

# Expression analysis of the entire MMP and TIMP gene families during mouse tissue development

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**Abstract** Matrix metalloproteinases (MMPs) and adamalysins (ADAMs) cleave many extracellular proteins, including matrix, growth factors, and receptors. We profiled the RNA levels of every MMP, several ADAMs, and inhibitors of metalloproteinases (TIMPs and RECK) in numerous mouse tissues during development and in the uterus during pregnancy. Observations include: most secreted MMPs are expressed at low to undetectable levels in tissues, whereas membrane-bound MMPs, ADAMs and inhibitors are abundant; almost every proteinase and inhibitor is present in the uterus or placenta at some time during gestation; the mouse collagenases mColA and mColB are found exclusively in the uterus and testis; and each tissue has its unique signature of proteinase and inhibitor expression. © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Key words:** Matrix metalloproteinase; Adamalysin; Tissue inhibitor of metalloproteinase; Mouse tissue development; Real-time polymerase chain reaction

## 1. Introduction

Proteases are enzymes that can hydrolyze peptide bonds of proteins, and there are over 550 members in each of the mouse and human genomes [1]. These enzymes are classified according to the composition of their catalytic site as aspartic, cysteine, metallo, serine, or threonine proteases, and are further divided into clans and families based on structural similarity [2]. The family of matrix metalloproteinases (MMPs) consists of 24 human or 23 mouse zinc-dependent endopeptidases that collectively can degrade all proteinaceous components of the extracellular matrix, thereby facilitating tissue remodeling and cell migration. These enzymes can also cleave and activate growth factors and cytokines, shed their receptors from the cell surface, and disrupt the interaction of growth factors and receptors with associated proteins [3,4]. Through these varied actions, MMPs have a profound effect

on the pericellular environment, promoting or disrupting tissue integrity.

Sub-classification of the MMPs was originally based on their matrix substrate, but now MMPs have a number designation (MMP-1–28), based on the order in which they were identified, and are classified according to protein structure [5]. MMP-7 and human MMP-26 have the minimal domain structure that is shared by all MMPs: an amino-terminal signal sequence (pre), a propeptide domain, and a catalytic domain containing the zinc binding site. The remaining MMPs contain at least one of a hemopexin-like domain, a fibronectin-like domain, a recognition motif for furin-like serine proteases, a vitronectin-like insert, a transmembrane domain, or a glycosylphosphatidylinositol (GPI) anchor.

Another family of metalloproteinases (MPs) is the 32 integrins and metalloproteinases, or adamalysins (ADAMs), proteins containing extracellular disintegrin, metalloproteinase, and cysteine-rich, EGF-like domains, followed (in most) by transmembrane and cytoplasmic regions. Many ADAMs are found only in the testes, where they potentially play roles in spermatogenesis and subsequently in fertilization, and only about half the family have the HEXXHXXGXXH catalytic core required for MP activity. Only a minority of ADAMs have both a broad tissue distribution and the capacity to be proteolytically active [6].

Activity of both the MMPs and ADAMs is inhibited principally by the four tissue inhibitors of metalloproteinase (TIMPs). Inhibition results from the TIMP protein binding to the active site of the MMPs in a 1:1 stoichiometric ratio. For the most part, each of the TIMPs can non-selectively bind to all MMPs, with one exception being the inability of TIMP-1 to inhibit effectively several matrix-bound MMPs (MMP-14, -15, -16, -24) [7]. In addition, the membrane-anchored glycoprotein RECK can inhibit MMP-9 [8], MMP-2 and MMP-14 [9], and mice with a RECK null mutation die in utero due to defects in collagen formation and vascular development [9]. The balance or imbalance of MPs with inhibitor levels is therefore a potential predictor of extracellular matrix (ECM) production and/or degradation.

MMPs, ADAMs and TIMPs are implicated as regulators of many physiological and pathological processes, including, but by no means limited to, tissue development, placenta formation, ovulation, wound healing, cancer progression, and neural degeneration [2,3,5,10]. Historically, only a small number of MMPs have been studied in each of these processes, in part because of the relative ease of measurement for these

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**Abbreviations:** MP, metalloproteinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ADAM, a disintegrin and metalloproteinase, or adamalysin; pc, post coitum; qPCR, quantitative real-time polymerase chain reaction; C<sub>T</sub>, cycle threshold; GPI, glycosylphosphatidylinositol

MMPs but also because many MPs have only recently been identified.

We used hybridization-probe-based quantitative real-time polymerase chain reaction (qPCR) to determine mRNA levels of a large number of MPs and inhibitors. With this technique, during each PCR cycle a gene-specific fluorogenic probe hybridizes to its target cDNA and is digested by endonuclease activity of the polymerase generating a fluorescent signal; the amount of fluorescence is proportional to the amount of cDNA amplified. The cycle number (termed cycle threshold, or  $C_T$ ) at which amplification entered the exponential phase was determined and this number used as an indicator of the amount of mRNA in each tissue, i.e. a lower  $C_T$  indicates a higher quantity of starting RNA. This study represents the first comprehensive and simultaneous analysis of every mouse MMP, TIMP, several proteolytic ADAMs and RECK, in a variety of mouse tissues throughout development.

## 2. Materials and methods

### 2.1. Tissue collection and RNA isolation

Tissues were collected from CD-1 mice (Charles River, Lachine, QC, Canada), with the exception of the lung tissue, which was collected from C57/Black6 mice (Charles River). Female mice, 7–8 weeks of age, were paired with males to generate pregnant animals for collection of staged embryonic and postnatal tissues with noon of the

day of the vaginal plug being designated day 0.5 post coitum (pc). Female animals were killed at various days pc to recover embryos, uteri and placentas, while newborn mice were killed at various days post partum. A range of tissues from multiple embryos and newborn mice were dissected and pooled, including brain, heart, lung, skeletal muscle, kidney and liver. In addition, testis, spleen, thymus and adrenal gland were collected from newborn mice. Tissues were frozen on dry ice and stored at  $-70^{\circ}\text{C}$ . All animals were cared for and killed in accordance with guidelines of the Canadian Council for Animal Care.

### 2.2. RNA isolation, reverse transcription and qPCR

Total RNA was extracted using the single-step guanidinium isothiocyanate method, as described previously [11], followed by incubation with 20 U of DNase I, RNase-free (Roche, Lewes, UK) for 10 min at  $37^{\circ}\text{C}$  and heat inactivation at  $70^{\circ}\text{C}$  for 15 min. One microgram of total RNA was reverse transcribed using 2  $\mu\text{g}$  random hexamers (Amersham Biosciences, Amersham, UK) and 200 U of Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK), according to the supplier's instructions. qPCR reactions were done on the ABI Prism 7700 (Applied Biosystems, Warrington, UK) according to previously described methods [11], with each reaction containing 5 ng of reverse-transcribed RNA in 25  $\mu\text{l}$ .

Sequences for most primers and probes are described elsewhere [12], but we also designed and used primers and probes for MMP-27, ADAM-10, -12, -15, -17, -19, -28, -33, RECK, and a different set of mColA, mColB, and MMP-10 primers and probes (Table 1); these were designed using Primer Express 1.0 software (Applied Biosystems). 18S rRNA (primers and probes from Applied Biosystems) was used as an endogenous control to account for differences in the extraction and reverse transcription of total RNA.

Table 1  
Primer and probe sequences of genes analyzed by qPCR

Gene	Primer/probe	Sequence
<i>mColA</i>	Forward	CGTGGACCAACAGCAGTGAA
	Reverse	GAGTGAGCCCAAGGGAGTGA
	Probe	TCAACTTGTTCTATGTTACGGCTCATGAACTGG
<i>mColB</i>	Forward	TGGACCGACAACAATGAGGAT
	Reverse	TGGGAGAGTCCAAGGGAGTG
	Probe	as mColA
<i>MMP-10</i>	Forward	CCTGCTTTGTCCTTTGATTTCAGT
	Reverse	CGGGATTCCAATGGGATCT
	Probe	TCCTATTCTTTAAAGACAGGTACTTCTGGCGCA
<i>MMP-27</i>	Forward	AGGATAATAAAGTGCTTCCAGGA
	Reverse	AAGAAATAGAGGAATCCATTATGTTGG
	Probe	TCGCCTCCGTGTGGATGCTGTC
<i>ADAM10</i>	Forward	GTGCCAGTACAGGCTCTTTGTC
	Reverse	CACAGTAGCCTCTGAGTCATTACATG
	Probe	ACTATCACTCTGCAGCCGGGCTCTCC
<i>ADAM12</i>	Forward	ATCAGTGTCTTCGGCGTTCA
	Reverse	GGCAATTCTTCTGTGTTACATACC
	Probe	CCATGCAGTGCCACGGCCG
<i>ADAM15</i>	Forward	GACCACTCCACAAGCATCTTAGG
	Reverse	GGGAGAATCATGGTCCAAACC
	Probe	TGCCTCCTCGATTGCCCATGAATTG
<i>ADAM17</i>	Forward	AAGTGCAAGGCTGGGAAATG
	Reverse	CACACGGGCCAGAAAGGTT
	Probe	CCTGCGCATGCATTGACACTGACAAC
<i>ADAM19</i>	Forward	CGGGCCACCTCGAA
	Reverse	CCGTTTCATTCTGCGAGGTT
	Probe	TGGGCCCTTCAGTTTACACATCAGACCA
<i>ADAM28</i>	Forward	TACTGCTTGAAGGGCAAATGTC
	Reverse	TGTCCACCTTCATTCTGCTT
	Probe	TCCAGGAACCAAGGTGCAATACATCATGTTAC
<i>ADAM33</i>	Forward	CAGGCACTGTCTCAGAAATGCTACCT
	Reverse	CTATTGCAAACCCACCGTTA
	Probe	TGGAACGTTGCTTGACTGCCTGCC
<i>RECK</i>	Forward	GGCCTCACCGTGCACTTG
	Reverse	TGGAGGAACGCTTTGCAGTT
	Probe	CTGGACTAGACCCTGACCACCCACAACCTG

Sequences for the genes not included in this table can be found in [12].

Sequences are shown in a 5' to 3' orientation; probes contain a carboxyfluorescein fluorescent reporter on the 5' end and a carboxytetramethylrhodamine quencher on the 3' end.

### 2.3. Analysis

The  $C_T$  at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue, i.e. a lower  $C_T$  indicated a higher quantity of starting RNA.

### 3. Results and discussion

We utilized TaqMan®-based qPCR to profile the RNA levels of every mouse MMP and TIMP, and several proteolytic ADAMs, in various mouse tissues during development. To aid in the visual presentation of these data, results are shown as the  $C_T$  value, which provides an accurate approximation of the level of expression of each gene. In previous reports we

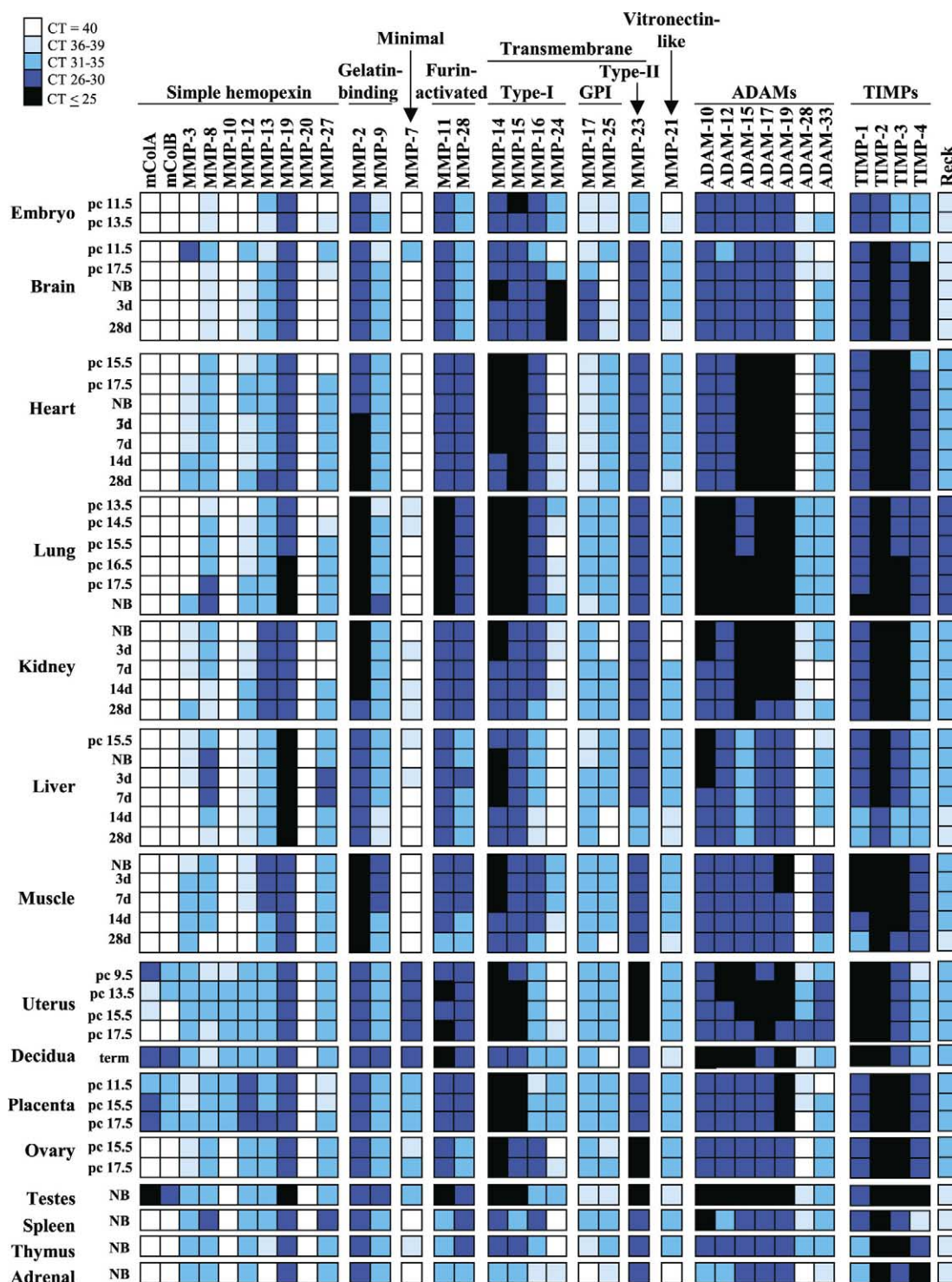


Fig. 1. The relative mRNA levels for all mouse MMPs and TIMPs and several ADAMs in mouse tissues throughout development. Classification of expression levels was determined from the  $C_T$  of each gene as either very high ( $C_T \leq 25$ ), high ( $C_T = 26-30$ ), moderate ( $C_T = 31-35$ ), low ( $C_T = 36-39$ ), or not detected ( $C_T = 40$ ); see legend for color scheme.



determined absolute transcript numbers for each gene, which required synthesizing and quantifying RNA transcripts and then making serial dilutions of its cDNA covering the range of  $10^8$  to  $10^1$  transcripts [11,13]. However, we observed that for different genes, when equal numbers of transcripts were analyzed, the differences in  $C_T$  value were never greater than five. We therefore consider the  $C_T$  value a reliable overall indicator of relative RNA abundance.

Fig. 1 shows the results with MMPs organized by structural similarity as opposed to numerical order. This highlights the low abundance in non-reproductive tissues of MMP-7, the simplest MMP in terms of domain structure, and all of the simple hemopexin MMPs except MMP-19. However, many of these genes, while expressed at relatively low levels in most normal tissues, are elevated in the uterus and testes, and in various pathologies [2,3,5,10]. There is complete absence of expression for MMP-20 (enamelysin) in all tissues, although this gene has only ever been detected in tooth pulp tissue and odontoblasts [14,15]. Conversely there is essentially ubiquitous and abundant expression of the gelatinases, the type I MT-MMPs, most ADAMs, and the TIMPs. Substrates for the MT-MMPs and ADAMs include other proteases, or growth factors and associated proteins, and the presence of these enzymes does not imply ECM degradation, but instead supports the suggestion by Pagenstecher et al. [16] that cells and tissue are in an 'alert state' awaiting the availability of a substrate.

This study represents the first extensive analysis of every member of the MMP and TIMP family concurrently during tissue development, although there have been numerous studies that have looked at one, or a few genes together, with the majority of these having results in agreement with ours. When mColA and mColB, the mouse orthologues of human MMP-1, were identified, their expression was found only in the placenta and absent in the embryo, kidney, ovary, and lung [17]. We further show that transcripts for these genes are also absent in the brain, heart, muscle, and liver, but *are* present at abundant levels in the testes, a tissue not previously examined. The mColA and mColB genes are present in mouse and rat and presumably reflect a recent duplication event during mammalian evolution [1]. It is interesting therefore that the expression of these two genes seems to be restricted exclusively to reproductive tissues, whereas the distant human relative plays a more fundamental role in tissue remodeling processes.

Pagenstecher et al. [16] used RNase protection assays to simultaneously analyze nine MMPs and three TIMPs in adult mouse brain, kidney, liver, and spleen. Like us, they detected MT1-MMP (MMP-14), stromelysin-3 (MMP-11), and macrophage metalloelastase (MMP-12) mRNAs in all tissues, with those for stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), matrilysin (MMP-7) and collagenase (MMP-13) absent in all. Here, we further show that MMP-3, -7, and -10 transcripts are absent in most tissues, but are present in reproductive tissues, in agreement with others [18,19]. However, in contradiction to the Pagenstecher report, we detected MMP-13 RNAs in most tissues, with the highest levels in the kidney.

It has previously been shown that gelatinase A (MMP-2) is most abundantly expressed in kidney and spleen [16], while another study showed it as highest in the lung, muscle and kidney [20], in complete agreement with our data. We additionally show that MMP-2 mRNA is very abundant in the

heart, a tissue that was not included in the other studies. Gelatinase B (MMP-9), on the other hand, is lowly expressed, but its RNA is also present in all tissues.

Many of the transmembrane MMPs are highly expressed in most tissues. When mouse MT1-MMP (MMP-14) was first characterized, its RNA was most abundant in the placenta, kidney, heart, and lung [21], as we show, while MT5-MMP (MMP-24) was originally characterized as being predominant in the brain and testes [22], and our data demonstrate that it is weakly or not expressed in other tissues. There have not yet been broad tissue profiles for the remaining MT-MMPs until this study. MT2- and MT3-MMP (MMP-15 and -16) transcripts, like MMP-14, are abundant and ubiquitous, while transcripts of the two GPI-anchored MT-MMPs, MT4- and MT6-MMP (MMP-17 and -25) are in all tissues, although at lower levels. MMP-23, a type II transmembrane protein that is soluble in its active form, is expressed in all tissues but at the highest levels in the uterus, ovary, and kidney, in agreement with others [23].

ADAMs are transmembrane MPs that are distinct from the MMPs in that they also have an extracellular disintegrin domain and a cytoplasmic domain that can associate with intracellular proteins. Five of the seven ADAMs that we profiled are abundantly expressed in most tissues, and four of these, ADAM12, ADAM19 [24], ADAM15 [25], and ADAM17 [26], have been shown by others to have identical tissue distribution as ours. Our data also support the observations that ADAM28 is expressed in only a few tissues, notably the epididymis and the lung [27], and that ADAM33 is widely expressed [28].

The MP inhibitors were found in all tissues examined. Of the four TIMPs, TIMP-2 is the most constitutively expressed at high levels, while the other three have a more specific pattern of high expression. In fact, the expression patterns we describe for all TIMPs are identical to previously published patterns [29,30]. The constitutive and very abundant expression of at least one TIMP in every tissue may provide a crucial checkpoint for tissue degradation. When the RECK gene was first identified, it was observed to be expressed in a wide variety of human tissues [8], and we show that its transcripts are also present in a wide variety of mouse tissues, though at most sites RECK is expressed at a lower level than any of the four TIMPs. Interestingly, mice with a RECK null mutation die in utero due to poor collagen formation and vascular defects [9], whereas mice lacking TIMP-1, -2, or -3 are viable. Our results do not suggest a pattern of RECK expression distinct from the TIMPs to explain this embryonic lethality.

This study is also the first to describe the tissue distribution of MMP-27 in any species. The primers and probe for this gene were based on the predicted sequence for the *Rattus norvegicus* MMP-27 sequence (GenBank accession number XM235795) and have not been examined in the mouse. Using this primer set we detected an amplicon that has a sequence identical to a portion of the rat sequence. This transcript was detected in most tissues, with the exception of the brain, with highest expression in the liver. There have been no functional studies done for this putative protein.

A notable finding from this study is that the transcripts for most MMPs, all ADAMs, and all TIMPs are present at some time in the uterus, placenta, or decidua. This reflects the extensive tissue generation and remodeling that occurs during

placenta formation. Between days 5 and 10 pc fibrillar collagen and fibronectin are replaced by type IV collagen and laminin, which is later degraded by the invading trophoblast cells [31], while at parturition there is a massive breakdown in the uterine ECM [32]. mColA and mColB transcripts are present initially in the uterus (days 9.5–13.5 pc), with their expression later changing to the placenta, while mRNA levels for the other major collagenase, MMP-13, increases in the placenta towards parturition. Another interesting observation is the low expression of ADAM28 in the early uterus, with its levels substantially elevated at day 17.5 pc, implicating this enzyme as a putative mediator of parturition.

Each tissue exhibits unique patterns of MP and TIMP expression. In the brain, there are substantial gains in the levels of MMP-24, ADAM33 and TIMP-4 transcripts from day 11.5 to postnatal day 28, and complete losses of MMP-3, MMP-7, MMP-27, and ADAM28 RNAs. As neural development progresses, there is production of both fibronectin and laminin to facilitate the migration of glial and neuronal cells [33,34]. It may be that the presence of MMP-3, -7, -27 and ADAM28 at early times limits matrix deposition, and their disappearance, along with the increase in TIMP-4 expression, facilitates matrix stability. On the other hand, the increase in MMP-24 expression may be associated with neurite extension and axonal growth [35].

In the development of the heart, the prenatal stage is characterized by myocyte proliferation while postnatally there is myocyte hypertrophy and interstitial cell proliferation [36]. Growth of the myocytes is in part regulated by the presence of collagen [37], and it is interesting to note that around the time of parturition, there is an increase in the expression of several MMPs, including MMP-2, -3, -12, -27, and -24, suggesting a breakdown of ECM components leading to termination of myocyte growth stimuli.

Development of both the lung and kidney is characterized by branching – the bronchioles in the lung, and the ureteric bud in the kidney. Both the lung and kidney have high levels of MMP-2, MMP-14, ADAM15, 17, 19, TIMP-2, and -3 RNAs throughout development, and both have an increase in MMP-12 levels over time. However, in the lung, there is a unique decrease in MMP-7 and increase in MMP-8 expression, while the kidney exhibits a decrease in MMP-8, -14, and ADAM10, but an increase in MMP-21 and -25. Regulation of MP activity is essential in branching morphogenesis as mice with a null mutation in TIMP-3 show reduced branching of bronchioles [38].

The liver and spleen are early sites of hematopoiesis in the fetus, and both of these tissues have high mRNA levels of MMP-14, -19, ADAM10, and TIMP-2. It should be noted that in the liver there is a transient phase of high MMP expression from newborn to 7 days, with peaks in MMP-8, -14, -27 and -28 RNA levels. These enzymes potentially mediate the changes from a fetal ECM to an adult ECM and promote liver development, since the fetal ECM inhibits growth of hepatocytes [39].

Skeletal muscle development is principally characterized by differentiation and fusion of myoblast cells and three major mediators of this process are meltrins  $\alpha$ ,  $\beta$ , and  $\gamma$  (ADAM12, ADAM19, and ADAM9) [40]. In this study, RNA levels of these ADAMs are lower in the muscle than in other tissues, although this may be because the samples were from newborn onward, after the time period of myogenesis. There is, how-

ever, elevated ADAM19 in the newborn and 3-day-old mice, possibly representing the last stages of myogenesis.

In conclusion, this report demonstrates high RNA levels of many MT-MMPs, ADAMs and TIMPs, but low levels of secreted MMPs in most differentiated tissues. However, at early times in development numerous MPs are elevated, with the exact proteases depending on the tissue, supporting the hypothesis that MPs are high during active tissue generation, but low in stable tissue unless there is pathology. The high levels of membrane-associated MPs may act to maintain cells in an active state, awaiting substrates such as other proteases or growth factors, while high expression of the TIMPs may maintain tissue integrity. In addition, almost every proteinase and inhibitor is present in the uterus or placenta during gestation; mColA and mColB are found exclusively in the uterus and testis; and each tissue has its unique signature of proteinase and inhibitor expression.

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