Distinct roles of two alternative splice variants of matrilin-2 in protein oligomerization and proteolysis

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Abstract. Matrilin-2 (matn2) contains a unique domain, between the second von Willebrand factor A (vWFA) domain and the C-terminal coiled-coil domain, with no sequence homology with other family members. Complementary DNA (cDNA) sequence analysis of matn2 expression in both mice and humans revealed an alternative splice site in the region of the unique domain, which forms a short and a long splicing variant (containing an additional 19 amino acids). However, the expression heterogeneity of the alternative spliced variants, and the roles of the unique domain in oligomerization and proteolysis of matn2 are unknown. In this study, we examined the expression of the two alternative splice variants of matn2 in several skeletal and non-skeletal tissues by reverse transcription-polymerase chain reaction. Both splice variants of matn2 were detected at the mRNA level in all tissues studied. To explore the biochemical significance, several minigene constructs containing the second vWFA domain, the unique domain (with either a long or short form) and the coiled-coil domain of mouse mini matn2 were generated. Ectopic expression of these constructs demonstrated that the long form of matn2 is capable of self-assembling into several oligomeric forms, including a tetramer, trimer, pentamer or multimer; but the short form is only capable of forming a tetramer, trimer or dimer. Moreover, we observed that the splice variants of matn2 are important in modulating matn2 cleavage when co-expressed with matrilin-1 or matrilin-3. These results indicate that the two alternative splice variants have distinct roles in the processes of post-translational modification of matn2, which may have an impact on the homeostasis of the matrilin filamentous network of the extracellular matrix.

Introduction

Matrilin-2 (matn2) is the largest member of the matrilin family, with multiple domains of extracellular matrix protein, and plays a role in the formation of filamentous network structures and connects different molecules, such as collagens, other matrilins, aggrecan, small leucine-rich proteoglycans or cartilage oligomeric matrix protein (COMP), to form the extracellular macromolecular network with adaptor functions (1-3). Based on the analysis of the mouse matn2 sequence, the precursor containing 956 amino acids consists of an N-terminal putative signal peptide, two von Willebrand factor type A-like domains (vWFA) connected by 10 epidermal growth factor-like (EGF) repeats, one unique domain and a series of heptad repeats at its C-terminal coiled-coil domain; the predicted molecular mass is approximately 106.8 kDa (2-3). The vWFA domain is one of the ubiquitously distributed structural modules. There are seven vWFA domains in all four members of the matrilin family. The vWFA domain-containing molecules have been identified ubiquitously in the extracellular matrix, cell membrane and nucleus, and they participate in cell adhesion, protein-protein interaction and the formation of multiprotein complexes (4-5). The EGF domain is another ubiquitously distributed structural module consisting of 30 to 40 amino acid residues (5-7). The main structure is a two-stranded β-sheet followed by a loop to a C-terminal short two-stranded sheet. The EGF domain has six evolutionarily conserved cysteine residues, which indicates that this domain is involved in forming disulfide bonds. The EGF domain proteins include many extracellular matrix, membrane and signal pathway proteins (5-7). Multiple functions of both vWFA and EGF domains of matrilins have

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Table I. Primers used in this study.

Primers	Primer sequences (5'-3')	PCR purpose
1	TAA TAC GAC CTA TAG GG	T7, amplifying inserts from pCDNA3.1
2	CTA GAA GGC AAC AGT CGA GG	BGH, amplifying inserts from pCDNA3.1
3	CGC GGC CGC ATG GAC GGG ATT TTC TGC	Clone matn2
4	GGC TCG AGT CTG TAT TTT AGG CGA TT	Clone matn2
5	GGT CCT GGC TTT CAC AGA TGC CTT CTT	Delete unique region
6	CAT CTG TGA AAG CCA GGA CCA ATG CAA	Delete unique region
7	GAT GCA CTG AAG GCC CAA TTG AC	Amplifying 57 bp splicing, 1st PCR
8	TGA GTC ATT TCT TCT AAG CGC TGG G	Amplifying 57 bp splicing, 1st PCR
9	TGC CTC TGA GCC CAT TGA CAA G	Amplifying 57 bp splicing, 2nd PCR
10	TTT GCA TTG GTC CTG GCT TTC TT	Amplifying 57 bp splicing, 2nd PCR
11	GCA TCT TTT GTC ATC ATC GTC CTT	Add FLAG tag
12	GAT GAC AAA AAG AGA TGC ACT GAA GGC	Add FLAG tag

been extensively investigated (8-11). The coiled-coil domain at the C-terminus is a nucleation site for the potential homo- and hetero-oligomerization.

Matn2 contains a unique domain without any sequence homology to other known molecules (1-3). The unique domain is located between the second vWFA domain and the coiled-coil domain (1-3). In mouse matn2, the domain consists of 75 amino acid residues (1-2,11). Sequence analysis of expressed complementary DNA (cDNA) indicated that the unique domain of either mouse or human matn2 has 19 triplets alternatively retained or spliced out (1,2,11,12). While the function of the other domains of matn2 including vWFA, EGF and coiled-coil has been extensively studied, the biological role of the unique domain is unclear. In this study, we examined the alternative splicing expression of matn2 in several tissues from newborn mice, and then explored the biochemical properties of protein oligomerization and proteolysis by ectopic expression, in which the recombinant construct expressed either the long or short alternative splice form of the unique domain of matn2.

Materials and methods

RNA extract from animal tissues, and reverse transcription polymerase chain reaction (RT-PCR). The various skeletal tissues, including skull, knee joint, rib, spinal column, and several non-skeletal tissues, such as kidney, brain, skin, ear, stomach and colon, were harvested from newborn C57BL/6 mice. Tissue weighing approximately 20 mg was homogenized, and total RNA was isolated using an RNeasy Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using Superscript II kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To ensure the sensitivity, nested PCR was performed using first the distal primers and then the proximal primers (Table I) for mouse matn2. To avoid false results, mini matn2 with and without 57-bp splicing plasmids was used as a positive control and water was used to replace total RNA as a negative control. The study was approved by the ethics committee of Guangdong Medical College, Zhanjiang, China.

Preparation of mini matrilin gene constructs. Mini matn2 cDNA fragments were cloned from the newborn C57BL/6 mouse rib library. Suitable primers (Table I) introduced a 5'-terminal *Not*I and a 3'-terminal *Xho*I restriction enzyme site into the expression vector pcDNA3.1/v5-His (Invitrogen). To ensure the secretion from cells, a signal peptide cDNA sequence of matrilin-1 (matn1) was added into the 5'-terminal of the matn2 cDNA fragment (13,14). To study the function of the unique domain, a genetically engineered deletion of the unique domain mutant of mini matn2 was linked by overlapping PCR with the described primer sets (Table I). To examine the interaction between matn2 and matn1 or matrilin-3 (matn3), the whole length of matn1 and mini matn3 constructs from previous experiments were used in this study (13,14). In addition, a genetically engineered FLAG tag was introduced into all the mini matn2 cDNA constructs, which allowed the identification of the recombinant protein. Therefore, all the matn2 constructs contained the FLAG tag and the V5 tag, but the matn1 and matn3 constructs only contained the V5 tag (Fig. 1). The sequences of the recombinants were confirmed by DNA sequencing.

Transfection of plasmid DNA. Plasmids containing mini matn2, matn1 and mini matn3 were transfected or co-transfected into COS-1 cells using Lipofectamine 2000 (Life Technologies, Invitrogen) according to manufacturer's instructions. Briefly, COS-1 cells were trypsinized and counted. Each 60 mm plate was seeded with $6x10^5$ cells overnight, and reached 70% confluence in DMEM supplied with 10% FBS (Life Technologies). On the following day, the cells were rinsed with DMEM three times, and then transfected/ co-transfected with plasmids/Lipofectamine 2000 mix as described previously (13,14). For a single transfection 4.5 μg of plasmid was used, and 3 μg of each plasmid was used when two plasmids were co-transfected. Seventy-two hours after transfection, the conditioned media was collected for western blotting analysis (13,14).

SDS-polyacrylamide gel electrophoresis and western blotting. Conditioned media was mixed with 2X SDS reduced or
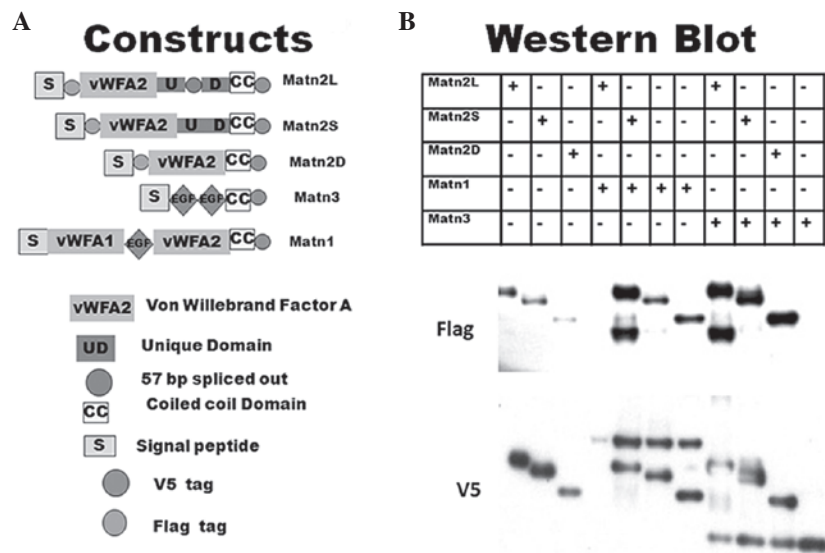
Figure 1. (A) Schematic drawings of the domain structure of recombinant matrilin variants and (B) analysis of recombinant matrilin products. The recombinant proteins of matn2 single transfection and matn2 co-transfection with matn1 or matn3 were separated on a 4-15% gel, blotted to a membrane and incubated with antiserum FLAG tag (B, upper panel, only detecting recombinant matn2 product), and incubated with antiserum V5 tag (B, lower panel, detecting all matrilin products) under reducing conditions.

non-reduced gel loading buffer. The reduced loading buffer contained 5% β-mercaptoethanol. After boiling for 10 min, samples were loaded onto 4-15% gradient gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred onto immobilon-polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The blots were blocked with 5% non-fat milk (Bio-Rad). The monoclonal antibody against V5 tag (Invitrogen, diluted 1:5000) and the polyclonal antibody against FLAG tag (Affinity BioReagents, Pierce, Rockford, IL, USA; diluted 1:1000) were used as the primary antibodies, respectively. The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (H+L) (Bio-Rad, diluted 1:5000). Visualization of immunoreactive proteins was achieved using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Amersham, UK) and then by exposing the membrane to Kodak X-Omat AR film. The molecular weights of the immunoreactive proteins were determined with two sets of protein markers (13).

Results

Tissue distribution of mouse matn2 splice variants. To examine expression of matn2 splice variants, forward and reverse primers located upstream and downstream of the splicing region in the unique domain were used. RT-PCR products of long and short splice variants were matn2L (337 bp) and matn2S (280 bp), respectively. The long and short splice variants of matn2 at the mRNA level were observed in all the mouse skeletal and non-skeletal tissues examined (Fig. 2). The skeletal tissues included knee, sterna, skull, rib and backbone, and non-skeletal tissues included kidney, skin, heart, stomach, small and large intestine. The results indicated that the long and short splicing variants are expressed in all examined tissues, and may be important in the extracellular matrix network.
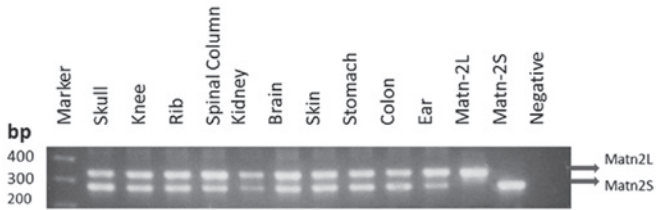

Figure 2. Differential expression of the variable unique region of matrilin-2 was assessed in newborn mouse tissues by RT-PCR. RT-PCR yielded two bands corresponding to matn2L (337 bp) and matn2S (280 bp), respectively. Both splice variants of unique domain of matrilin-2 mRNA were observed in all the mouse tissues examined.

Alternative splicing variants affect homo-oligomerization. Matn2L and matn2S were cloned into the expression vector and matn2L and matn2S constructs were generated. Matn2L contained the entire unique domain of matn2, while matn2S lacked the 57-bp sequence that encodes 19 amino acid residues in the unique domain (Fig. 1A). A FLAG tag was ligated to the N-terminus, and a V5 tag and 6 His tags were fused into the C-terminus of the minigene. The recombinant protein was identified with antibody against these tags (Fig. 1A). To express these recombinant splicing variants, the constructs with either matn2L or matn2S were transfected into COS-1 cells. The secreted protein products in the conditioned medium were examined by a western blotting analysis of the FLAG tag (Fig. 1B, upper panel) and V5 Tag (Fig. 1B, lower panel), respectively.

The long form of matrilin-2 (matn2L), under non-reduced conditions, showed 4 bands: a dominant band at 156 kDa, consisting of the matn2L tetramer (see Table II); a weak band at 117 kDa (trimer); a faint band at 196 kDa (pentamer); and a multimer (Fig. 3A, lane 1; and 3B, upper panel, lane 1). In the reduced gel, only one band at approximately 39 kDa (monomer) was detected (Fig. 3B, lower panel, lane 1). Under

Table II. Calculated molecular weight of matn1, -2 and -3 homo- and hetero-oligomers.

	Monomer kDa	Dimer kDa	Trimer kDa	Tetramer kDa
Matn2L	39	78	117	156 (M2)4
				188 (M2)2(M1)2
				204 (M2)1(M1)3
				137 (M2)3(M3)1
Matn2S	37	74	111	148 (M2)4
				184 (M2)2(M1)2
				202 (M2)1(M1)3
				131 (M2)3(M3)1
Matn2D	32	64	96	124 (M2)4
				172 (M2)2(M1)2
				199 (M2)1(M1)3
				102 (M2)3(M3)1
Matn1	55			
Matn3	20			

Matn2L, matrilin-2 long; Matn2S, matrilin-2 short; Matn2D, matrilin-2 deletion; Matn1, matrilin-1; Matn3, matrilin-3.

non-reduced conditions, the short form (matn2S) exhibited 3 bands: a predominant band at 148 kDa (tetramer); a band at 111 kDa (trimer), a weak band with 74 kDa (dimer) (Fig. 3A, lane 3, and 3B, lane 2). Only a 37-kDa (monomer) band was observed under the reduced conditions (Fig. 3B, lower panel,

lane 2). To further determine the role of the unique domain of matn2 in the oligomerization, a deletion mutant construct (matn2D) was generated and expressed the mutated protein in the COS-1 cells. Only a band of approximately 124 kDa (tetramer) was observed (Fig. 3A, lane 5, and 3B, upper panel, lane 3) on the blot under non-reduced conditions. As expected, there was a 32-kD band as a monomer of the mutant matn2D on the reduced blot (Fig. 3B, lower panel, lane 3). The results demonstrated that the long form (matn2L) produced different oligomeric forms from the short form (matn2S) and suggested that the alternative splicing mechanism in matn2 expression plays a role in modulating stochiometry of the matn2 oligo-merization. These different oligomeric forms, matn2L and matn2S, may play different roles in the filamentous network.

Splice variants of matn2 do not affect hetero-oligomerization with matn1 and matn3. To examine the heterogenic oligomer-ization of matn2 with matn1 and 3, each of the mini matn2 constructs (matn2L and matn2S) was co-transfected with matn1 and mini matn3. When matn2L was co-transfected with matn1 (Fig. 3A, upper panel) and blotted with antiserum against FLAG tag, 2 additional bands (approximately 188 and 204 kDa) were found higher than the homotetramer (Fig. 3A, lane 2), compared with the single construct transfection (Fig. 3A, lane 1). According to the molecular weight calculations (Table II), the major 188-kDa product was a form of (Matn1)2(Matn2L)2 hetero-oligomer, and the weak band (approximately 204 kDa) was a form of (Matn1)3(Matn2L)1 hetero-oligomer (Fig. 3A, upper panel, lane 2). When matn2S was co-transfected with matn1 (Fig. 3A), there were also 2 additional bands observed, whose sizes (approximately 184 and 202 kDa) (Fig. 3A, lane 4) were larger than the homotetramer products shown in the
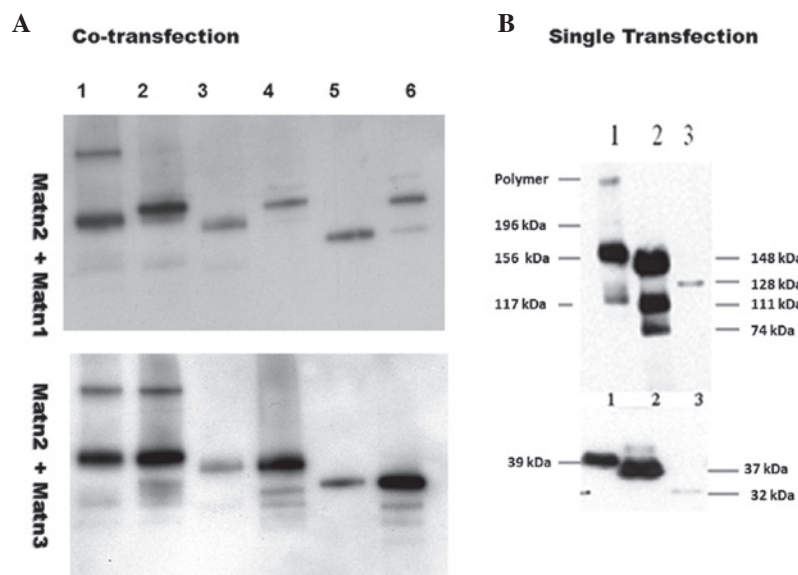

Figure 3. Co-transfection of matn2 with matn1 (Fig. 3A, upper panel) and matn3 (Fig. 3A, lower panel) and single transfection matn2 (Fig. 3B). Co-transfection matn2 with matn1 (Fig. 3A, upper panel), side by side comparison of matn2 single transfection (lane 1, 3 and 5) with matn2 co-transfection with matn1 (lane 2, 4 and 6). Two heterotetramers of (Matn1)2(Matn2)2 and (Matn1)3(Matn2)1 were found. Co-transfection of matn2 with matn3 (Fig. 3A, lower panel), side by side comparison of matn2 single transfection (lane 1, 3 and 5) with matn2 co-transfection with mini matn3 (lane 2, 4 and 6). One heterotetramer of (Matn2L)3(Matn3)1 was found. In the single transfection under non-reduced conditions (Fig. 3B, upper panel), lane 1 (matn2L) contained 4 tetramer products at 156 kDa, a trimer at 117 kDa, a pentamer at 196 kDa and a higher order multimer; lane 2 (matn2S) contained 3 tetramer products at 148 kDa, a trimer at 111 kDa and a dimer at 74 kDa; lane3 (matn2D) contained only a tetramer at 124 kDa. Upon reduction, all of these bands were reduced to 39 kDa (matn2L), 37 kDa (matn2S) and 32 kDa of the monomer (lower panel).
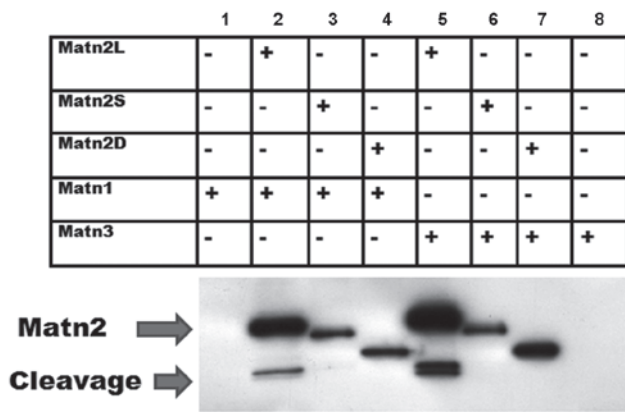
Figure 4. Analysis of matn2 cleavage products. Using SDS-PAGE analysis, matn2 co-transfections with matn1 or matn3 were separated on a 4-15% gel, blotted to a membrane and incubated with antiserum for FLAG tag. There were no cleavage products in the single transfection with matn1 (lane 1) and matn3 (lane 8). After matn2L co-transfection with matn1, there was a strong band at approximately 26 kDa, and a faint 24-kDa product (lane 2). For matn2L co-transfection with matn3, those two products were almost equal (lane 5). In matn2S, there was a very faint 24-kDa product in the co-transfection with matn1 (lane 3), but it was difficult to detect this faint band when matn2S was co-transfected with matn3 (lane 6). However, when the unique region was deleted, there were no cleavage products in matn2D (lanes 4 and 7).

single construct transfection (Fig. 3A, lane 3). The 184-kDa form, (Matn1)2(Matn2S)2, was a prominent product, and the 202-kDa form, (Matn1)3(Matn2S)1, was a less prominent product of the hetero-oligomer. Furthermore, when the matn2D mutant was co-transfected with matn1 (Fig. 3A), there were two bands higher than the homotetramer, and the other band was lower than the homotetramer (Fig. 3A, lane 6) when compared with single transfection (Fig. 3A, lane 5). The dominant product (approximately 172 kDa) was a hetero-oligomeric form of (Matn1)2(Matn2D)2, the band with 199 kDa was a (Matn1)3(Matn2D)1 hetero-oligomer and the 96 kDa product was a trimeric form of matn2D (Fig. 3A, lane 6).

When matn2L was co-transfected with matn3 (Fig. 3A, lower panel), under non-reducing conditions and detected with FLAG tag, there was a weak band (approximately 137 kDa) between the homotetramer and homotrimer (Fig. 3A lower panel, lane 2), compared with single transfection (Fig. 3A lower panel, lane 1). The product was believed to be a heterotetrameric form of (Matn2L)3(Matn3)1 (Fig. 3A, lower panel, lane 2). When matn2S was co-transfected with matn3 (Fig. 3A, lower panel, lane 4), there was also an additional band (131 kDa) between the homotetramer and homotrimer, which was believed to be a (Matn2S)3(Matn3)1 hetero-oligomer (Fig. 3A, lower panel, lane 4). Matn2D was co-transfected with matn3 (Fig. 3A, lower panel, lane 6), and 3 additional bands were observed that were smaller than the homotetramer (Fig. 3A, lower panel, lane 6). They were thought to represent a 105-kDa hetero-oligomer (Matn2D)3(Matn3)1, a 96 kDa homotrimer and a 64-kDa homodimer. These results indicated that the unique domain of matn2 may not be important to assemble hetero-oligomers with matn1 and matn3 *in vitro*.

Alternative splicing variants have an impact on proteolysis. Using SDS-PAGE analysis of the recombinant FLAG tag,

there were two cleavage products when mini matn2 was co-transfected with matn1 or mini matn3, but no cleavage product was observed in matn2 single transfection (Fig. 1B, upper panel). Those products were not recognized by V5 tag (Fig. 1B, lower panel). The molecular sizes of the products were approximately 26 and 24 kDa, respectively. When matn2L was co-transfected with matn1, there was a dominant cleavage product (approximately 26 kDa), and a very faint 24-kDa cleavage product (Fig. 4, lane 2). When matn2L was co-transfected with matn3, the density of two cleavage products was found to be almost equal (Fig. 4, lane 5). Furthermore, only a very faint 24-kDa cleavage product was observed when matn2S was co-transfected with matn1 (Fig. 4, lane 3), but it was difficult to find this faint band when matn2S was co-transfected with matn3 (Fig. 4, lane 6). However, the unique region of the matn2 was deleted completely; no cleavage product was observed (Fig. 4, lanes 4 and 7). These results demonstrated that the unique domain of matn2 is critical for proteolysis and two alternative splicing variants of matn2 have different abilities in the processes of post-translational modification.

Discussion

Matn2 is a novel family member of the filament-forming oligomeric adapter proteins that are involved in the development and homeostasis of the extracellular matrix network (1-3). At present, the information on the biological function of matn2 is limited. Matn2 is one of the major components of basement membranes and a putative adaptor molecule of the extracellular matrix, which can form both collagen-dependent and collagen-independent filamentous networks (1-3,14,15), and has been involved in the reorganization of tissue architecture in liver cirrhosis and hepatocellular carcinoma (16), the balance of keratinocyte and fibroblasts in response to wounding (17), and participated in peripheral nerve regeneration (18), and is downregulated in the early phase of muscle injury and then increased in its late phase (19). Matn2 expression was elevated in the majority of cases of human sporadic pilocytic astrocyoma (20), liver cirrhosis and hepatocellular carcinoma (16). Seven putative Smad-binding sites exist within the human matn2 promoter as well as exon I (17). Although the functions of this filamentous network adaptor of the matn2 molecule are accumulating, gene-deficient mice show no gross abnormalities during embryonic or adult development, with a normal lifespan (15). Future studies with separated isoform gene-deficient mice may provide further information on the distinct roles of splicing variants of this molecule.

Matn2 is an inherent component of all types of dense and loose connective tissue, bone, cartilage, some epithelia and in a variety of organs (1-3,15). In this study, we found that both long and short splice variants of the unique domain of matn2 at the mRNA level were expressed in all the mouse tissues examined, including skeletal tissues such as knee, sterna, skull, rib and backbone, and non-skeletal tissues such as kidney, skin, heart, stomach, small and large intestine.

Matn2 is a multiple domain oligomeric molecule that is capable of self-assembly and co-assembly with a variety of other members of the family (1-3). The heptad repeat coiled-coil domain at the C-terminus is believed to respon-

sible for the oligomerization of the molecule (6-7). Certain data showed that the second vWFA domain also plays a role in regulating matrilin oligomeric formation (13,14,21). In the present study, we demonstrated that the splicing of the unique domain of matn2 played an essential role in homo-oligomerization, but not hetero-oligomerization with other members. The short form of the splicing variant lacks 19 residues (EPEPVTIKIKDLLSCSNFA) in the unique region, which includes one cysteine. This cysteine may play a role in forming the covalent disulfide linkage responsible for pentamer and multimer homo-oligomerization. However, this cysteine appears to not be involved in the covalent disulfide bond formation between matn2 and others (matn1, matn3), as both long and short variants have the same forms of hetero-oligimers. To further confirm the role of this cysteine in the oligomerization of matn2, it is necessary to mutate the cysteine and examine homo-oligomerization of the long and short splicing variants in future studies. In addition, the coiled-coil domain of matn2 may be the only domain involved in the hetero-oligomerization.

Proteolysis is a major post-translational modification used to modify the function of proteins (22-24). Proteolysis is very complex. This complexity is three-fold. First, in the physiological condition, there are a mixture of different homo-oligomers and hetero-oligomers (8,11,13,16,17). These oligomers are participants of the extracellular matrix network (1-3). Second, the oligomeric proteins have a bouquet-like structure and bind to a variety of different molecules (11,22). Therefore, the avidity of their interactions is dependent on the number of subunits and domains present (22-24). The balance between the synthesis and degradation is important to homeostasis of the extracellular network (25). The adaptor functions of matrilins may be modulated by both physiological and pathophysiological proteolysis that causes the loss of single subunits and thereby decreases the binding avidity (22-24). Third, the cleavage occasionally releases protein fragments that have completely new functions (1-3). In this study, the splicing variants of the matn2 unique domain were shown to have a distinct role in protein cleavage, particularly when they are co-expressed with matn1 or matn3. Different activity was observed in the two splicing variants in *in vitro* experiments, indicating that the two splicing variants may have different biological functions. The splicing region in the unique domain may be important for matn2 to form a variety of oligomeric forms and to provide a functional diversity in the extracellular matrix proteins. In the future, investigating the proteolytic processing and the functions of cleavage products may provide some important information of the balance on matn2 filamentous network in the extracellular matrix.

In conclusion, the expression, splicing, assembly and proteolysis of extracellular matrix molecules are often determined by complex regulatory processes including transcriptional and translational events, as well as post-translational modifications. However, there are only a few studies stating that matn2 is involved in reorganization of tissue architecture of the extracellular matrix. In this study, we demonstrated that the splicing of the unique domain of matn2 was observed in all mouse tissue examined, and played an essential role in regulating oligomerization and proteolysis.

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