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REGULAR ARTICLE

Contrasting expression of membrane metalloproteinases, MT1-MMP and MT3-MMP, suggests distinct functions in skeletal development

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Abstract Membrane-type 1 matrix metalloproteinase (MT1-MMP) is the most ubiquitous and widely studied of the membrane-type metalloproteinases (MT-MMPs). It was thus surprising to find no published data on chicken MT1-MMP. We report here the characterization of the chicken gene. Its low sequence identity with the MT1-MMP genes of other species, high GC content, and divergent catalytic domain explains the absence of data and our difficulties in characterizing the gene. The absence of structural features in the chicken gene that have been suggested to be critical for the activation of MMP-2 by MT1-MMP; for the effect of MT1-MMP on cell migration and for the recycling of MT1-MMP suggest these features are either not essential or that MT1-MMP does not perform these functions in chickens. Comparison of the expression of chicken MT1-MMP with MT3-MMP and with MMP-2 and MMP-13 has confirmed the previously recognized co-expression of MT1-MMP with MMP-2 and MMP-13 in fibrous and vascular tissues, particularly those surrounding the developing long bones in other species. By contrast, MT3-MMP expression differs markedly from that of MT1-MMP and of both MMP-2 and MMP-13. MT3-MMP is expressed by chondrocytes of the developing articular surface. Similar expression patterns of this group of MT-MMPs and MMPs have been observed in mouse embryos and suggest distinct and specific functions for MT1-MMP and MT3-MMP in skeletal development.

Keywords MT1-MMP · MT3-MMP · Skeletal development · Cartilage · Chicken

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Introduction

The matrix metalloproteinases (MMPs) are a large family of zinc-dependent proteinases that have long been suggested to play a central role in extracellular matrix remodeling associated with embryonic development and morphogenesis (Murphy et al. 1999; Nagase and Woessner 1999). The generation of mice deficient in several individual MMPs (MMP-2, MMP-3, MMP-7, MMP-9, MMP-11, and MMP-12) has however shown that, although each might play specific and important roles in matrix remodeling, none are critical for skeletal development (Itoh et al. 1997; Masson et al. 1998; Mudgett et al. 1998; Puente et al. 2003; Vu et al. 1998; Wilson et al. 1997). A subset of MMPs, the membrane bound MMPs (MT1-MMP to MT6-MMP), has been described. These enzymes share structural homology with the other MMPs but (with the exception of MT4-MMP and MT6-MMP, which have a glycosylphosphatidylinositol anchor) possess an additional single-pass transmembrane domain (Nagase and Woessner 1999). Generation of two mouse strains deficient in MT1-MMP has demonstrated the essential role of this enzyme in skeletal development (Holmbeck et al. 1999; Zhou et al. 2000). Both strains have a similar phenotype with severe defects in craniofacial, axial, and appendicular skeletogenesis. The small size of the mice (30%–40% of control littermate weight at 3 weeks) and the defective skeletal development appear to be attributable to decreased chondrocyte mitosis and/or vascular resorption. Thickened growth plates and delayed development of the secondary ossification centers have been observed.

In situ localization of MT1-MMP in mouse embryos has demonstrated prominent expression in fibrous tissues around the cartilaginous anlagen of developing bones, in developing tendons, and in ligament and fascial sheaths between muscle compartments. Blood vessels, particularly

those associated with cartilage vascularization in developing embryos, have demonstrated prominent labeling. MT1-MMP and MMP-2 appear to be co-expressed and are usually observed in the same tissue location (Apte et al. 1997; Kinoh et al. 1996) consistent with a proposed activation of MMP-2 by MT1-MMP (Morrison et al. 2001; Sato et al. 1996). Recent identification of MT1-MMP substrates suggests that this class of proteinase also functions in cell signaling processes and pericellular matrix remodeling (Tam et al. 2004).

MT3-MMP has been less well studied. It has been shown to be expressed in the developing chicken skeleton and developing neural tube, perichondrium, and muscle (Yang et al. 1996). The present studies describe the characterization of the chicken MT1-MMP gene and the demonstration of prominent but distinct expression of both MT1-MMP and MT3-MMP and of MMP-2 and MMP-13 in the developing chicken and mouse skeleton.

Materials and methods

Cloning chicken MT1-MMP RNA was extracted from freshly dissected chicken embryo limbs by homogenization (PRO 200 homogenizer, Scientific, Oxford, Conn.) in TRIzol (Invitrogen, Carlsbad, Calif.) and further purified by high salt and isopropanol precipitation. The quality of total RNA purified was assessed by agarose electrophoresis and quantified by absorbance at 260 nm. RNA was denatured with 40% DMSO (dimethylsufoxide) at 65°C, and reverse transcription was conducted in the presence of 10% DMSO by using Superscript II reverse transcriptase (Invitrogen) at 45°C. The polymerase chain reaction (PCR) was conducted with *Taq* DNA polymerase in the presence of 10% DMSO with an annealing temperature of 55°C. A series of new degenerate primers designed from the conserved regions of known MT1-MMPs of all species were used to amplify the potential chicken MT1-MMP cDNA fragments. Two primers, viz., 5′-CCT (A/C)GI TG (C/T) GGI GTI CCI GA-3′ and 5′-TCA CCG TG(A/G) AAI CC(T/C) TCI CG(A/G) AA-3′, derived from conserved sequences of all known MT1-MMP ^{91}PRCGVPD^{97} and ^{181}FAEGHGD^{188} peptides resulted in an expected PCR product of ~280 bp. Cloning and sequencing of the PCR product suggested that it was a new chicken MMP gene, and that it was most closely related to human and bovine MT1-MMP sequences. Further 5′- and 3′-end cDNA sequences were generated by using nested PCR and rapid amplification of cDNA ends (RACE) similar to that used for cloning other chicken MMP cDNAs (Yang et al. 1996; Yang and Kurkinen 1998). Nested PCR was conducted with r*Tth* DNA Polymerase, XL (Applied Biosystems, Foster City, Calif.). All the PCR products were gel-purified, polished, and ligated into pZero-2 pre-digested with *Eco*RV. Sequencing was conducted in a local sequence core facility by using ABI's BigDye Terminators reagents with the inclusion of 5% DMSO in the sequencing reaction.

RNA expression Reverse-transcription-coupled PCR was performed as described previously (Yang and Kurkinen 1994). Briefly, 1 μg total RNA was denatured with random primers and nucleotides at 65°C for 10 min and chilled on ice. A master mixture of Superscript II and reaction buffer was added to a final volume of 20 μl, and the reaction was incubated at 42°C for 40 min. TE buffer (40 μl TRIS/EDTA buffer) containing RNase was added, and the cDNA mixture was heated to 75°C for 5 min and stored at -20°C. A sample of 1 μl cDNA was used for PCR (with the primers shown in Table 1); all amplification reactions included 5% DMSO as a co-solvent to enhance the PCR efficiency. Each cDNA pool was first normalized by PCR by using D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers for only 22–25 cycles and cDNA fragments of genes of interest amplified for 25–30 cycles.

RNA in situ hybridization Chicken and mouse MT1-MMP, MT3-MMP, MMP-13, and MMP-2 cDNA fragments were derived from 3′ untranslated regions or regions corresponding to the C-terminal region of the protein and were cloned into pZero-2 vector (Invitrogen). Digoxigenin (DIG)-labeled sense or antisense RNA probes were synthesized from the linearized cDNA fragments in the pZero-2 vector by using DIG-labeled UTP with either T7 or SP6 RNA polymerase. Chicken and mouse embryos at various stages

Table 1 PCR primers (*GAPDH* D-glyceraldehyde-3-phosphate dehydrogenase, *MMP* metalloproteinase, *MT1 MMP* membrane-type 1 matrix metalloproteinase, *Col X* collagen X)

Primer type	Sequence
cGAPDH	5′-ATG GTG AAA GTC GGA GTC AAC GGA-3′ and 5′-TCA CTC CTT GGA TGC CAT GTG GAC-3′
cMMP-13	5′-CTT GTT GCT GCT CAT GAA TTT G-3′ and 5′-ATG CAG TTC CAG ACT GAC ATA CA-3′
cMT1-MMP	5′-GAC GGC GGC TTC GAT ACC A- 3′ and 5′- ATC GCG GAG CAG GGA TTT G-3′
cMT3-MMP	5′-GTA ACA AGT TCT GGG TTT TCA AGG-3′ and ACA TCA CAC CCA CTCTTG CAT AG-3
cMMP-2	5′-CGT AATGAT GGA TTC CTC TGG-3′ and 5′-CTG GGT CCT CGG AGT GCT CTA-3′
cCol X	5′-ATC ATC AGC TTC TGC TCA CTC ACC-3′ and 5′-GCC CTT GTT CAC CCC TCA TCT-3′

of development were fixed in 4% paraformaldehyde in phosphate-buffered saline, processed, embedded in paraffin and sectioned. The 7-μm sections were mounted on Probe-On slides (Fisher Scientific, Pittsburgh, Pa.). RNA in situ hybridization was conducted in a 50% formamide-based hybridization solution at 60°C overnight and washed at high stringency in 2×standard sodium citrate (SSC), 0.1% CHAPS, three times. Hybridized probes were detected by immunohistochemistry with anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche, Indianapolis, Ind.). Chromogenic staining was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate, alkaline phosphatase substrates. Sections were also stained with toluidine blue by routine procedures.

Results

Cloning and sequencing of chicken MT1-MMP

We had been previously unsuccessful at isolating the chicken MT1-MMP gene based on the use of degenerate primers from other species. This was surprising considering the relative abundance of this gene product in other species and the success with the cloning of other chicken MMPs. The difficulty was primarily attributable to the much greater sequence variability such that a number of degenerate primers designed from conserved regions of MT1-MMP from other species failed to amplify the chicken gene, together with the high GC content of the chicken MT1-MMP cDNA. Use of a high DMSO concentration during both reverse transcription and PCR enabled amplification with one pair of primers (cDNA was not able to be amplified by using degenerate primer pairs designed from several other conserved regions of MT1-MMP). The remaining sequence was generated by using nested primers and RACE technology (Fig. 1a). Although containing only slightly more than 60% identity in amino acid sequence, the chicken MT1-MMP gene has highest sequence identity with other MT1-MMPs confirming its identity (Table 2). By comparison, chicken MT3-MMP has greater than 90% amino acid sequence identity with the same gene in other species. Frog and zebrafish MT1-MMP have greater identity with the human than chicken (similar to the MT3-MMP sequence identity between these species, Table 3).

The highly conserved MT1-MMP catalytic domain shows sequence variation in chickens that is not seen with other species, including a deletion of 5 amino acid residues (^{164}YAYIR^{168} Fig. 1b). A similar sequence of amino acids to those in human MT1-MMP occur in the same region of human MT2 and MT3-MMP and constitute 5 amino acids of the 8-amino-acid region termed the MT-loop. Chicken MT1-MMP also has a 3-amino-acid deletion and several amino acid substitutions in the hinge region. As a result only one (T^{300}) of the three threonine and two serine residues suggested to be O-gylcosylyated in human MT1-MMP (T^{291}, T^{299}, T^{300}, S^{301}, and S^{304}; Remacle et al. 2006; Wu et al. 2004) is present in chicken MT1-MMP. The chicken MT1-MMP also has a large insertion of 14 amino acids in the second hinge (also termed stalk) region adjacent to the transmembrane domain. The cytoplasmic domain of the chicken MT1-MMP also exhibits sequence differences from that of other species. The chicken MT1-MMP arginine residue in the cytoplasmic domain is substituted for the tyrosine residue seen in other species. Recent studies have shown that this tyrosine residue (Y^{573}) in human MT1-MMP undergoes Src-dependent phosphorylation and has been suggested to influence cell migration (Nyalendo et al. 2007) and MT1-MMP recycling (Uekita et al. 2001). The chicken MT1-MMP also has only a partially conserved carboxy-terminal PRV sequence (Fig. 1b). In MT1-MMP from other species, the carboxy-terminal sequence is DKV and has been shown to be required for MT1-MMP recycling (Wang et al. 2004).

MMP expression during chicken limb development

MT3-MMP and MT1-MMP were shown to be expressed by several tissues of the chicken embryo including lung, skin and brain. Expression, however, was most prominent in skeletal tissues (Fig. 2a). MT1-MMP and MT3-MMP were both expressed from early to late skeletal development. Both MT1-MMP and MT3-MMP expression tended to reach a peak in association with hypertrophic differentiation in the cartilage anlagen, as indicated by type X collagen and MMP-13 expression at 11–12 days as (Fig. 2b).

In situ detection of MT1-MMP, MT3-MMP, MMP-2, and MMP-13 expression in developing chicken and mouse skeleton

MT1-MMP and MT3-MMP showed distinct expression patterns in the developing chicken skeleton. In most sites where MT3-MMP expression was prominent, MT1-MMP expression was absent or weak. Similarly, where MT1-MMP expression was strong, MT3-MMP expression was usually absent (Fig. 3). MT1-MMP expression was prominent in vascular and fibrous tissues of the developing limb and joint. The meniscus, perichondrium, ligament, and cartilage canals showed strong expression (Fig. 3a,g). Of note, a fibrous non-cartilaginous tissue covers the cartilage surface of articulating joints in chickens. This can be seen clearly as a blue-stained tissue in contrast to the underlying metachromatic (purple, proteoglycan-rich) cartilage in toluidine-blue-stained sections (Fig. 3e,j). MT3-MMP expression was prominent in cartilage near the articular

a

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gccgccatggcgcccgctctgctgctgctgctcctctgctgcgccgccccccccccgccgccgccttccggcccgaggcgtggctgcag   90
     M  A  P  A  L  L  L  L  L  L  C  C  A  A  P  P  P  A  A  A  F  R  P  E  A  W  L  Q
cagtacggctatctgccccccggcgacctccgcgcccaccccccacttcggcccattcggtatcggccgcgttggccgccatgcagcgc  180
Q  Y  G  Y  L  P  P  G  D  L  R  A  H  P  P  T  S  A  H  S  V  S  A  A  L  A  A  M  Q  R
ttctacgggctgcgcgtcaccggaagtgtcgacccggaaacgctgcgggccatgaagcgcccccgctgtggggtcccggaccgctttggg  270
F  Y  G  L  R  V  T  G  S  V  D  P  E  T  L  R  A  M  K  R  P  R  C  G  V  P  D  R  F  G
gcggaggtgaaggccaacgtgaggcgccgccgttacgccattcagggctccaagtgggagcagcgcgacatcaccttctgccttcagaac  360
A  E  V  K  A  N  V  R  R  R  R  Y  A  I  Q  G  S  K  W  E  Q  R  D  I  T  F  C  L  Q  N
cacaccccgaaggtgggggaggcggccacccgcgctgccatcctccgcgccttcggggtgtgggcgtccgtcaccccactgcgcttccgg  450
H  T  P  K  V  G  E  A  A  T  R  A  A  I  L  R  A  F  G  V  W  A  S  V  T  P  L  R  F  R
gaagtgccccccggcgccgcccccccgccgacatcgtcctcttcttcgccgagggcttccacggcgacagctccccttcgacggcgag  540
E  V  P  P  G  A  A  P  P  A  D  I  V  L  F  F  A  E  G  F  H  G  D  S  S  P  F  D  G  E
gggggttcctggcccacgcctacttccccgggccccacatcgggggggacacgcacttcgacggcgccgagccctggaccacgcgcaac  630
G  G  F  L  A  H  A  Y  F  P  G  P  H  I  G  G  D  T  H  F  D  G  A  E  P  W  T  T  R  N
gacgacctcagcggtcacgacgtgttcctggtggcgctgcacgagctgggccacgcgctgggcctggagcactccagcgacccctcggcc  720
D  D  L  S  G  H  D  V  F  L  V  A  L  H  E  L  G  H  A  L  G  L  E  H  S  S  D  P  S  A
gtcatggcgcccttctaccaatggatggacaccgcccccttcgtgctgcccgacgacgaccgccgcggcatccagcagctctacgggccg  810
V  M  A  P  F  Y  Q  W  M  D  T  A  P  F  V  L  P  D  D  D  R  R  G  I  Q  Q  L  Y  G  P
ggtcccaacatgccccccccggaccccccgcggcacagcgctgccccacgaccccgaccggccgccccacggccccccctatgggccccgc  900
G  P  N  M  P  P  P  D  P  R  G  T  A  L  P  H  D  P  D  R  P  P  H  G  P  P  Y  G  P  R
atctgcgacggcggcttcgataccatcgcggtgctcaggggggagatgttcgtgttcaaggagcggtggctgtggcggctgcgggagcgc  990
I  C  D  G  G  F  D  T  I  A  V  L  R  G  E  M  F  V  F  K  E  R  W  L  W  R  L  R  E  R
cgggtgctgcccggttacccccctccctatggggcagctgtggcccggactgccccacagcatcgacgccgcctatgagaggaaggacggc 1080
R  V  L  P  G  Y  P  L  P  M  G  Q  L  W  P  G  L  P  H  S  I  D  A  A  Y  E  R  K  D  G
aagttcgtcttcttcaaaggcgggcggcagtgggtgttctcggaggcggcgctgcagccgggcttcccgcgcgctctgccggacgtgggc 1170
K  F  V  F  F  K  G  G  R  Q  W  V  F  S  E  A  A  L  Q  P  G  F  P  R  A  L  P  D  V  G
cgggggctgccggagcgcatcgacgccgcgctgctgtggctgcccagcggggccacgtacctcttccggggcgacaagtactaccggttc 1260
R  G  L  P  E  R  I  D  A  A  L  L  W  L  P  S  G  A  T  Y  L  F  R  G  D  K  Y  Y  R  F
aatgaggagacggagtcggtggaccccgattaccccaaaagcatttccgtgtggggcggcgtccccgaatcaccccaaggagcatttatg 1350
N  E  E  T  E  S  V  D  P  D  Y  P  K  S  I  S  V  W  G  G  V  P  E  S  P  Q  G  A  F  M
gggtcggatgacgcctacacgtactttgtgaagggctcccgctattggcagttcgacaaccgccagctgcgcgtcaccccgggttacccc 1440
G  S  D  D  A  Y  T  Y  F  V  K  G  S  R  Y  W  Q  F  D  N  R  Q  L  R  V  T  P  G  Y  P
aaatccctgctccgcgattggctgggctgcccggagccccgcccaccaccccgccctggccccgcccttcctcttccccgccggaaacg 1530
K  S  L  L  R  D  W  L  G  C  P  E  P  R  P  P  P  R  P  G  P  A  P  S  S  S  P  P  E  T
ggcgccgggggggggggtggggaaacggaagtcatcgtcatcgaagtgggcggggaaggggcggggcccggagcggtggccacgccccctg 1620
G  A  G  G  G  G  G  E  T  E  V  I  V  I  E  V  G  G  E  G  A  G  P  G  A  V  A  T  P  L
gcgctgctgggggggccgggggctgctggcggccgtgctgtggttccgccgccgggggggcgcccaagaaactgctgcgctgtcagcgc 1710
A  L  L  G  G  A  G  G  L  L  A  A  V  L  W  F  R  R  R  G  A  P  K  K  L  L  R  C  Q  R
tccctcctgccccgcgtttaggccacgccccccccacccccccccagacctgcgtggccagcccactcccccaccacccctcccgtgttg 1800
S  L  L  P  R  V  *
tcaatgggagagaggccacgcccacactgcccaagccacgcccacaatcacacaccacgcccccgacctgcgtggccacgcccactcccc 1890
cctccacccctcccgtgttgtc                                                                      1980
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Fig. 1 **a** Nucleotide and deduced amino acid sequence of full-length cDNA encoding chicken MT1-MMP. **b** Sequence alignment of chick (*ch*), human (*hu*), and bovine (*bov*) MT1-MMPs (*brown bars* deletions in the chicken enzyme, *green triangles* threonine or serine residues suggested to be glycosylated in human MT1-MMP, *orange bars* a 14-amino-acid insertion in the chicken enzyme, *yellow stars* LL residues required for enzyme recycyling, *purple star* Y^{573} of human MT1-MMP that has been replaced by R in the chicken enzyme). Note that the numbering is affected by the insertion in the chicken (*orange star* carboxy-terminal PRV in chicken and DKV in other species. Sequence alignment was performed by using online software (Corpet 1988)

surface but was not seen in the overlying fibrous tissue. MT3-MMP was weakly expressed in cells of the cartilage canals but was prominent in chondrocytes surrounding these canals (Fig. 3b,h). Chondrocytes expressing MT3-MMP appeared to be smaller and more closely packed than the underlying proliferating chondrocytes associated with growth cartilage, where MT3-MMP was more weakly expressed (compare Fig. 3h,j). MMP-13 and MMP-2 showed a similar expression pattern to MT1-MMP. Expression was prominent in fibrous tissues covering the articular cartilage, in the perichondrium, meniscus, ligament, and cartilage canals. MMP-13 was also expressed in chondrocytes near the articular surface, which also expressed MT3-MMP (Fig. 3c,i), and in more distal chondrocytes associated with endochondral bone formation. MMP-2 expression in articular chondrocytes was extremely weak compared with the strong expression in the overlying non-cartilaginous fibrous tissue (Fig. 3d).

The site of the primary ossification center in the tibia showed expression of MT1-MMP in the invading vascular tissue and in surrounding hypertrophic chondrocytes (Fig. 3k). MT3-MMP showed a similar expression pattern in cells in the region of invading blood vessels and developing bone marrow adjacent to the growth cartilage and in some hypertrophic chondrocytes in this region. It was not prominently expressed in most of the vascular

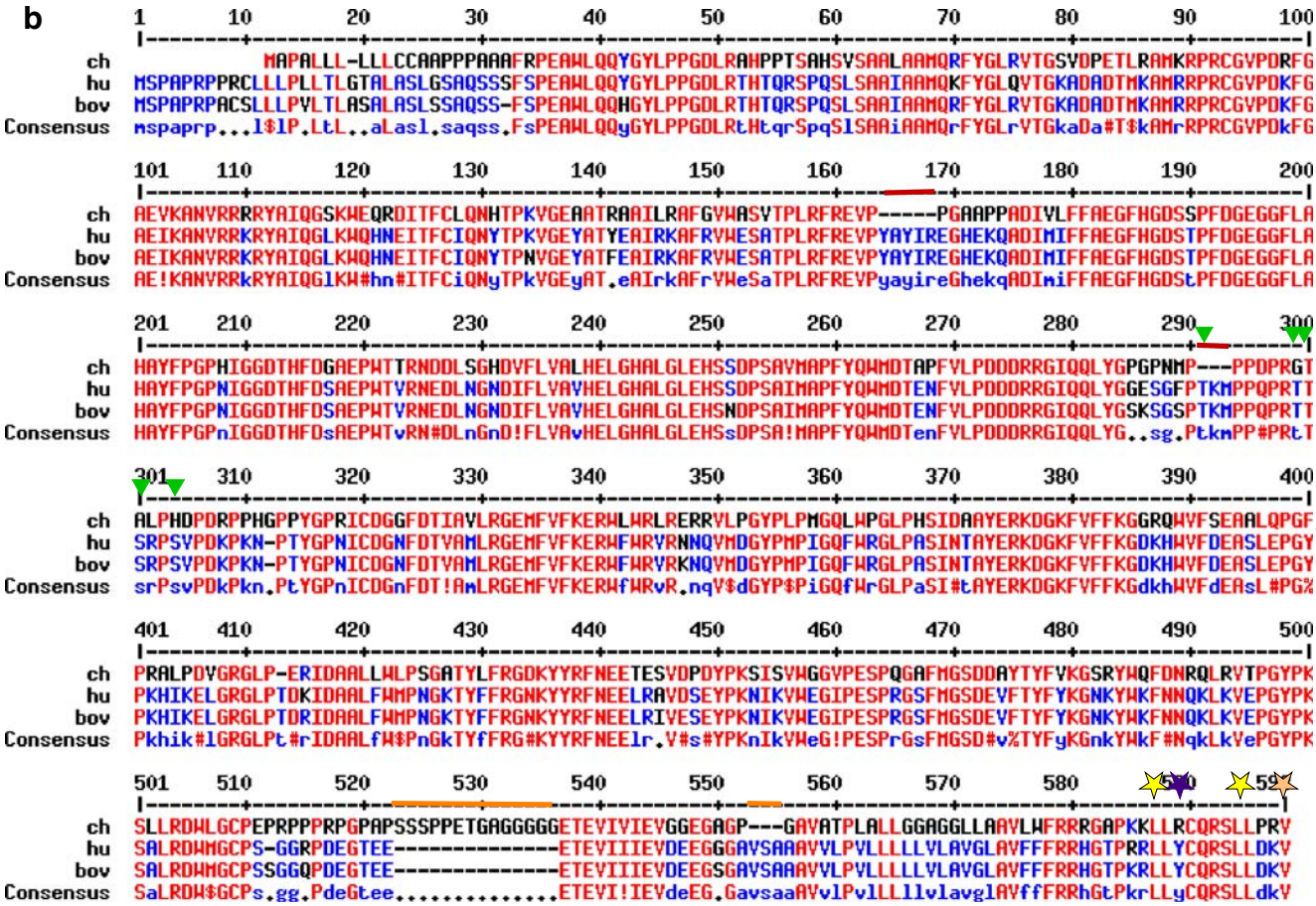


Fig. 1 (continued)

tissue. MMP-13 also showed expression in cells of the invading vasculature and scattered hypertrophic chondrocytes similar to MT1-MMP and MT3-MMP.

The pattern of MT1-MMP and MT3-MMP seen in mouse embryos was similar to that observed in the chicken embryo. As shown in Fig. 3p, MT1-MMP expression was prominent in perichondrial tissue surrounding developing cartilaginous anlagen and within the joint space. In contrast, MT3-MMP expression was largely confined to chondrocytes near the cartilage surface (Fig. 3q). Similarly in the developing spine, MT1-MMP expression was most prominent in the perichondrium and developing disk (Fig. 3s). MT3-MMP expression was most prominent in the devel-

Table 2 Sequence identity of chicken MT1-MMP with other MT-MMPs and with MT1-MMP from other species

cMT1-MMP identity to:	Amino acid identity (%)
hMT1-MMP	64
hMT2-MMP	51
hMT3-MMP	50
hMT4-MMP	40
hMT5-MMP	40
Dog MT1-MMP	64
Frog MT1-MMP	63
Rat MT1-MMP	64
Cow MT1-MMP	64
Mouse MT1-MMP	64
Rabbit MT1-MMP	63

Table 3 Sequence identity of chicken MT3-MMP with other MT-MMPs and MT3-MMP from other species

cMT3-MMP identity to:	Amino acid identity (%)
hMT1-MMP	55
hMT2-MMP	54
hMT3-MMP	94
hMT4-MMP	38
hMT5-MMP	37
Dog MT3-MMP	93
Frog MT3-MMP	92
Rat MT3-MMP	95
Cow MT3-MMP	96
Mouse MT3-MMP	94
Rabbit MT3-MMP	94

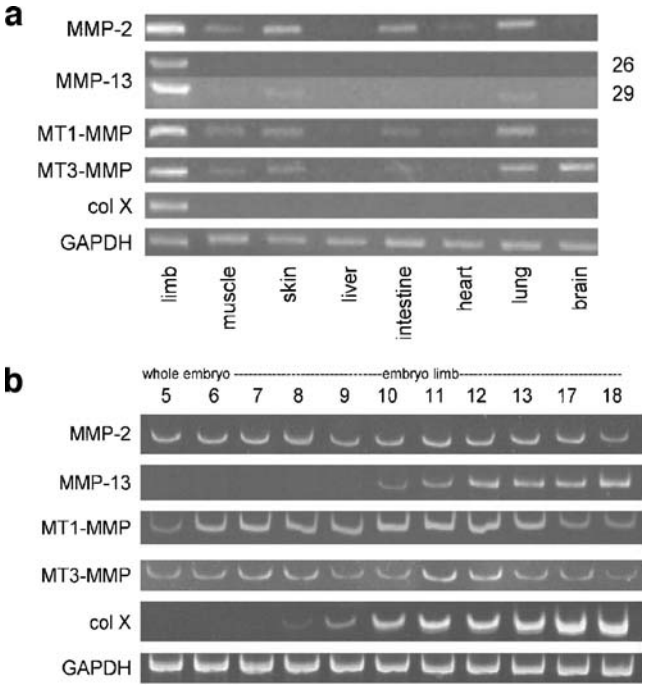


Fig. 2 Tissue and development-specific expression of MT1-MMP and MT3-MMP. RNA extracted from tissues of 14-day chicken embryos (**a**) or embryo and embryo limbs at various stages of development (**b**) were analyzed for MMP-2, MMP-13, MT1-MMP, MT3-MMP, Col X, and GAPDH expression by reverse transcription/polymerase chain reaction (RT-PCR) with specific primers; 26 cycles (*26*) of PCR amplification were used, except for MMP-13 in **a** where results were obtained by using 29 cycles (*29*) to demonstrate expression in non-skeletal tissues

oping vertebrae, particularly in the region of the presumptive endplate (Fig. 3t).

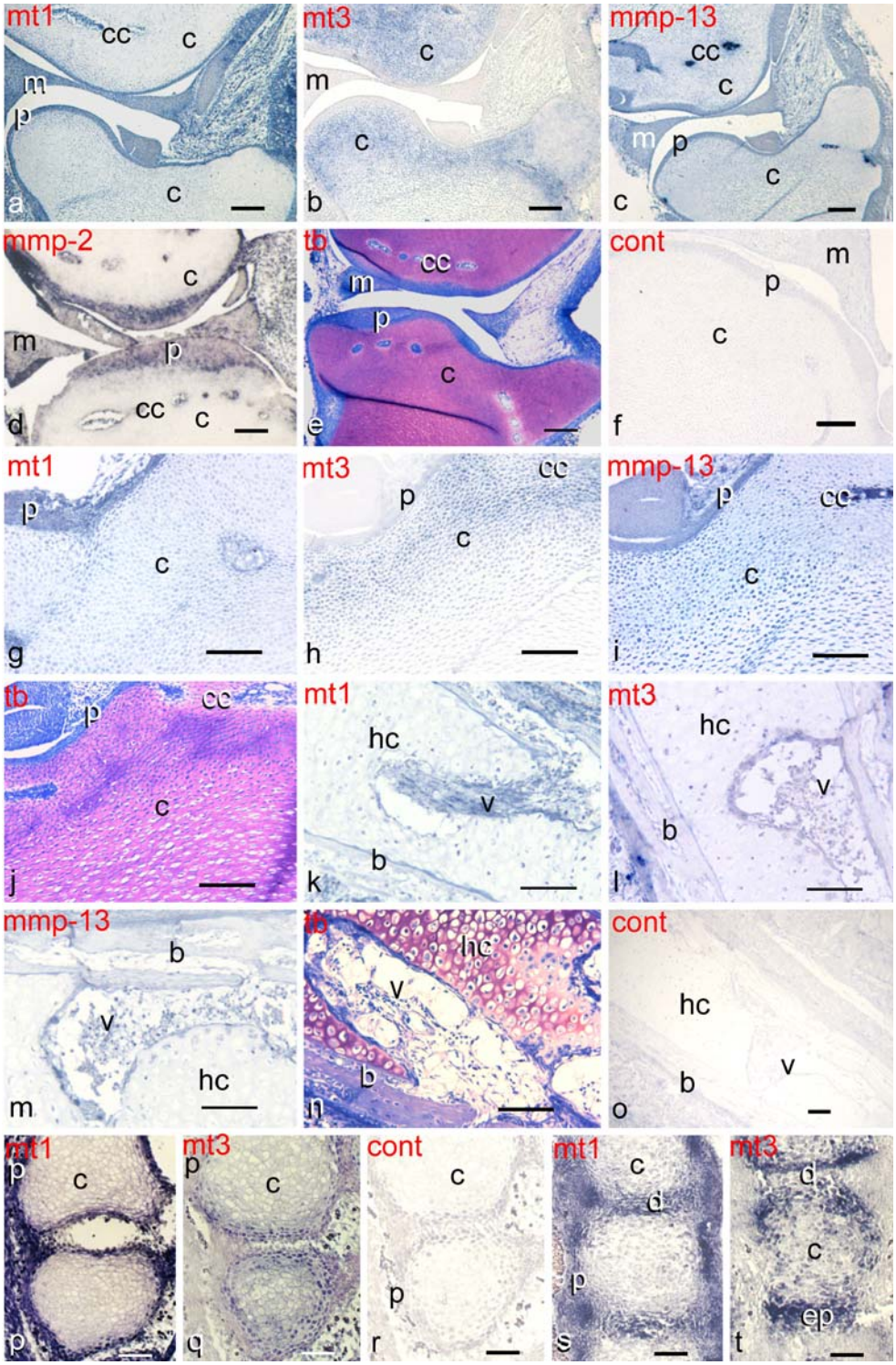
Discussion

Although the chicken MT1-MMP is clearly homologous with MT1-MMP of other species, we have found much lower sequence identity than seen between these other species, thereby explaining difficulties associated with its isolation and characterization. The high GC content of the chicken MT1-MMP gene is indicative of its presence, like approximately half the chicken genes, in one of the many microchromosomes. The high recombination rate of microchromosomes also explains the relatively low sequence identity of MT1-MMP genes with those of other species (Burt 2002). Although most domain structures and critical amino acid sequences, such as the furin cleavage sites, catalytic domain, hinge region, hemopexin domain, and transmembrane and cytoplasmic domains of chicken MT1-MMP are conserved as expected, several features of the amino acid sequence thought to be critical for specific functions of this enzyme are absent or substantially different in chicken MT1-MMP. The most prominent of these is a 5-amino-acid deletion in the MT-loop. The MT-loop is an 8-amino-acid insertion in the catalytic domain of several MT-MMPs (Fernandez-Catalan et al. 1998). It forms a pocket in the MT-MMP catalytic domain that interacts with TIMP2 and appears to facilitate the efficient activation of MMP-2. Mutations in this domain of human MT1-MMP impair the activation of proMMP-2 (English et al. 2001). Although in situ hybridization has demonstrated the co-expression of MT1-MMP and MMP-2, the truncated MT-loop of MT1-MMP suggests that it would not function as an efficient activator of MMP-2 in chickens. The hinge region of chicken MT1-MMP also shows extensive differences in amino acid sequence, including a 3-amino-acid deletion. This results in the presence of only one of the three threonine and two serine residues suggested to be O-glycosylated. Published data suggest that glycosylation restricts the autocatalytic degradation of MT1-MMP (Remacle et al. 2006) and possibly facilitates proMMP-2 activation (Wu et al. 2004). The level of glycosylation in these studies is probably influenced by overexpression of MT1-MMP in cell lines. The glycosylation of a single threonine residue in chicken MT1-MMP might be sufficient to restrict autocatalysis or other functions associated with this post-translational modification of the enzyme.

The presence of a large insertion in the second hinge region of chicken MT1-MMP is consistent with the

Fig. 3 Localization of MT1-MMP, MT3-MMP, MMP-13, and MMP-2 expression in developing chicken and mouse skeleton. Parafin sections of 14-day chicken embryo legs or 16-day mouse embryos were examined for MT1-MMP (**a**, **g**, **k**, **p**, **s**), MT3-MMP (**b**, **h**, **l**, **q**, **t**), MMP-13 (**c**, **i**, **m**), or MMP-2 (**d**) expression by in situ hybridization or stained with toluidine blue (**e**, **j**) to show morphology. Sites of probe binding are shown as *dark blue deposits*. Similar regions of the femoro-tibial joint of 14-day chick embryos are shown in **a–f** at low power and **g–j** at higher power. Strong expression of MT1-MMP, MMP-13, and MMP-2 is seen in the meniscus (*m*), perichondrium (*p*), and cartilage canals (*cc*). In contrast, strong expression of MT3-MMP is seen in chondrocytes (*c*) near the articular surface but is not detected in the perichondrium and meniscus. Sites of vascular resorption in the primary ossification center of the same chicken tibias are shown in **k–o**. Expression of MT1-MMP, MT3-MMP, and MMP-13 is prominent in hypertrophic chondrocytes (*hc*) surrounding resorbing cartilage and cells associated with invading blood vessels and developing bone marrow abutting the cartilage surface. Strong expression of MT1-MMP and MMP-13 but relatively weak expression of MT3-MMP is seen in forming bone marrow and associated with blood vessels (*v*) at the primary ossification center. MT1-MMP and MT3-MMP expression in 16-day mouse tarsometatarsal joint (**p–r**) and developing spine (**s**,**t**). MT1-MMP expression is prominent in perichondrium (*p*) and fibrous tissue of the developing tarsometatarsal joint and perichondrium (*p*), surrounding the fibrous tissue and immature intervertebral disk (*d*) of the developing spine. MT3-MMP expression is prominent in chondrocytes near the articular surface of the developing joint and chondrocytes of the endplate (*ep*) of developing vertebrae but is not detectable in the perichondrium, fibrous tissues, or developing intervertebral disk. Sense probes for MT1-MMP (**f**), MT3-MMP (**o**, **r**) gave no signal. *Bars* 250 μm (**a-f**), 100 μm (**g-o**), 60 μm (**p-t**)

heterogeneity of the sequence and the length of this domain observed in other species, such as *Xenopus* and the takifugu. The function of this domain and effects of domain length remain to be determined. Of note, this variable domain is adjacent to a highly conserved region and possible critical structure composed of glutamate and hydrophobic amino acids and lies at the start of the membrane insertion domain. In the cytoplasmic domain, the tyrosine residue (Y^{573}) of human MT1-MMP, which has been shown to undergo Src-dependent phosphorylation (Nyalendo et al. 2007), is substituted with an arginine in chicken MT1-MMP. Studies suggest that the phosphoryla-



tion of this tyrosine residue plays a key role in cell migration and MT1-MMP recycling. Mutation or deletion of ^{571}LLY^{573} and the neighboring ^{578}LL^{579} has been shown to prevent the internalization of human MT1-MMP in cultured cell lines, although the introduction of a Y573A mutation alone does not have a significant effect on internalization (Uekita et al. 2001). Although ^{573}Y is not conserved in chicken MT1-MMP, both the ^{571}LL^{572} and ^{578}LL^{579} sequences are conserved. The substitution of the tyrosine residue in the cytoplasmic domain of chicken MT1-MMP for arginine indicates that tyrosine phosphorylation is not critical for MT1-MMP recycling or cell migration in the chicken. The sequence differences between chicken and human MT1-MMP also suggest that phosphorylation of this tyrosine residue might not play the key role in recycling and cell migration that has been proposed. The adjacent carboxy-terminal sequence DKV has also been shown to be required for MT1-MMP recycling in human MT1-MMP (Wang et al. 2004). The carboxy-terminal sequence in chicken MT1-MMP is PRV, thereby suggesting the aspartate residue in the human sequence might not be critical for recycling. Future investigation of the effects of chicken MT1-MMP on MMP-2 activation, enzyme recycling, and cell migration might provide a more detailed understanding of structures of MT1-MMP critical for its role in these processes.

Cloning chicken MT1-MMP has enabled a comparison of its expression with that of MT3-MMP and other MMPs in the developing chicken limb. Both MT1-MMP and MT3-MMP have prominent and distinct tissue-specific expression in the developing skeleton. MT1-MMP expression is most prominent in fibrous tissues of the developing limb and shows a similar expression pattern to MMP-13 and MMP-2. In contrast, MT3-MMP expression is prominent in chondrocytes and not detectable in fibrous tissues of the developing skeleton. The association of MT1-MMP and MT3-MMP with skeletal development has been suggested previously (Kinoh et al. 1996; Yang et al. 1996; Zhou et al. 2000); however, the cell-specific expression described in the present study differs from that reported previously. In the initial description of the chicken MT3-MMP gene, we described its expression in the neural tube, in developing muscle, and in chondrocytes and the perichondrium of the developing skeleton. In the present study, we have demonstrated that skeletal expression of MT3-MMP is most prominent in, and possibly restricted to, chondrocytes, particularly those of the developing articular cartilage in mouse and chicken embryos. In contrast to earlier studies, expression has not been detected in the perichondrium. Riboprobes have been employed in the present study, in contrast to the cDNA probes used previously. The increase in sensitivity has facilitated greater stringency in hybridization and may explain the absence of hybridization signal in the perichondrium and other fibrous tissues in the present studies. The patterns of MT1-MMP and MT3-MMP expression are similar in chicken and mouse embryos, consistent with the specific and distinct functions of these enzymes.

MT1-MMP has been shown to play a prominent role in cell matrix interaction. MT1-MMP substrates include collagen, (Holmbeck et al. 2004), CD44 (Kajita et al. 2001), αvβ3 integrin (Deryugina et al. 2002), cell-surface transglutaminase (Zhou et al. 2000), and proMMPs (Knauper et al. 2002). Proteolytic modification of the first four substrates suggests MT1-MMP and probably other members of this enzyme family play a key role in the regulation of cell phenotype by specific modulation of pericellular matrix molecular dynamics. The capacity of MT1-MMP to activate the pro-form of other MMPs, particularly MMP-2, suggests that these enzymes provide a focal, transcriptionally controlled mechanism for the regulation of proteolytic cascades. MT1-MMP has also been shown to modify chemokine activity (McQuibban et al. 2001) and cellular migration (Koshikawa et al. 2000). Co-localization of MMP-2 and MMP-13 expression with MT1-MMP is consistent with the suggested role of MT1-MMP in the activation of the pro-forms of these enzymes (Knauper et al. 2002; Zhou et al. 2000). The functional significance of MT1-MMP expression in skeletal development has been established with the generation of MT1-MMP null mice (Holmbeck et al. 1999; Zhou et al. 2000). However, considering the prominent expression of MT1-MMP in the developing skeleton, as confirmed here, its limited or lack of effect on embryonic development is surprising. The MT1-MMP-null mice show progressive disruption of postnatal growth plate function and abnormalities of the skeleton and associated soft connective tissues, attributable to the loss of collagenolytic activity (Holmbeck et al. 2004). The functions of MT1-MMP in the embryo, like all MMPs studied to date, can thus be substituted by other enzymes. However, MT3-MMP is not likely to substitute for MT1-MMP activity, as indicated by the distinct expression patterns of MT1-MMP and MT3-MMP.

Throughout normal development, changes in cell matrix interactions and the reshuffling of membrane-anchored adhesion molecules, receptors, and ligands occur in concert with changes in cell phenotype or activation state. It is becoming apparent that cell-surface proteinases are capable of directing the modulation of cell-surface receptor-ligand interaction and thus of orchestrating changes in cell phenotype. The well-characterized transition from prehypertrophic to hypertrophic chondrocyte associated with endochondral ossification and possibly also the much less well understood, but no less critical, commitment of chondrocytes to an

articular phenotype are two stages of chondrocyte development in which changes in cell-surface or pericellular matrix organization may be essential to an orderly transition in phenotype. The localization of both MT1-MMP and MT3-MMP expression in hypertrophic chondrocytes suggests a role for pericellular proteinase activity in chondrocyte hypertrophy. The function of MT3-MMP in skeletal development is unknown. Its expression in articular chondrocytes in both the chicken and mouse clearly contrasts with that of MT1-MMP and suggests that MT3-MMP might play a role in the growth and/or development of articular cartilage.

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