAX, paired axial; TIE, transforming growth factor-β inhibitory element; Sp1, transcription factor Sp-1; SAF-1, serum amyloid A-activating factor 1; CCAAT, CCAAT-binding proteins; CIZ, Crk-associated substrate (Cas)-interacting zinc finger protein; SBE, signal transduction and activators of transcription-binding element; ZBP-89, the 89 kDa zinc-binding protein; SPRE, stromelysin-1 platelet-derived growth factor-responsive element; NF-E1, nuclear factor-E1 (p45). Note an unusual polyadenylation site (PA) in MMP-26 [16].

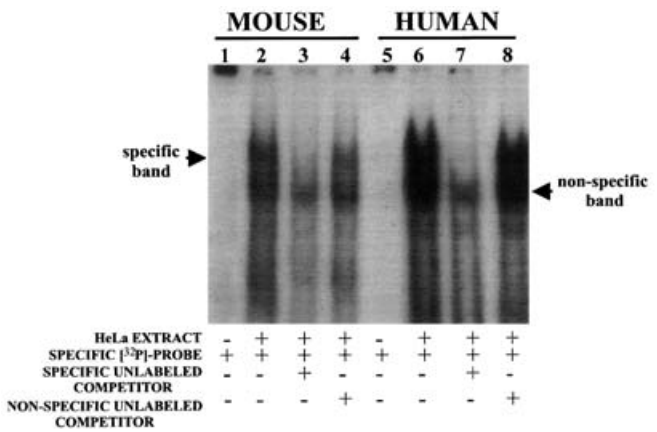

Figure 5 EMSA of the Tcf-4-binding motif of the MMP-21 promoter

^{32}P-labelled double-stranded mmp21{−212-f}/mmp21{−212-r} (human) and M-mmp21tcf-f/M-mmp21tcf-r (mouse)-specific probes were co-incubated with HeLa nuclear extract (lanes 2–4 and 6–8). Unlabelled oligonucleotides used as specific [mmp21{−212-f}/mmp21{−212-r} (human) and M-mmp21tcf-f/M-mmp21tcf-r (mouse)] (lanes 3 and 7), and non-specific [mmp21{−212-m-f}/mmp21{−212-m-r} (human) and M-mmp21tcf-m-f/M-mmp21tcf-m-r (mouse)] (lanes 4 and 8) competitors were added in a 100–150-fold molar excess. Lanes 1 and 6, specific double-stranded [^{32}P]-labelled oligonucleotides alone.

a higher transcriptional activity compared with the shorter pLUC21{−167} and, especially, pLUC21{−153} plasmids. This may be due to the positively acting Tcf-4, Pax and RBP-Jκ transcription-factor-binding sites located between positions −120 and −212.

To evaluate further the significance of the Tcf-4- and RBP-Jκ-binding sites, we introduced mutations in the respective sequence regions of the pLUC21{−212} and pLUC21{−153} reporter plasmids. The resulting mutant pLUC21{−212 m} and pLUC21-{−153 m} reporter plasmids, expressing the inactivated Tcf-4- and RBP-Jκ-binding sites respectively, were each transfected into MCF7 cells. These studies demonstrated that modifications of the Tcf-4- and, especially, the RBP-Jκ-binding sites inhibited the transcriptional activity of the chimaeric constructs.

To examine further the effects of Tcf-4, a downstream target of the Wnt pathway, we co-transfected MCF7 cells with the pLUC21{−212} construct and the recombinant plasmids expressing LEF-1, β-catenin-S33Y, GSK3β, axin and mutant E-cadherin with the cytoplasmic domain lacking the β-catenin interaction region. Co-expression of LEF-1 with β-catenin-S33Y mutant strongly up-regulated the transcriptional activity of the chimaeric luciferase promoter. Similarly, the expression of mutant E-cadherin, bearing the cytoplasmic tail with the β-catenin-binding site deleted, stimulated the transcriptional activity of the pLUC21{−212} plasmid. In contrast, the transcriptional activity of the mutant pLUC21{−212 m} construct bearing the functionally inert Tcf-4 site was not affected in MCF7 cells by β-catenin-S33Y, LEF-1, GSK3β, axin or mutant E-cadherin. As expected, the transcriptional activity of the pLUC21{−212} plasmid was repressed when this plasmid was co-transfected with GSK3β and axin, in which both are the repressors of the Wnt pathway [28]. The presence of the LEF-1/Tcf-4 regulatory element in the promoter suggests the possibility that MMP-21 is a direct target of β-catenin transactivation. The Wnt pathway probably plays an important role in regulating the expression of MMP-21 in cells and tissues.

Single-nucleotide polymorphism in the human MMP-21 gene

There is a 572C/T single-nucleotide polymorphism of the MMP-21 gene, which alters an amino acid within the N-terminal part of the catalytic domain from Ala^{191} to Val^{191} [15]. Genotyping of 572C/T polymorphism was performed using genomic DNA from 49 male and female healthy individuals. For these purposes, the 124 bp fragment of the MMP-21 gene bearing 572C/T polymorphism was amplified by PCR using individual genomic DNA as template. The amplified fragment was then digested with *Sac*II endonuclease to discriminate the *Sac*II-sensitive ccgC^{572}gg samples from the *Sac*II-resistant ccgT^{572}gg DNA fragments (Figure 7). In the MMP-21 gene polymorphism, the frequency of the 572C/572C genotype was substantially higher in healthy donors when compared with the 572C/572T genotype. To our surprise, no 572T/572T genotype was observed within the

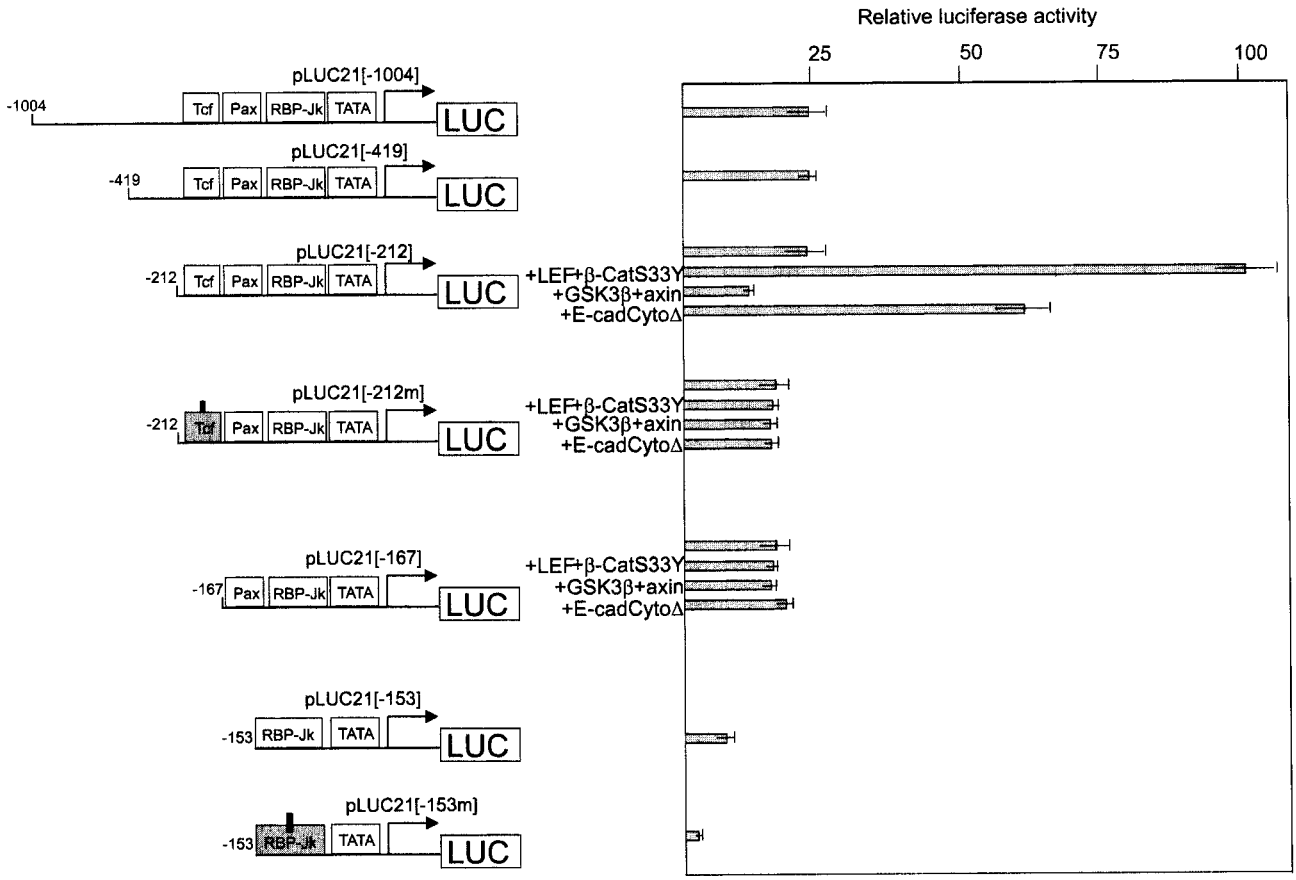

Figure 6 Functional analysis of the MMP-21 promoter–luciferase chimaeric genes

The individual chimaeric pLUC plasmids (schematically shown on the left panel) were co-transfected into breast carcinoma MCF7 cells with the pβgal control plasmid, which was used as an internal control for transfection efficiency. Luciferase activity was determined using coelenterazine as a substrate. The background luciferase activity of the control promoterless pLUC-null plasmid was subtracted from the activity of the chimaeric pLUC constructs. The luciferase activity was expressed in relative units defined as the ratio of *Renilla* luciferase to β-galactosidase activity. The regulatory motifs are shown within boxes. Mutations inactivating the Tcf-4 and RBP-Jκ motifs are shown as shaded boxes pierced with a thick line. Where indicated, the chimaeric constructs were co-transfected with the respective plasmids expressing Tcf-4 (LEF-1), β-catenin-S33Y, GSK3β, axin or mutant E-cadherin with the cytoplasmic domain lacking the β-catenin interaction region (E-cadCytoΔ). Results are presented as means $\pm$ S.E.M. for at least three independent transfection experiments performed in quadruplicate.

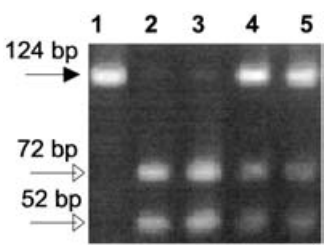

Figure 7 The 572C/T polymorphism in the human MMP-21 gene

The 124 bp fragment (solid arrow) of the MMP-21 gene was amplified in PCR using the genomic DNA from healthy individuals, and the mmp21-13-f and mmp21-12-r oligonucleotides as the forward and reverse primers respectively. Next, the amplified fragment was digested with *Sac*II endonuclease for 3 h at 37 °C to generate the 72 and 52 bp fragments (open arrows). The digested fragments were separated by electrophoresis on 3 % agarose to discriminate the *Sac*II-sensitive ccgC^{572}gg samples from the *Sac*II-resistant ccgT^{572}gg DNA samples. The 124 bp fragment was amplified using the human MMP-21 gene (accession no. AF520613) as template (lane 1) and then cleaved with *Sac*II (lane 2). Lane 3, a 572C/572C homozygote genomic sample; lanes 4 and 5, two 572C/572T heterozygote genomic samples.

available pool of genomic DNAs. Further studies are in progress in our laboratory to address the question of whether or not there is any association of the 572C/T single-nucleotide polymorphism with different cancer types.

Expression of the MMP-21 gene in cells and tissues

To gain insight into the functional role of MMP-21, expression of the proteinase gene was evaluated by PCR amplification of the first-strand cDNA panels and by reverse transcriptase–PCR of the mRNA pool isolated from human normal and cancerous cells and tissues. Amplification of glyceraldehyde-3-phosphate dehydrogenase was performed to control the amount of cDNA. The MMP-21 mRNA was identified in foetal brain, kidney and liver (results not shown). In adults, MMP-21 was found primarily in ovary, kidney, liver, lung, placenta, brain and peripheral blood leucocytes (results not shown). Expression of MMP-21 was also identified in lung carcinomas (LX-1 and GI-117) and breast carcinoma (GI-101). Additional studies are needed to identify the clinical significance of the expression of the MMP-21 gene. Overall, our findings and the results of other authors suggest a role of MMP-21 in embryogenesis and, quite probably, in certain types of cancer. To support this suggestion, the expression of MMP-21 was analysed in mouse tissues. In agreement with the results obtained with human tissues, MMP-21 was largely identified in mouse brain and liver (results not shown). To corroborate further the potential role of MMP-21 in embryogenesis, we tested the expression of MMP-21 in mouse embryo. To our surprise, the MMP-21 expression was relatively low in a 7-day-old embryo, increased in 11-day-old embryo samples, but decreased

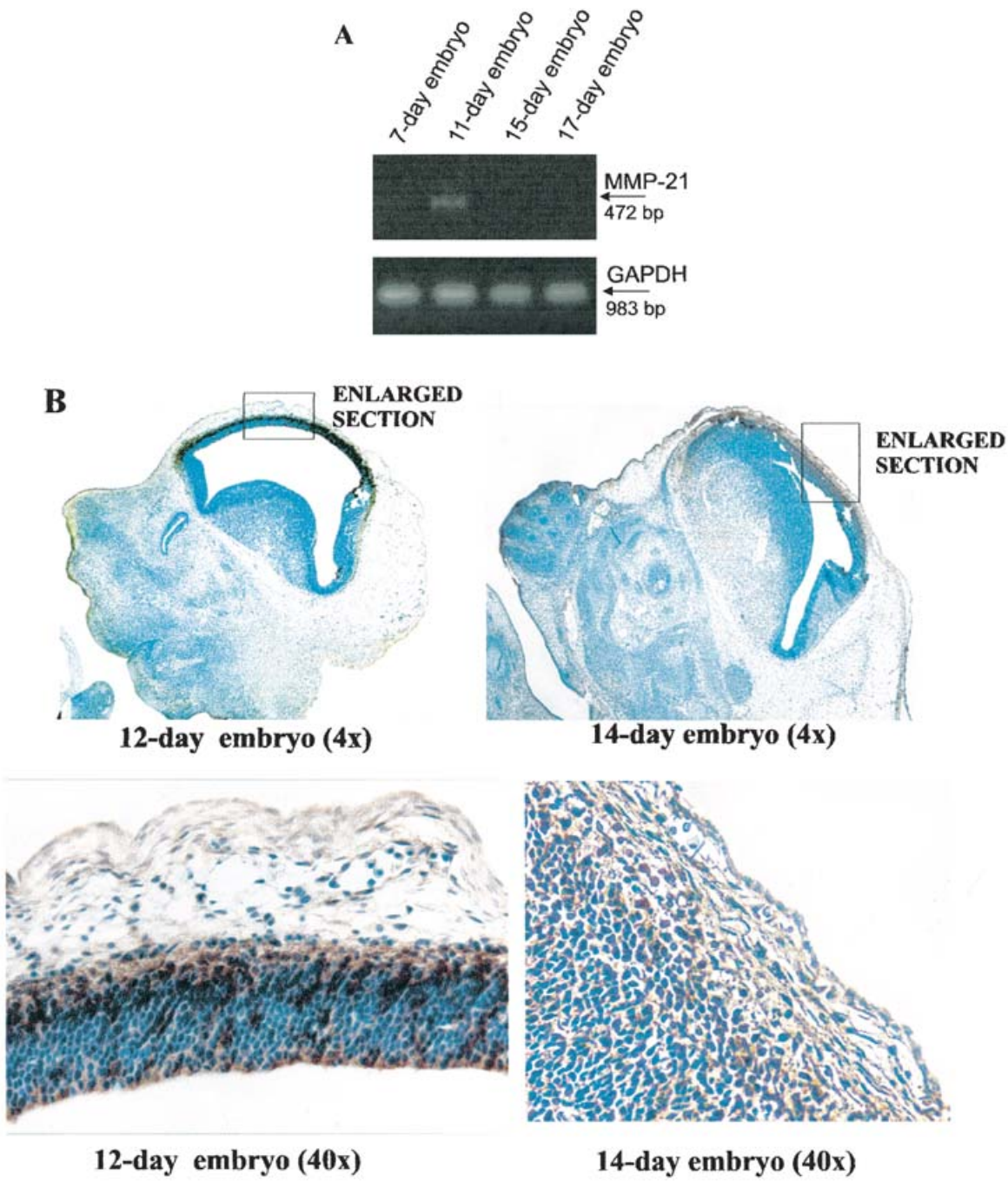

Figure 8 Transient expression of the MMP-21 gene in mouse embryogenesis

(**A**) Expression of MMP-21 was evaluated by PCR amplification of the multiple tissue cDNA mouse panel I isolated from 7-, 11-, 15- and 17-day mouse embryo (ClonTech Laboratories). The M-mmp21-6-f forward and M-mmp21-end-r reverse primers were employed to generate a 472 bp fragment of mouse MMP-21. The PCR was analysed on 2 % agarose gel. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used to ascertain equal loading and integrity of the cDNA in each reaction. (**B**) Paraffin-embedded 12-day (left upper and lower panels) and 14-day (right upper and lower panels) mouse embryos were sectioned and stained with the RP3MMP-21 anti-human MMP-21 rabbit antibody followed by a biotin-labelled goat anti-rabbit secondary antibody, streptavidin–HRP and the diaminobenzidine substrate. Staining with control rabbit antibody was negative (results not shown). Magnification: $\times 4$ (upper panels) and $\times 40$ (lower panels). The square boxes denote the enlarged area. Note the expression of MMP-21 in the neopallial cortex (future cerebral cortex) of the 12-day embryo.

in the 15- or 17-day-old embryo material (Figure 8A), thereby suggesting a role for MMP-21 in the development of a mouse. Moreover, our immunostaining studies confirmed the presence of MMP-21 in the neuronal cells forming the roof of the neopallial cortex (the future cerebral cortex) in a 12-day-old embryo. In agreement with the results of PCR, immunostaining demonstrated a decrease in MMP-21 in the roof of the neopallial cortex of a 14-day-old embryo (Figure 8B). In general, our results are in agreement with the potential role of MMP-21 in the development of brain tissues as proposed by other authors [15].

Recombinant MMP-21 from bacterial cells

To elucidate whether the isolated cDNA gene of MMP-21 encodes the corresponding enzyme with proteolytic activity, we expressed the cloned cDNA in *E. coli*. The 912 and 909 bp constructs encoding for the Glu^{25}-Gly^{327} and the Glu^{25}-Gly^{326} sequences of the prodomain–catalytic domain construct of human and mouse MMP-21 respectively were both subcloned into the pET21(+) expression vector under the control of the T7 promoter. Each of the constructs was transformed into *E. coli* BL21(DE3) competent cells. The expression of MMP-21 was induced by isopropyl β-D-thiogalactoside in transformed cells. Cell extracts were analysed by SDS/PAGE for the presence of MMP-21. The insoluble fraction of the *E. coli* extracts contained the denatured MMP-21 proteins, with the expected molecular mass of 33 kDa. Mouse and human MMP-21 was solubilized in 8 M urea and purified by ion-exchange chromatography on a Mono Q column (Figure 9A). This allowed us to isolate the homogeneous samples of human and mouse MMP-21. The RP3MMP-21 antibody was efficient in detecting mouse MMP-21 and, especially, human MMP-21 by Western blotting. Control rabbit IgG failed to associate with MMP-21 specifically (results not shown). In contrast with many other MMPs, both human and mouse MMP-21 constructs failed to demonstrate any gelatinolytic activity in gelatin zymography (results not shown).

APMA was employed to induce activation of the purified and refolded human MMP-21. After treatment with APMA, the samples were analysed by SDS/PAGE to confirm the conversion of the 33 kDa prodomain–catalytic domain construct into the activated MMP-21 form. We observed partial activation and conversion of MMP-21 into the 26 and 22 kDa molecular-mass forms, representing the active MMP-21 constructs. This conversion was completely blocked by EDTA (Figure 9B). The results support the functionality of the cysteine switch in MMP-21.

To confirm proteolytic activity of MMP-21, we used APMA-activated MMP-21 samples in the cleavage of α1-anti-trypsin, a protein that is known to be highly sensitive to the MMP cleavage [32] (Figure 9C). These results demonstrated that the latent MMP-21 construct was fully inert in cleaving α1-anti-trypsin. In turn, the APMA-activated MMP-21 was capable of cleaving α1-anti-trypsin, but with relatively low efficiency. The cleavage of α1-anti-trypsin by activated MMP-21 was fully blocked with a broad-range hydroxamate inhibitor of MMP activity, GM6001 (10 μM).

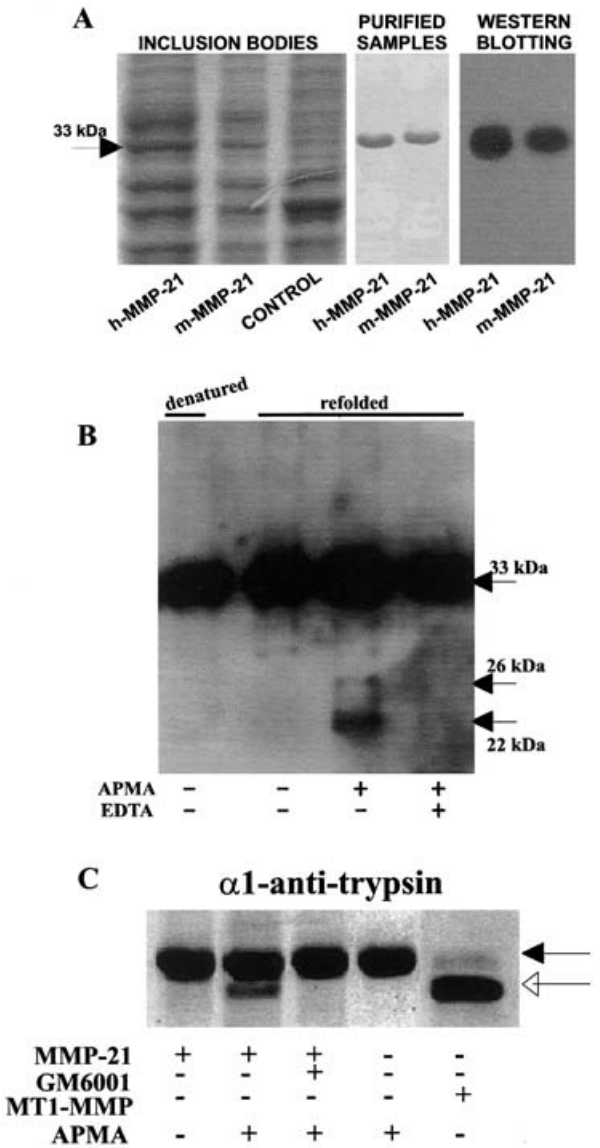

Figure 9 Expression of MMP-21 in *E. coli*

(**A**) The prodomain–catalytic domain constructs of human and mouse MMP-21 (h-MMP-21 and m-MMP-21 respectively) were expressed in *E. coli*, purified from inclusion bodies and analysed by SDS/PAGE (12 % gel) and Western-blot analysis was performed with the RP3MMP-21 anti-human MMP-21 rabbit antibody. *E. coli* cells transformed with the original vector were used as control. An arrow points to the MMP-21 band (33–34 kDa). (**B**) The purified denatured sample of the prodomain–catalytic domain human MMP-21 construct (200 ng) was refolded and then incubated with 2 mM APMA to generate the activated form of MMP-21 with the expected molecular mass in the range 22–23 kDa. The samples were evaluated by Western-blot analysis employing the antibody RP3MMP-21 specific to MMP-21. Where indicated, 25 mM EDTA was added to block proteolytic activity of MMP-21. Arrows point to the latent construct (33 kDa), the intermediate activation form (26 kDa) and the active species (22 kDa) of MMP-21. (**C**) α1-Anti-trypsin (0.5 μg; solid arrow) was incubated with the purified and refolded MMP-21 (20 ng), or the individual recombinant catalytic domain of MT1-MMP (20 ng) for 2 h at 37 °C in 20 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.01 % Brij 35. Where indicated, 2 mM APMA and 10 μM GM6001 were added to the samples. The samples were analysed by SDS/PAGE (12 % gel) to identify the cleavage product (open arrow).

DISCUSSION

Identification of the expanding roles for MMPs in complex regulatory processes has stimulated the search for genes encoding proteinases with unique functions, regulation and expression patterns. There has been a significant effort to identify novel members of the MMP family [18,25,33–38]. Recently, we identified novel genes encoding human and mouse MMP-21 and submitted the respective nucleotide sequences to the GenBank® with the accession numbers AF520613 and AY124569 respectively. Human and mouse MMP-21 is the orthologue of X-MMP and Cy-MMP, previously cloned in *X. laevis* and *C. pyrrhogaster* respectively [13,14]. While the present paper was being written, Ahokas et al. [15] independently and very recently reported the discovery, cloning and partial characterization of human MMP-21. The results presented in this paper confirm and significantly extend their findings, thereby considerably increasing our knowledge of the unconventional regulation of the MMP-21 gene and protein. Thus extensive computer search through the known sequences of the human genome failed to detect any additional, novel members of the MMP superfamily, suggesting that MMP-21 appears to be the last uncharacterized human MMP.

Similar to X-MMP, the gene that is under tight conditional regulation in *Xenopus* development [13,39], the transient expression of human and mouse MMP-21, is probably highly tissue- and cell-specific, which indicates a potential role in embryogenesis, especially in brain development. The promoter structure and regulation of MMP-21 exhibit a few transcription factor-binding motifs and are strikingly different from all other known MMPs. Existence of the structural motifs involved in the binding of transcription factors Tcf-4, Pax and RBP-Jκ (Notch) indicates a stringent tissue-specific pattern of MMP-21 regulation. Thus the presence of the Tcf-4 transcription-factor-binding site and our experimental results indicate that MMP-21 is a probable target of β-catenin transactivation and the Wnt pathway [28]. The Wnt signal-transduction pathway is involved in various differentiation events during embryonic development and leads to tumour formation when aberrantly activated. The Wnt signal is transmitted to the nucleus by multifunctional β-catenin: in the absence of Wnt signal, β-catenin is constitutively degraded in proteosomes, whereas in the presence of Wnt signal, β-catenin is stabilized and can associate with transcription factors of the Tcf/LEF family. The Tcf/LEF–β-catenin complexes activate specific Wnt target genes.

Similarly, the presence of the Pax-binding motif in the promoter raises the possibility that MMP-21 plays some role in embryogenesis and tissue development [31]. The Pax gene family encodes transcription factors that have been highly conserved through evolution. Mutations of the Pax genes cause profound developmental defects in humans. Pax proteins might regulate transcription of bcl-2 and p53, suggesting, concurrently, that MMP-21 might have a role in apoptosis. Furthermore, the existence of the regulatory motif of the Notch signalling in the promoter of the human MMP-21 gene again suggests a role for MMP-21 in development. Notch signalling is known to play a key role in the normal development of many tissues and cell types, including, through neural stem cell differentiation, central and peripheral nervous system, through diverse effects on differentiation, survival and proliferation that are highly dependent on cellular context and signal strength [30,40]. Aberrant Notch signalling has been proved to be involved in a variety of neoplasms [29]. Importantly, a critical role was established for Notch signalling in lymphocyte development, i.e. for the development of T-cell progenitors and the reciprocal suppression of B-cell development in the thymus [41,42]. It is quite probable that Notch regulates subsequent survival and cell fate decisions in developing T-cells. The potential Notch-dependent regulation and expression of MMP-21 in T-cells observed in our research as well as in the work of Ahokas et al. [15] suggest that this proteinase may play a unique role in lymphocyte development and survival. Furthermore, transient up-regulation of MMP-21 in mouse embryo neuronal cells correlates well with Notch functions, suggesting a functional role for MMP-21 in glial cells in the developing brain [43].

Our studies demonstrated the proteolytic activity of recombinant MMP-21 derived from *E. coli*. The physiological substrates of MMP-21 as well as the function of the proteinase in normal or malignant tissues are, however, unknown. Additional studies are needed to elucidate the clinical significance of MMP-21 as well as its role in foetal development and malignant progression.

A. Y. S. was supported by grants from National Institutes of Health (CA77470 and CA83017), Susan G. Komen Breast Cancer Foundation (9849) and the University of California Breast Cancer Program (5JB-0094A).

REFERENCES

1 Egeblad, M. and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer **2**, 161–174
2 Massova, I., Kotra, L. P., Fridman, R. and Mobashery, S. (1998) Matrix metalloproteinases: structures, evolution, and diversification. FASEB J. **12**, 1075–1095
3 Nagase, H. and Woessner, Jr, J. F. (1999) Matrix metalloproteinases. J. Biol. Chem. **274**, 21491–21494
4 Gu, Z., Kaul, M., Yan, B., Kridel, S. J., Cui, J., Strongin, A., Smith, J. W., Liddington, R. C. and Lipton, S. A. (2002) S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science **297**, 1186–1190
5 Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H. and Seiki, M. (2001) Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J. Cell Biol. **153**, 893–904
6 McQuibban, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I. and Overall, C. M. (2000) Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. Science **289**, 1202–1206
7 Ratnikov, B. I., Rozanov, D. V., Postnova, T. I., Baciu, P. G., Zhang, H., DiScipio, R. G., Chestukhina, G. G., Smith, J. W., Deryugina, E. I. and Strongin, A. Y. (2002) An alternative processing of integrin α(v) subunit in tumor cells by membrane type-1 matrix metalloproteinase. J. Biol. Chem. **277**, 7377–7385
8 Pei, D., Kang, T. and Qi, H. (2000) Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. J. Biol. Chem. **275**, 33988–33997
9 Park, H. I., Ni, J., Gerkema, F. E., Liu, D., Belozerov, V. E. and Sang, Q. X. (2000) Identification and characterization of human endometase (matrix metalloproteinase-26) from endometrial tumor. J. Biol. Chem. **275**, 20540–20544
10 Park, H. I., Turk, B. E., Gerkema, F. E., Cantley, L. C. and Sang, Q. X. (2002) Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. J. Biol. Chem. **277**, 35168–35175
11 Marchenko, N. D., Marchenko, G. N. and Strongin, A. Y. (2002) Unconventional activation mechanisms of MMP-26, a human matrix metalloproteinase with a unique PHCGXXD cysteine-switch motif. J. Biol. Chem. **277**, 18967–18972
12 Overall, C. M. and Lopez-Otin, C. (2002) Strategies for mmp inhibition in cancer: innovations for the post-trial era. Nat. Rev. Cancer **2**, 657–672
13 Yang, M., Murray, M. T. and Kurkinen, M. (1997) A novel matrix metalloproteinase gene (XMMP) encoding vitronectin-like motifs is transiently expressed in *Xenopus laevis* early embryo development. J. Biol. Chem. **272**, 13527–13533
14 Suzuki, A. S., Tadano, Y., Yamamoto, T., Abe, S. I. and Tajima, T. (2001) Expression of a novel matrix metalloproteinase gene during *Cynops* early embryogenesis. Biochem. Biophys. Res. Commun. **288**, 380–384
15 Ahokas, K., Lohi, J., Lohi, H., Elomaa, O., Karjalainen-Lindsberg, M. L., Kere, J. and Saarialho-Kere, U. (2002) Matrix metalloproteinase-21, the human orthologue for XMMP, is expressed during fetal development and in cancer. Gene **301**, 31–41
16 Marchenko, G. N., Marchenko, N. D., Leng, J. and Strongin, A. Y. (2002) Promoter characterization of the novel human matrix metalloproteinase-26 gene: regulation by the T-cell factor-4 implies specific expression of the gene in cancer cells of epithelial origin. Biochem. J. **363**, 253–262
17 Marchenko, G. N., Ratnikov, B. I., Rozanov, D. V., Godzik, A., Deryugina, E. I. and Strongin, A. Y. (2001) Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem. J. **356**, 705–718
18 Marchenko, G. N. and Strongin, A. Y. (2001) MMP-28, a new human matrix metalloproteinase with an unusual cysteine-switch sequence is widely expressed in tumors. Gene **265**, 87–93
19 Pei, D. and Weiss, S. J. (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature (London) **375**, 244–247
20 Velasco, G., Pendas, A. M., Fueyo, A., Knauper, V., Murphy, G. and Lopez-Otin, C. (1999) Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. J. Biol. Chem. **274**, 4570–4576
21 Ohnishi, J., Ohnishi, E., Jin, M., Hirano, W., Nakane, D., Matsui, H., Kimura, A., Sawa, H., Nakayama, K., Shibuya, H. et al. (2001) Cloning and characterization of a rat ortholog of MMP-23 (matrix metalloproteinase-23), a unique type of membrane-anchored matrix metalloproteinase and conditioned switching of its expression during the ovarian follicular development. Mol. Endocrinol. **15**, 747–764
22 Impola, U., Toriseva, M., Suomela, S., Jeskanen, L., Hieta, N., Jahkola, T., Grenman, R., Kahari, V. M. and Saarialho-Kere, U. (2003) Matrix metalloproteinase-19 is expressed by proliferating epithelium but disappears with neoplastic dedifferentiation. Int. J. Cancer **103**, 709–716
23 Mauch, S., Kolb, C., Kolb, B., Sadowski, T. and Sedlacek, R. (2002) Matrix metalloproteinase-19 is expressed in myeloid cells in an adhesion-dependent manner and associates with the cell surface. J. Immunol. **168**, 1244–1251

24 Stracke, J. O., Hutton, M., Stewart, M., Pendas, A. M., Smith, B., Lopez-Otin, C., Murphy, G. and Knauper, V. (2000) Biochemical characterization of the catalytic domain of human matrix metalloproteinase 19. Evidence for a role as a potent basement membrane degrading enzyme. J. Biol. Chem. **275**, 14809–14816
25 Lohi, J., Wilson, C. L., Roby, J. D. and Parks, W. C. (2001) Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J. Biol. Chem. **276**, 10134–10144
26 Saarialho-Kere, U., Kerkela, E., Jahkola, T., Suomela, S., Keski-Oja, J. and Lohi, J. (2002) Epilysin (MMP-28) expression is associated with cell proliferation during epithelial repair. J. Invest. Dermatol. **119**, 14–21
27 Westermarck, J. and Kahari, V. M. (1999) Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J. **13**, 781–792
28 Barker, N. and Clevers, H. (2000) Catenins, Wnt signaling and cancer. Bioessays **22**, 961–965
29 Allenspach, E. J., Maillard, I., Aster, J. C. and Pear, W. S. (2002) Notch signaling in cancer. Cancer Biol. Ther. **1**, 466–476
30 Baron, M., Aslam, H., Flasza, M., Fostier, M., Higgs, J. E., Mazaleyrat, S. L. and Wilkin, M. B. (2002) Multiple levels of Notch signal regulation (review). Mol. Membr. Biol. **19**, 27–38
31 Chi, N. and Epstein, J. A. (2002) Getting your Pax straight: Pax proteins in development and disease. Trends Genet. **18**, 41–47
32 Rozanov, D. V., Ghebrehiwet, B., Ratnikov, B., Monosov, E. Z., Deryugina, E. I. and Strongin, A. Y. (2002) The cytoplasmic tail peptide sequence of membrane type-1 matrix metalloproteinase (MT1-MMP) directly binds to gC1qR, a compartment-specific chaperone-like regulatory protein. FEBS Lett. **527**, 51–57
33 de Coignac, A. B., Elson, G., Delneste, Y., Magistrelli, G., Jeannin, P., Aubry, J. P., Berthier, O., Schmitt, D., Bonnefoy, J. Y. and Gauchat, J. F. (2000) Cloning of MMP-26. A novel matrilysin-like proteinase. Eur. J. Biochem. **267**, 3323–3329
34 Leontovich, A. A., Zhang, J., Shimokawa, K., Nagase, H. and Sarras, Jr, M. P. (2000) A novel hydra matrix metalloproteinase (HMMP) functions in extracellular matrix degradation, morphogenesis and the maintenance of differentiated cells in the foot process. Development **127**, 907–920
35 Llano, E., Pendas, A. M., Freije, J. P., Nakano, A., Knauper, V., Murphy, G. and Lopez-Otin, C. (1999) Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase A overexpressed in brain tumors. Cancer Res. **59**, 2570–2576
36 Llano, E., Pendas, A. M., Aza-Blanc, P., Kornberg, T. B. and Lopez-Otin, C. (2000) Dm1-MMP, a matrix metalloproteinase from *Drosophila* with a potential role in extracellular matrix remodeling during neural development. J. Biol. Chem. **275**, 35978–35985
37 Uria, J. A. and Lopez-Otin, C. (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res. **60**, 4745–4751
38 Velasco, G., Cal, S., Merlos-Suarez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J. and Lopez-Otin, C. (2000) Human MT6-matrix metalloproteinase: identification, progelatinase A activation, and expression in brain tumors. Cancer Res. **60**, 877–882
39 Damjanovski, S., Amano, T., Li, Q., Pei, D. and Shi, Y. B. (2001) Overexpression of matrix metalloproteinases leads to lethality in transgenic *Xenopus laevis*: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev. Dyn. **221**, 37–47
40 De Strooper, B. and Annaert, W. (2001) Where Notch and Wnt signaling meet. The presenilin hub. J. Cell Biol. **152**, F17–F20
41 Guidos, C. J. (2002) Notch signaling in lymphocyte development. Semin. Immunol. **14**, 395–404
42 Allman, D., Aster, J. C. and Pear, W. S. (2002) Notch signaling in hematopoiesis and early lymphocyte development. Immunol. Rev. **187**, 75–86
43 Frisen, J. and Lendahl, U. (2001) Oh no, Notch again! Bioessays **23**, 3–7

Received 28 January 2003/4 March 2003; accepted 5 March 2003
Published as BJ Immediate Publication 5 March 2003, DOI 10.1042/BJ20030174