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Matrix Metalloproteinase-9 Dependent Mechanisms of Reduced Contractility and Increased Stiffness in the Aging Heart

Rugmani Padmanabhan Iyer^{1,2}, Ying Ann Chiao³, Elizabeth R. Flynn^{1,2}, Kevin Hakala^{1,4}, Courtney A. Cates^{1,2}, Susan T. Weintraub^{1,4}, and Lisandra E. de Castro Brás^{1,5,*}

¹San Antonio Cardiovascular Proteomics Center

²Mississippi Center for Heart Research, Department of Physiology and Biophysics, Jackson, MS 39216

³Department of Pathology, University of Washington, Seattle, WA 98195

⁴Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

⁵Brody School of Medicine, Department of Physiology, East Carolina University, Greenville, NC 27834

Abstract

Purpose—Matrix metalloproteinases (MMPs) collectively degrade all extracellular matrix (ECM) proteins. MMP-9 has the strongest link to development of cardiac dysfunction. Aging associates with increased MMP-9 expression in the left ventricle (LV) and reduced cardiac function. We investigated the effect of MMP-9 deletion on the cardiac ECM in aged animals.

Experimental-Design—We used male and female middle-aged (10-16 month old) and old (20-24 month old) wild type (WT) and MMP-9 null mice (n=6/genotype/age). LVs were decellularized to remove highly abundant mitochondrial proteins that could mask identification of relative lower abundant components, analyzed by shotgun proteomics, and proteins of interest validated by immunoblot.

Results—Elastin microfibril interface-located protein (EMILIN)-1 decreased with age in WT (p<0.05), but not in MMP-9 null. EMILIN-1 promotes integrin-dependent cell adhesion and EMILIN-1 deficiency has been associated with vascular stiffening. Talin-2, a cytoskeletal protein, was elevated with age in WT (p<0.05), and MMP-9 deficiency blunted this increase. Talin-2 is highly expressed in adult cardiac myocytes, transduces mechanical force to the ECM, and is activated by increases in substrate stiffness. Our results suggest that MMP-9 deletion may reduce

* Address for correspondence: Lisandra E. de Castro Brás, Ph.D., Department of Physiology, The Brody School of Medicine, East Carolina University, 600 Moye Blvd, Greenville 27834 NC, Phone: +1 252 744 0391; Fax: +1 252 744 3460, decastrobrasl14@ecu.edu.

Author contributions

L.E.C.B. developed the initial project concept and designed the experiments, R.P.I., Y.A.C., E.F., C.A.C., and L.E.C.B. performed the experiments, K.H. and S.T.W. performed the HPLC and LC-MS experiment design and performance, quality control, and data analysis. R.P.I. and L.E.C.B. wrote the paper. All authors reviewed the paper.

Competing Financial Interests

The authors declare no competing financial interests.

age-related myocardial stiffness, which may explain improved cardiac function in MMP-9 null animals.

Conclusions—We identified age-related changes in the cardiac proteome that are MMP-9 dependent, suggesting MMP-9 as a possible therapeutic target for the aging patient.

Keywords

matrix metalloproteinase; MMP-9; aging; left ventricle; cardiac stiffness; EMILIN; Talin

Introduction

Heart failure is a major cause of morbidity and mortality in the elderly. Aging is an independent cardiovascular risk factor and is associated with cardiac dysfunction that results from several elements, including increased myocardium stiffness due to structural remodeling [1]. Left ventricular remodeling comprises changes in the volume, mass, composition, and structure of the left ventricle (LV). These changes are all important predictors of LV function and are more prominent with advancing age. An important component of cardiac age-related structural changes is remodeling of the extracellular matrix (ECM) [2]. The cardiac ECM *i)* provides a scaffold for cellular migration, proliferation, and differentiation [3], *ii)* delivers mechanical and structural stability and tissue compliance [4], *iii)* transmits mechanical forces and signals to myocardial fibers to regulate cell alignment and blood flow [4], and *iv)* is essential for proper cardiac structural integrity and pump function [5]. Additionally, the composition and arrangement of the cardiac ECM are main determinants of the specialized electrical conduction system properties [6]. Age-associated alterations in anisotropic conduction velocity result in reentrant arrhythmias and have pro-arrhythmic effects by reducing the threshold for ventricular fibrillation [7]. This phenomenon is linked to the realignment of myofibrils and contributes to increased myocardial wall thickness [8–9]. Thus, age-associated changes in the cardiac ECM profoundly affect cardiovascular function with striking impact on patient clinical outcomes.

The rate of ECM remodeling is defined by the balance of ECM synthesis and degradation. ECM synthesis is conducted by the cardiac cells; fibroblasts and smooth muscle cells synthesize the majority of the structural ECM proteins while endothelial cells and myocytes secrete basement membrane components [10]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that can degrade all structural elements of the ECM. The majority of MMPs are synthesized in their inactive pro-form; after stimulation, activated MMPs process the cardiac ECM proteins [4]. Therefore, MMPs are important regulators of matrix turnover in the heart, and contribute to physiological and pathological cardiac remodeling. Elevated levels of MMP-9, a 92 kDa gelatinase, are observed in several cardiovascular diseases, such as atherosclerosis, hypertension, and acute myocardial infarction [11]. Moreover, advancing age is linked to enhanced MMP-9 plasma and tissue levels, as well as a decline in LV function [12–14]. In a murine model of cardiac aging, MMP-9 deletion prevented age-associated cardiac dysfunction and MMP-9 levels correlated with inflammatory cytokines [12–15]. In addition, with age MMP-9 deletion reduces TGF- β signaling-induced periostin and connective tissue growth factor (CTGF) expression in the

heart, and attenuates myocardial fibrosis by increasing MMP-8 expression to regulate myocardial collagen turnover and deposition [16]. Thus, an understanding of upstream cardiac ECM regulatory factors such as MMP-9 and its substrates will help understand age-related ECM and myocyte remodeling and its clinical implications.

We and others have shown that while systolic function is relatively preserved with advancing age, diastolic function declines with age and this is observed in both experimental models and humans [12, 16-19]. Furthermore, middle-aged mice do not show diastolic dysfunction compared to old mice; however old mice present a decline in the mitral ratios of early to late diastolic filling velocities (E/A ratios) compared to their young counterparts, which is attenuated by MMP-9 deletion [12, 16]. Collectively, these data suggest that age-associated myocardial remodeling that leads to diastolic dysfunction occurs between middle and old age, and MMP-9 is an important modulator of cardiac remodeling. Accordingly, this study investigated the composition and changes of the cardiac ECM that are MMP-9 dependent in middle-aged and old mice that could link to age-related cardiac dysfunction.

Material and Methods

Animals and tissue collection

Animal procedures were performed according to the “Guide for the Care and Use of Laboratory Animals” (NIH Notice Number: NOT-OD-12-020) and were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and at the University of Mississippi Medical Center. Both male and female C57BL/6J WT and MMP-9 null (Null) mice were used in this study (n=6/genotype/age). WT and MMP-9 null colonies were bred in-house as homozygous colonies and were maintained in the same room since birth. The MMP-9 null mice were generated in Zena Werb’s laboratory and backcrossed by Lynn Matrisian’s laboratory and are on the C57BL/6J background [20, 21]. Animals were housed at a controlled temperature ($22 \pm 2^\circ\text{C}$) on a 12 h light/dark cycle, fed standard laboratory mice chow *ad libitum*, and had free access to tap water. Two age groups were analyzed: middle-aged (10 to 16 month old) and old (20 to 24 month old).

Mice were anesthetized with 5% isoflurane and the coronary vasculature flushed with 0.9 M saline, after which the hearts were excised. The whole left ventricle (LV) was isolated and either used for tissue decellularization or sliced into 3 transverse sections and the middle-section fixed in 10% zinc formalin, processed in paraffin, and sectioned at 5 μm for immunofluorescence analysis.

Tissue decellularization

The left ventricles were incubated in distilled water with 1x protease inhibitor cocktail (PI) (cOmplete Mini tablets, Roche) at room temperature for 30 min. The water was decanted and replaced with decellularization buffer (1% sodium dodecyl sulfate in phosphate buffered saline with 1x PI). Samples were left at room temperature in an orbital shaker until tissue was completely decellularized (three to four days). The decellularization buffer was replaced daily. Samples were considered decellularized when tissue was translucent. The

decellularized LV was washed three times in distilled water with 1x PI for 5 min, and then left overnight in fresh 1x PI/water to remove all remnants of the decellularization buffer. The tissues were homogenized (speed 6, 1 min 30 sec, Power Gen 1000, Fisher Scientific) in Protein Extraction Reagent 4 (15 μ L buffer per mg tissue, Sigma Aldrich), centrifuged at 14'000 RPM for 10 min at 4°C and the supernatant transferred to a new tube. The insoluble pellet was solubilized in 30 μ L dimethyl sulfoxide and added to the supernatant, all protein extracts were stored at -80°C. Total protein concentrations were quantified using Bradford reagent (Bio-Rad) according to the manufacturer specifications.

Mass spectrometry

Sixty micrograms of protein per sample were separated in a 12% bis tris gel (n=3 male and 3 female/genotype/age). The gel was run in 1x MOPS buffer until proteins were stacked and resolved in approximately 1 cm of gel. Each gel lane was excised and divided into 2 slices. Each slice was individually de-stained and dehydrated and the proteins digested *in situ* with trypsin (Promega). The digests were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS on a Thermo Fisher LTQ Orbitrap Velos mass spectrometer fitted with a New Objective Digital PicoView 550 NanoESI source. On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC micro HPLC: column, PicoFrit™ (New Objective; 75 μ m i.d.) packed to 15 cm with C18 adsorbent (Vydac; 218MSB5, 5 μ m, 300 Å); mobile phase A, 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA); mobile phase B, 90% acetonitrile/0.5% HAc/0.005% TFA; gradient 2 to 42% B in 30 min; flow rate, 0.4 μ L/min. Precursor ions were acquired in the Orbitrap in profile mode at 60,000 resolution (m/z 400); data-dependent collision-induced dissociation (CID) spectra of the six most intense ions in the precursor scan above a set threshold were acquired at the same time in the linear trap. Mascot (versions 2.3.02; Matrix Science) software was used to search the uninterpreted CID spectra against a combination of the mouse subset of the NCBI database [Mus. (145,083 sequences)] and a database of common contaminants (179 sequences). Methionine oxidation was considered as a variable modification; trypsin was specified as the proteolytic enzyme, with one missed cleavage allowed. A secondary search of the CID spectra using X! Tandem, cross correlation of the X! Tandem and Mascot results and determination of protein and peptide identity probabilities were accomplished by Scaffold (version 3; Proteome Software). The thresholds for acceptance of peptide and protein assignments in Scaffold were 95% and 99.9%, respectively, with minimum 2 unique peptides. The results for the individual slices were combined for presentation purposes. Subcellular locations were identified using Uniprot database and bioDBnet was used to identify the UniGene ID corresponding to each protein. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [22] via the PRIDE partner repository with the dataset identifier PXD002488 and 10.6019/PXD002488.

Immunoblotting

Proteins of interest were further analyzed by immunoblotting using non-decellularized samples that were sex, age, and genotype matched. Equal amount of protein (10 μ g) were loaded onto 4-12% Bis-Tris gels and run by SDS-PAGE (n=6/group). Proteins were transferred to a nitrocellulose membrane which was treated with the MemCode™ Reversible

Protein Stain Kit (Pierce, Thermo Scientific), to check for efficiency of protein transfer and for use as a loading control. De-stained membranes were blocked for 1 h at room temperature with 5% non-fat milk (Bio-Rad) and were hybridized overnight at 4°C with primary antibody. Primary antibodies used were anti-EMILIN 1 (1:200, sc-50430, Santa Cruz) and anti-Talin-2 (1:10'000, NBP1-95139, Novus Biologicals). After 1 h incubation with a secondary antibody, positive signaling was detected by chemiluminescent using an ECL substrate (GE Healthcare). HUVEC cells lysate (5 µg) was used as specificity control for Talin-2 antibody, since this cells only express Talin-1.[23] Immunoblots were densitometrically analyzed using GE Image Quant LAS4000 luminescent image analyzer (GE Healthcare). The signal intensity of each sample was normalized to the total protein in its respective lane.

Immunofluorescence

Sections were deparaffinized, dehydrated, and heat-incubated with antigen retrieval solution (Target retrieval solution, Dako) for approximately 15 min as described previously (22153350). Tissue was blocked with horse serum (Vector Laboratories, Marion, IA, USA) and incubated overnight at 4°C with primary antibody in blocking serum. A primary antibody specific for EMILIN-1 (1:100, sc-50430, Santa Cruz), and Talin-2 (1:100, NBP1-95139, Novus Biologicals) was used at 4°C overnight. The sections were incubated with respective secondary antibodies and positive staining was determined using HistoMark Black (KPL 54-75-00). Images were captured randomly from both free wall and septum of the LV by a group-blinded researcher. A minimal of 10 images were acquired per animal. Images were taken at 40X magnification with cellSens software (Olympus Life Sciences, Center Valley, PA, USA) across the whole tissue section to rule out the possibility that regional differences could contribute to different staining patterns. Quantification was performed using Image-Pro software (Media Cybernetics, Bethesda, MD, USA) as the percentage of positively stained area (green) to total area (pixel intensity in positive area/ field of view) from all images acquired. Data was presented as mean±SEM.

Statistical Analysis

Data are reported as mean±SEM. Total spectrum counts, immunofluorescence, and immunoblot intensities (arbitrary units) were analyzed by two-way ANOVA, followed by the Student Newman-Keuls when the Bartlett's variation test passed, or by the Kruskal-Wallis non-parametric test when the Bartlett's variation test did not pass, and the Dunn's multiple comparison post-test was used when differences were observed. A p<0.05 was considered significant.

Results

Decellularization of the LV enriched for ECM proteins

We identified a total of 245 proteins by mass spectrometry, of which 43 were only identified in the WT group and 42 only in the Null group (Supplemental Table 1 and Figure 1A). Of all proteins identified, 63 proteins (26%) were extracellular and secreted proteins (Supplemental Table 1 and Figure 1B), demonstrating that the decellularization process enriched the samples for ECM proteins. In addition to ECM proteins, the decellularization

process also highlighted cytoskeletal proteins (43 proteins, 17%, Figure 1B). Supplemental table 1 lists all proteins identified per group and sub-cellular location.

We used bioDBnet to identify the corresponding UniGene ID to each protein. The gene list was uploaded into the Database for Annotation, Visualization and Integrated Discovery v6.7 (DAVID) to detect functionally related groups within the list of identified proteins. The Functional Classification Tool clustered the proteins into 87 groups. The 3 protein clusters with the highest enrichment scores are listed on Table 1. The highest enrichment score was 25.1 for the cluster of contractile proteins, which included mostly cytoskeletal proteins, followed by 2 clusters of ECM proteins with 12.45 and 11.2 scores. The enrichment p-value was calculated for each term and terms were then clustered into gene functional groups. DAVID analysis gave further evidence that the decellularization process enriched samples for cytoskeletal and ECM proteins.

WT and MMP-9 null mice displayed distinct age-related and MMP-9 related proteomic changes

We used total spectrum counts and exclusive unique peptide counts to perform relative quantification and identify age and MMP-9-related changes. The proteins identified by mass spectrometry showed changes that are either age dependent or MMP-9 dependent. Sixteen ECM and ECM-related proteins showed age dependent changes and 12 proteins showed MMP-9 dependent changes, as listed in Table 2. Collagen type III levels decreased with age in the WT group and MMP-9 deletion removed this effect. EMILIN-1 showed decreased expression in WT old mice compared to middle-aged ($p < 0.05$) and this effect was eradicated by MMP-9 deficiency. Dynamin-like protein and talin-2 expression were enhanced in WT old mice compared to middle-aged (both $p < 0.05$), but not in the null mice. Fibronectin, a well-known MMP-9 substrate, presented both age and MMP-9 dependent changes. In MMP-9 null mice, fibronectin decreased with age ($p < 0.05$), an effect that was not observed in WT animals. Cytoskeleton proteins known to interact with ECM proteins during formation of focal adhesions and cell-matrix communication, such as catenin (cadherin associated protein)- $\alpha 3$ [24] and sorbin and SH3 domain-containing proteins [25] showed decreased expression in the old MMP-9 null mice compared to same genotype middle-age, indicative of an MMP-9 effect.

EMILIN-1 and talin-2 levels change with age in an MMP-9 dependent manner

To validate the MS results, we quantified EMILIN-1 and talin-2 by immunoblot using non-decellularized samples that were sex, age, and genotype-matched, and immunohistochemistry. EMILIN-1, an adhesive glycoprotein, promotes integrin-dependent cell adhesion, migration, and proliferation, and EMILIN-1 deficiency has been associated with stiffening in vessel walls and hypertension [26]. By immunoblot, the protein levels of EMILIN-1 were decreased by 2.5-fold in WT with age and this effect was eradicated by MMP-9 deletion (Figure 2A and 3A). As observed in the MS data, talin-2 showed a 2.2-fold increase in protein levels in the WT group with age and this effect was blunted by MMP-9 deficiency (Figure 2B and 3B). Talin-2 is an integrin activator that is highly expressed in adult cardiac myocytes and links ECM to the actin cytoskeleton.

Discussion

The goal of this study was to investigate MMP-9 driven ECM changes in the LV that precede age-associated cardiac dysfunction. The major findings of our study were: 1) tissue decellularization enriched samples for ECM and cytoskeletal proteins, 2) identification of MMP-9 dependent changes in ECM protein expression in aged animals by mass spectrometry, 3) advancing age is associated with an increase in contractile LV proteins, 4) absence of MMP-9 ameliorated age-related ECM changes, 5) EMILIN-1, a promoter of cellular migration and proliferation showed reduced levels in aged mice, and 6) absence of MMP-9 attenuated talin-2 levels in aged mice. Taken together, these findings reveal direct roles for MMP-9 in regulation of age-dependent cardiac ECM remodeling.

By mass spectrometry, we identified 15 LV proteins that presented age-dependent changes in the WT and MMP-9 null groups. Three proteins displayed decreased levels with age in the WT, these were collagen type III alpha 1, collagen type XV alpha 1, and EMILIN-1 (all $p < 0.05$ versus WT middle-age) and MMP-9 deletion eradicated these changes. Fetal tissues show increased expression of collagen type III alpha 1 compared to adult tissue, which has led some to hypothesize that collagen type III is a main contributor to the scarless phenotype observed in the early stages of gestation [27]. Reduced collagen type III in the aged LV is in accordance with reports of increased myocardial stiffness with age [1]. Dynamin 1-like, a mitochondrial fission/fusion protein, displayed an age dependent increase in the WT mice. This protein activates mitochondrial fragmentation leading to mitophagy and mitochondrial loss [28]. Aging is associated with a decline in viable cardiomyocytes and loss of cardiac function [29]. Overexpression of dynamin 1-like with increasing age may be an important contributor for cardiomyocyte loss. To the best of our knowledge, this study is the first to report a link between excessive mitochondria degradation and age-associated cardiomyocyte loss.

This study identified EMILIN-1 as one of the MMP-9 substrates in the decellularized LV tissue of aged mice. This result is in accordance with previous reports where EMILIN-1 was identified as an MMP-9 substrate in vascular tissue using proteomics approaches [30]. In humans, EMILIN-1 is highly expressed in aortic valves as early as the first trimester of gestation until adulthood [31], and EMILIN polymorphisms positively correlate with the occurrence of hypertension in the Mongolian population [32]. In the cardiovascular system, EMILIN-1 is secreted by myocytes, endothelial cells, vascular smooth muscle cells, and adventitial fibroblasts [31, 33]. EMILIN-1 is highly expressed in vessel walls, regulates elastogenesis, and binds to pro-transforming growth factor (TGF) β inhibiting its maturation by furin convertases [34, 35]. Active TGF β is a well-known stimulator of angiogenesis and ECM production [36], and TGF β levels positively correlate with adverse LV remodeling in hypertensive patients [37]. EMILIN-1 deficient mice have a systemic hypertensive phenotype and EMILIN-1 has been shown to regulate resting blood pressure in vascular smooth muscle cells by regulating the myogenic response in resistance arteries through TGF β [38]. Since cardiac MMP-9 levels increase with age [12], our data suggest that MMP-9 activity may be responsible for the age dependent decrease observed in EMILIN-1 levels. Furthermore, a reduction on EMILIN-1 expression will lead to a direct upsurge in

TGF β signaling, which in turn may explain the aged-associated pro-inflammatory arterial profile described by Lakatta's group [39-40].

Talin-2, a cytoskeletal protein, is expressed in cardiomyocytes, links the cytoplasmic domains of integrins to the actin cytoskeleton, transduces mechanical force to the ECM, and is activated by increased substrate stiffness [41-43]. Since EMILIN-1 deficiency leads to increased vessel wall stiffening [26], the observed age-dependent decline in EMILIN-1 levels may be a stimulus for talin-2 activation. We observed a 2.2-fold increase in talin-2 protein levels in the WT group with age and this effect was blunted by MMP-9 deficiency. To date, few clinical studies have characterized the roles of talin-2. Post-encephalitic epilepsy patients showed elevated levels of talin-2 in cerebrospinal fluid and serum, suggesting the involvement of talin-2 in development of epilepsy [44]. To the best of the authors' knowledge, only one clinical study reports the study of the talin family of proteins in the cardiovascular field. Moulik and colleagues showed that 2% percent of dilated cardiomyopathy patients present a missense mutation originating loss of cardiac ankyrin repeat protein ligation to talin-1 disrupting the normal cardiac stretch-based signaling [45]. In animal models, talin-2 has been shown to be a major player in maintaining normal cardiomyocyte function and structure and talin-2 knockout mice develop mild skeletal myopathy [46]. Talin-2-integrin activation leads to stimulation of cell-matrix adhesion, motor activation, and actin polymerization that generates traction force to induce cell migration [47-48]. In adult human skeletal muscle myoblasts, talin-2 is upregulated during muscle differentiation and localizes to costameres and intercalated disks generating stable adhesions required for the assembly of mature striated muscle [49]. In addition, increased expression of talin-2 leads to enhanced activation of myosin, which generates contractile force, and enhanced focal adhesion formation [43-50-51]. Age-related increased talin-2 expression may represent an indirect effect of increased energy demand in the aging heart. Cardiomyocytes intensify contractile output in response to increased substrate stiffness by augmenting their contraction stress [52]. LV stiffness correlates with increasing age both in humans and animal models [53-54]. Thus, age-associated cardiac substrate stiffness may stimulate talin-2 expression, which in turn would result in elevated cell-matrix traction forces, which would lead to increased substrate stiffness creating a vicious circle. MMP-9 inhibition has been reported to decrease migration in several cell types [55-57]; yet, if the underlying mechanism involves talin-2 has not been reported. Our data is the first to report a link between MMP-9 and talin-2. However, the mechanism through which MMP-9 regulates talin-2 remains to be explored in the future.

In summary, we identified age-dependent changes in the cardiac proteome that relate to age-associated myocardial reduced contractility and increased stiffness. We also identified MMP-9-dependent changes such that MMP-9 deletion eradicated age dependent effects, which may explain improved LV function in MMP-9 null animals. Our data suggest MMP-9 as a possible therapeutic target for the aging patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation list

CID	collision-induced dissociation
DAVID	database for annotation, visualization and integrated discovery
ECM	extracellular matrix
EMILIN-1	elastin microfibril interface-located protein
HPLC	high performance liquid chromatography
HPLC-ESI-MS/MS	HPLC electrospray ionization tandem mass spectrometry
LV	left ventricle
MMPs	matrix metalloproteinases
Null	MMP-9 null mice
PI	protease inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
WT	wild type

Clinical Relevance

Aging is an independent cardiovascular risk factor and is associated with cardiac dysfunction that can lead to heart failure. Cardiac remodeling comprises changes in the volume, mass, composition, and structure of the left ventricle; these are all important predictors of left ventricular function and are more prominent with advancing age. Age-associated changes in the cardiac extracellular matrix profoundly affect cardiovascular function with striking impact on patient clinical outcomes. In this study, we identified age-dependent changes in the cardiac proteome that relate to age-associated myocardial reduced contractility and increased stiffness. Additionally, we also showed that MMP-9 deletion eradicated matrix-age dependent effects, which may explain improved cardiac function in MMP-9 null animals. This study identified MMP-9 as a possible therapeutic target for the aging patient.

Figure 1. Decellularization of the LV enriched the proteome for ECM proteins

A. Mass spectrometry analysis identified 245 proteins across all groups. Presence of a protein in at least one sample per genotype was the criteria used to generate the Venn diagram. Of the 245 proteins identified, 160 proteins were common between wild type (WT) and MMP-9 null animals. Forty three proteins were identified solely in the WT group, while 42 proteins were identified only in the MMP-9 null groups. B. Of all proteins identified, 26% (63 proteins) were secreted and extracellular proteins and 17% were cytoskeletal proteins. Intracellular proteins, including cytoplasmic, nuclear, and mitochondrial, corresponded to 57% of the total proteins identified. The box shows examples of the ECM proteins identified.

Figure 2. EMILIN-1 and Talin-2 levels are age-related and MMP-9 dependent

A. EMILIN-1 levels decrease 2-fold with age in the WT group. MMP-9 deletion bunted this change. **B.** Talin-2 levels increase with age in the WT group, but not in the MMP-9 null mice. The left panels display total spectra and the right panels show densitometry units by immunoblot. Representative images of the immunoblots for each protein and for the total protein used as loading control are displayed above the respective densitometry graph. Ctr = HUVEC lysate used as negative control for the Talin-2 antibody. Data represent mean $\pm$ SEM, n=6/group, (3 male and 3 female animals/group). *p<0.05 versus respective middle-age; #p<0.05 versus same age WT animals.

A. EMILIN-1 **B. Talin-2** **Figure 3. EMILIN-1 and Talin-2 expression are MMP-9 dependent**

Representative images displaying EMILIN-1 (A) and Talin-2 (B) immunofluorescence staining by genotype and age group. The bar corresponds to 50 μ m. The right panels display percentage of positively stained area. Values are mean $\pm$ SEM, *p<0.05 versus respective middle-age; #p<0.05 versus old WT animals.

Table 1

The Database for Annotation, Visualization and Integrated Discovery (DAVID) identified 87 functional protein clusters. This table lists the 3 clusters with the highest enrichment scores. Terms identified for each cluster are also listed with the p-value for enrichment of annotation, the genes ID number associated with the term, and fold change.

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
1	25.1	GOTERM_CC_FAT	Contractile fiber	3.2E-40	Mm.35134, Mm.29733, Mm.117709, Mm.686, Mm.37638, Mm.291928, Mm.178, Mm.6712, Mm.416750, Mm.39046, Mm.13849, Mm.299774, Mm.27806, Mm.4103, Mm.259767, Mm.10728, Mm.250705, Mm.461064, Mm.422801, Mm.340090, Mm.297382, Mm.290003, Mm.340163, Mm.457983, Mm.1529, Mm.141157, Mm.386769, Mm.339542, Mm.234912, Mm.239871, Mm.204969, Mm.123110, Mm.34359, Mm.446279, Mm.27674, Mm.279361	2.5E1
		GOTERM_CC_FAT	Myofibril	2.5E-39	Mm.259767, Mm.39046, Mm.422801, Mm.27806, Mm.686, Mm.141157, Mm.239871, Mm.1529, Mm.340163, Mm.279361, Mm.291928, Mm.29733, Mm.299774, Mm.35134, Mm.34359, Mm.250705, Mm.13849, Mm.457983, Mm.297382, Mm.117709, Mm.27674, Mm.386769	2.5E1
		GOTERM_CC_FAT	Contractile fiber part	2.7E-35	Mm.339542, Mm.123110, Mm.204969, Mm.37638, Mm.446279, Mm.290003, Mm.4103, Mm.6712, Mm.373672, Mm.178, Mm.461064, Mm.416750, Mm.10728, Mm.259767, Mm.39046, Mm.422801, Mm.27806, Mm.686, Mm.141157, Mm.239871, Mm.340163, Mm.279361, Mm.291928, Mm.29733, Mm.299774, Mm.35134, Mm.34359, Mm.250705, Mm.13849, Mm.457983, Mm.297382, Mm.117709, Mm.27674	2.5E1
		GOTERM_CC_FAT	Sarcomere	3.9E-33	Mm.339542, Mm.123110, Mm.204969, Mm.37638, Mm.446279, Mm.290003, Mm.4103, Mm.6712, Mm.373672, Mm.178, Mm.461064, Mm.416750, Mm.10728, Mm.259767, Mm.39046, Mm.422801, Mm.27806, Mm.686, Mm.141157, Mm.239871, Mm.340163, Mm.291928, Mm.29733, Mm.299774, Mm.35134, Mm.34359, Mm.13849, Mm.457983, Mm.297382, Mm.117709, Mm.27674	2.5E1
		GOTERM_CC_FAT	I band	2.7E-20	Mm.339542, Mm.239871, Mm.204969, Mm.37638, Mm.446279, Mm.291928, Mm.29733, Mm.299774, Mm.35134, Mm.34359, Mm.373672, Mm.6712, Mm.13849, Mm.178, Mm.416750, Mm.39046, Mm.117709, Mm.27806, Mm.686, Mm.141157	2.4E1
		GOTERM_CC_FAT	Actin cytoskeleton	2.9E-19	Mm.440740, Mm.339542, Mm.123110, Mm.204969, Mm.37638, Mm.446279, Mm.290003, Mm.4103, Mm.373672, Mm.7353, Mm.461064, Mm.10728, Mm.340090, Mm.39046, Mm.422801, Mm.444692, Mm.141157, Mm.18962, Mm.1529, Mm.340163,	9.4E0

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
					Mm.279361, Mm.299774, Mm.250705, Mm.29933, Mm.457983, Mm.210815, Mm.297382, Mm.208601, Mm.34637, Mm.446592	
		SP_PIR_KEYWORDS	Actin-binding	3.4E-19	Mm.440740, Mm.123110, Mm.204969, Mm.37638, Mm.290003, Mm.461064, Mm.416750, Mm.10728, Mm.340090, Mm.259767, Mm.39046, Mm.422801, Mm.10117, Mm.340163, Mm.279361, Mm.295533, Mm.34359, Mm.250705, Mm.29933, Mm.457983, Mm.297382, Mm.261043, Mm.234912, Mm.27674, Mm.35789, Mm.446592, Mm.386769	1.1E1
		GOTERM_CC_FAT	Z disc	2.3E-18	Mm.339542, Mm.204969, Mm.239871, Mm.37638, Mm.446279, Mm.291928, Mm.29733, Mm.299774, Mm.34359, Mm.373672, Mm.6712, Mm.13849, Mm.178, Mm.416750, Mm.39046, Mm.117709, Mm.27806, Mm.141157	2.4E1
		GOTERM_MF_FAT	Actin binding	2.4E-18	Mm.440740, Mm.123110, Mm.204969, Mm.37638, Mm.290003, Mm.461064, Mm.416750, Mm.10728, Mm.340090, Mm.259767, Mm.39046, Mm.422801, Mm.10117, Mm.18962, Mm.340163, Mm.279361, Mm.295533, Mm.34359, Mm.250705, Mm.33645, Mm.29933, Mm.457983, Mm.297382, Mm.261043, Mm.234912, Mm.208601, Mm.27674, Mm.309975, Mm.35789, Mm.446592, Mm.386769	7.8E0
		GOTERM_MF_FAT	Cytoskeletal protein binding	8.3E-15	Mm.440740, Mm.339542, Mm.123110, Mm.204969, Mm.37638, Mm.290003, Mm.461064, Mm.416750, Mm.10728, Mm.340090, Mm.259767, Mm.39046, Mm.422801, Mm.10117, Mm.18962, Mm.340163, Mm.279361, Mm.295533, Mm.34359, Mm.250705, Mm.33645, Mm.29933, Mm.457983, Mm.297382, Mm.261043, Mm.234912, Mm.208601, Mm.27674, Mm.309975, Mm.35789, Mm.446592, Mm.386769	5.6E0
2	12.45	SP_PIR_KEYWORDS	Extracellular matrix	2.0E-28	Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.404771, Mm.4691, Mm.271644, Mm.277735, Mm.209715, Mm.290527, Mm.4339, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.172674, Mm.2509, Mm.256087, Mm.249555, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.193099, Mm.20348	1.4E1
		GOTERM_CC_FAT	Extracellular matrix part	1.6E-26	Mm.290527, Mm.4339, Mm.130388, Mm.10299, Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.172674, Mm.286892, Mm.273662, Mm.256087, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.4691, Mm.16773, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.271644, Mm.193099, Mm.20348, Mm.277735, Mm.209715	1.9E1
		GOTERM_CC_FAT	Extracellular matrix	3.1E-23	Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.334994, Mm.	8.1E0

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
					404771, Mm.4691, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.172674, Mm.2509, Mm.286892, Mm.256087, Mm.249555, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.193099, Mm.20348, Mm.330731	
		GOTERM_CC_FAT	Proteinaceous extracellular matrix	8.4E-23	Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.334994, Mm.404771, Mm.4691, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.290527, Mm.4339, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.2509, Mm.172674, Mm.286892, Mm.256087, Mm.249555, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.193099, Mm.20348, Mm.330731	8.2E0
		GOTERM_BP_FAT	Cell adhesion	2.1E-18	Mm.258065, Mm.1249, Mm.257437, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.334994, Mm.10728, Mm.4691, Mm.444692, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.18962, Mm.286375, Mm.279361, Mm.291928, Mm.299774, Mm.14455, Mm.288381, Mm.172674, Mm.2509, Mm.210815, Mm.256087, Mm.4159, Mm.22339, Mm.235570, Mm.7281, Mm.208601, Mm.280547, Mm.2423, Mm.34637, Mm.425599, Mm.233547, Mm.193099, Mm.2252, Mm.20348	5.7E0
		GOTERM_BP_FAT	Biological adhesion	2.2E-18	Mm.258065, Mm.1249, Mm.257437, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.334994, Mm.10728, Mm.4691, Mm.444692, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.18962, Mm.286375, Mm.279361, Mm.291928, Mm.299774, Mm.14455, Mm.288381, Mm.172674, Mm.2509, Mm.210815, Mm.256087, Mm.4159, Mm.22339, Mm.235570, Mm.7281, Mm.208601, Mm.280547, Mm.2423, Mm.34637, Mm.425599, Mm.233547, Mm.193099, Mm.2252, Mm.20348	5.6E0
		KEGG_PATHWAY	Focal adhesion	3.5E-18	Mm.37638, Mm.1249, Mm.258065, Mm.277792, Mm.32804, Mm.738, Mm.1949, Mm.334994, Mm.39046, Mm.277735, Mm.209715, Mm.290527, Mm.4339, Mm.1529, Mm.10299, Mm.279361, Mm.291928, Mm.295533, Mm.33645, Mm.2509, Mm.172674, Mm.256087, Mm.249555, Mm.22339, Mm.4159, Mm.7281, Mm.208601, Mm.2423, Mm.425599, Mm.181021, Mm.193099	7.2E0
		KEGG_PATHWAY	ECM-receptor interaction	1.2E-17	Mm.290527, Mm.4339, Mm.10299, Mm.1249, Mm.258065, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.172674, Mm.256087, Mm.249555, Mm.334994, Mm.22339, Mm.4159, Mm.7281, Mm.2423, Mm.	1.2E0

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
					425599, Mm.181021, Mm.193099, Mm.277735, Mm.209715	
		INTERPRO	Collagen triple helix repeat	3.2E-17	Mm.130388, Mm.286375, Mm.10299, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.286892, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.2423, Mm.233547, Mm.7562, Mm.181021, Mm.277735, Mm.209715	1.9E1
		SP_PIR_KEYWORDS	Collagen	3.7E-17	Mm.130388, Mm.286375, Mm.10299, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.286892, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.2423, Mm.233547, Mm.7562, Mm.181021, Mm.277735, Mm.209715	1.9E1
		SP_PIR_KEYWORDS	Cell adhesion	1.9E-15	Mm.1249, Mm.258065, Mm.257437, Mm.1949, Mm.236067, Mm.345891, Mm.10728, Mm.4691, Mm.444692, Mm.4339, Mm.18962, Mm.130388, Mm.286375, Mm.279361, Mm.291928, Mm.299774, Mm.14455, Mm.288381, Mm.2509, Mm.172674, Mm.256087, Mm.22339, Mm.4159, Mm.280547, Mm.34637, Mm.425599, Mm.233547, Mm.193099, Mm.20348	6.8E0
		GOTERM_CC_FAT	Extracellular region part	7.7E-15	Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.334994, Mm.4691, Mm.404771, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.172674, Mm.19131, Mm.2509, Mm.286892, Mm.88793, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.30063, Mm.193099, Mm.20348, Mm.330731	3.8E0
		SP_PIR_KEYWORDS	Secreted	1.0E-07	Mm.159651, Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.4691, Mm.404771, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.288381, Mm.172674, Mm.19131, Mm.2509, Mm.256087, Mm.249555, Mm.16422, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.30063, Mm.193099, Mm.20348	2.5E0
		GOTERM_CC_FAT	Extracellular region	1.3E-05	Mm.275831, Mm.159651, Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.334994, Mm.4691, Mm.404771, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.288381, Mm.172674, Mm.19131, Mm.2509, Mm.286892, Mm.88793, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.138455, Mm.	1.9E0

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
					7281, Mm.439678, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.30063, Mm.193099, Mm.20348, Mm.330731	
		SP_PIR_KEYWORDS	Signal	6.2E-03	Mm.159651, Mm.258065, Mm.1249, Mm.257437, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.4691, Mm.404771, Mm.16773, Mm.443385, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.19131, Mm.288381, Mm.172674, Mm.2509, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.280547, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.20388, Mm.30063, Mm.193099, Mm.20348, Mm.35811	1.4E0
		UP_SEQ_FEATURE	Signal peptide	1.6E-02	Mm.159651, Mm.258065, Mm.1249, Mm.257437, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.4691, Mm.404771, Mm.16773, Mm.443385, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.19131, Mm.288381, Mm.172674, Mm.2509, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.280547, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.20388, Mm.30063, Mm.193099, Mm.20348, Mm.35811	1.4E0
					Mm.339542, Mm.258065, Mm.1249, Mm.446279, Mm.373672, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.279782, Mm.404771, Mm.4691, Mm.259767, Mm.16773, Mm.389924, Mm.271644, Mm.4339, Mm.286375, Mm.18888, Mm.35134, Mm.14455, Mm.29933, Mm.288381, Mm.172674, Mm.19131, Mm.256087, Mm.16422, Mm.249555, Mm.22339, Mm.4159, Mm.138455, Mm.439678, Mm.425599, Mm.233547, Mm.181021, Mm.20388, Mm.30063, Mm.193099, Mm.20348	
		SP_PIR_KEYWORDS	Disulfide bond	2.1E-02	Mm.339542, Mm.258065, Mm.1249, Mm.446279, Mm.373672, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.279782, Mm.404771, Mm.4691, Mm.259767, Mm.16773, Mm.389924, Mm.271644, Mm.4339, Mm.286375, Mm.18888, Mm.35134, Mm.14455, Mm.29933, Mm.288381, Mm.172674, Mm.19131, Mm.256087, Mm.16422, Mm.249555, Mm.22339, Mm.4159, Mm.138455, Mm.439678, Mm.425599, Mm.233547, Mm.181021, Mm.20388, Mm.30063, Mm.193099, Mm.20348	1.4E0
		UP_SEQ_FEATURE	Disulfide bond	4.2E-02	Mm.339542, Mm.258065, Mm.1249, Mm.446279, Mm.373672, Mm.738, Mm.273662, Mm.236067, Mm.279782, Mm.404771, Mm.4691, Mm.259767, Mm.16773, Mm.389924, Mm.271644, Mm.4339, Mm.286375, Mm.18888, Mm.35134, Mm.14455, Mm.29933, Mm.288381, Mm.172674, Mm.19131, Mm.256087, Mm.16422, Mm.249555, Mm.22339, Mm.4159, Mm.138455, Mm.439678, Mm.425599, Mm.233547, Mm.181021, Mm.20388, Mm.30063, Mm.193099, Mm.20348	1.4E0
					Mm.440740, Mm.123110, Mm.258065, Mm.1249, Mm.257437, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.4691, Mm.404771, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.19131, Mm.288381, Mm.172674, Mm.2509, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.280547, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.20388, Mm.30063, Mm.193099, Mm.20348	
		SP_PIR_KEYWORDS	Glycoprotein	3.2E-01	Mm.440740, Mm.123110, Mm.258065, Mm.1249, Mm.257437, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.4691, Mm.404771, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.290527, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.19131, Mm.288381, Mm.172674, Mm.2509, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.439678, Mm.280547, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.20388, Mm.30063, Mm.193099, Mm.20348	1.1E0

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
3	11.2	UP_SEQ_FEATURE	Glycosylation site:N-linked (GlcNAc...)	8.1E-01	Mm.10299, Mm.286375, Mm.18888, Mm.288381, Mm.19131, Mm.172674, Mm.2509, Mm.210815, Mm.256087, Mm.249555, Mm.16422, Mm.4159, Mm.235570, Mm.22339, Mm.138455, Mm.439678, Mm.280547, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.7562, Mm.20388, Mm.30063, Mm.193099, Mm.20348, Mm.35811	
		SP_PIR_KEYWORDS	Extracellular matrix	2.0E-28	Mm.258065, Mm.1249, Mm.257437, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.345891, Mm.4691, Mm.271644, Mm.277735, Mm.209715, Mm.4339, Mm.10299, Mm.286375, Mm.288381, Mm.172674, Mm.19131, Mm.2509, Mm.256087, Mm.16422, Mm.22339, Mm.235570, Mm.4159, Mm.138455, Mm.439678, Mm.280547, Mm.425599, Mm.233547, Mm.181021, Mm.20388, Mm.30063, Mm.193099, Mm.20348, Mm.35811	9.1E-1
		GOTERM_CC_FAT	Extracellular matrix part	1.6E-26	Mm.290527, Mm.4339, Mm.130388, Mm.10299, Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.172674, Mm.286892, Mm.273662, Mm.256087, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.4691, Mm.16773, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.271644, Mm.193099, Mm.20348, Mm.277735, Mm.209715	1.9E1
		GOTERM_CC_FAT	Proteinaceous extracellular matrix	8.4E-23	Mm.258065, Mm.1249, Mm.277792, Mm.738, Mm.1949, Mm.273662, Mm.236067, Mm.334994, Mm.404771, Mm.4691, Mm.16773, Mm.271644, Mm.277735, Mm.209715, Mm.290527, Mm.4339, Mm.130388, Mm.10299, Mm.286375, Mm.18888, Mm.14455, Mm.2509, Mm.172674, Mm.286892, Mm.256087, Mm.249555, Mm.22339, Mm.235570, Mm.138455, Mm.7281, Mm.2423, Mm.425599, Mm.233547, Mm.181021, Mm.193099, Mm.20348, Mm.330731	8.2E0
		GOTERM_MF_FAT	Extracellular matrix structural constituent	9.6E-23	Mm.1249, Mm.10299, Mm.286375, Mm.277792, Mm.738, Mm.172674, Mm.286892, Mm.249555, Mm.334994, Mm.235570, Mm.404771, Mm.7281, Mm.2423, Mm.181021, Mm.271644, Mm.277735, Mm.209715	4.1E1
		GOTERM_CC_FAT	Collagen	4.3E-18	Mm.334994, Mm.235570, Mm.7281, Mm.290527, Mm.10299, Mm.2423, Mm.181021, Mm.277792,	4.5E1

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
					Mm.738, Mm.277735, Mm.286892, Mm.209715, Mm.24955	
		KEGG_PATHWAY	ECM-receptor interaction	1.2E-17	Mm.290527, Mm.4339, Mm.10299, Mm.1249, Mm.258065, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.172674, Mm.256087, Mm.249555, Mm.334994, Mm.22339, Mm.4159, Mm.7281, Mm.2423, Mm.425599, Mm.181021, Mm.193099, Mm.277735, Mm.209715	1.2E1
		INTERPRO	Collagen triple helix repeat	3.2E-17	Mm.130388, Mm.286375, Mm.10299, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.286892, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.2423, Mm.233547, Mm.7562, Mm.181021, Mm.277735, Mm.209715	1.9E1
		SP_PIR_KEYWORDS	Collagen	3.7E-17	Mm.130388, Mm.286375, Mm.10299, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.286892, Mm.249555, Mm.334994, Mm.235570, Mm.7281, Mm.2423, Mm.233547, Mm.7562, Mm.181021, Mm.277735, Mm.209715	1.9E1
		INTERPRO	Fibrillar Collagen, C- terminal	4.1E-14	Mm.334994, Mm.235570, Mm.7281, Mm.10299, Mm.2423, Mm.277792, Mm.277735, Mm.209715, Mm.249555	7.1E1
		SMART	COLF1	9.8E-14	Mm.334994, Mm.235570, Mm.7281, Mm.10299, Mm.2423, Mm.277792, Mm.277735, Mm.209715, Mm.249555	6.3E1
		SP_PIR_KEYWORDS	Hydroxylation	2.1E-13	Mm.130388, Mm.10299, Mm.277792, Mm.738, Mm.1949, Mm.2509, Mm.249555, Mm.7281, Mm.404771, Mm.2423, Mm.181021, Mm.233547, Mm.277735, Mm.209715	2.0E1
		UP_SEQ_FEATURE	Fibrillar collagen, NC1	3.2E-12	Mm.235570, Mm.7281, Mm.10299, Mm.2423, Mm.277792, Mm.277735, Mm.209715, Mm.249555	6.8E1
		UP_SEQ_FEATURE	Triple-helical region	1.2E-11	Mm.7281, Mm.2423, Mm.181021, Mm.1949, Mm.738, Mm.2509, Mm.277735, Mm.249555	4.2E1
		SP_PIR_KEYWORDS	Triple helix	1.4E-10	Mm.2423, Mm.181021, Mm.277792, Mm.738, Mm.2509, Mm.277735, Mm.249555	7.0E1
		GOTERM_MF_FAT	Platelet- derived growth factor binding	4.9E-10	Mm.7281, Mm.2423, Mm.277792, Mm.738, Mm.2509, Mm.277735, Mm.249555	5.6E1
		GOTERM_CC_FAT	Fibrillar collagen	1.5E-08	Mm.334994, Mm.290527, Mm.2423, Mm.277792, Mm.277735, Mm.249555	5.7E1
		PIR_SUPERFAMILY	Collagen alpha 1(I) chain	2.3E-07	Mm.10299, Mm.2423, Mm.277792, Mm.277735, Mm.249555	6.7E1
		UP_SEQ_FEATURE	N-terminal propeptide	2.7E-07	Mm.2423, Mm.277792, Mm.277735, Mm.209715, Mm.249555	7.1E1

Annotation Cluster	Enrichment Score	Annotation	Annotation term	p value	Genes	Fold change
		UP_SEQ_FEATURE	C-terminal propeptide	6.3E-07	Mm.10299, Mm.277792, Mm.277735, Mm.209715, Mm.249555	6.1E1
		GOTERM_BP_FAT	Collagen fibril organization	4.5E-06	Mm.7281, Mm.290527, Mm.10299, Mm.2423, Mm.209715, Mm.249555	2.3E1
		SP_PIR_KEYWORDS	Trimer	1.3E-05	Mm.2423, Mm.277792, Mm.738, Mm.249555	7.2E1
		UP_SEQ_FEATURE	VWFC	3.9E-05	Mm.4159, Mm.10299, Mm.2423, Mm.277735, Mm.249555	2.5E1
		INTERPRO	von Willebrand factor, type C	1.1E-04	Mm.22339, Mm.4159, Mm.10299, Mm.2423, Mm.277735, Mm.249555	1.2E1
		SMART	VWC	1.9E-04	Mm.22339, Mm.4159, Mm.10299, Mm.2423, Mm.277735, Mm.249555	1.1E1
		GOTERM_MF_FAT	Growth factor binding	4.5E-04	Mm.7281, Mm.2423, Mm.277792, Mm.738, Mm.2509, Mm.277735, Mm.249555	7.1E0
		UP_SEQ_FEATURE	Glycosylation site:O-linked	2.8E-03	Mm.2423, Mm.277735, Mm.249555	3.6E1
		GOTERM_MF_FAT	SMAD binding	3.0E-02	Mm.10299, Mm.277792, Mm.249555	1.1E1

Table 2

List of ECM and ECM-related proteins that are age dependent or MMP-9 dependent. Total spectrum counts were used to calculate differences between middle-aged and old animals and between genotype. Direction of change, up: increased with age or higher in MMP-9 null compared to WT and down: decreased with age or lower in MMP-9 null compared to WT.

Age-dependent changes (middle-age $\times$ old)		
Protein	Wild Type	MMP-9 null
catenin (cadherin associated protein)-α3	no change	down <i>b</i>
collagen type III, alpha-1	down <i>a</i>	no change
collagen type VI, alpha-6 isoform 1	no change	down <i>b</i>
collagen type XV, alpha-1	down <i>a</i>	no change
dynamin 1-like, isoform CRA_b	up <i>a</i>	no change
EMILIN-1	down <i>a</i>	no change
fibrillin-1	no change	down <i>b</i>
fibrillin-1 precursor	no change	down <i>b</i>
fibronectin 1, isoform CRA_d	no change	down <i>b</i>
fibulin 5	no change	down <i>b</i>
junction plakoglobin	no change	down <i>b</i>
laminin gamma-1, isoform CRA_a	no change	down <i>b</i>
laminin gamma-1, isoform CRA_b	no change	down <i>b</i>
sorbin and SH3 domain-containing protein 1	no change	down <i>b</i>
sorbin and SH3 domain-containing protein 2 isoform 1	no change	down <i>b</i>
talin-2	up <i>a</i>	no change
Genotype-dependent changes (WT $\times$ MMP-9 null)		
Protein	Middle-age	Old
catenin (cadherin associated protein)-α3	no change	down <i>d</i>
collagen type VI alpha-1	down <i>c</i>	down <i>d</i>
collagen type VI alpha-2	no change	down <i>d</i>
collagen type VI alpha-3 isoform 1	down <i>c</i>	down <i>d</i>
collagen type VI alpha-3 isoform 2	down <i>c</i>	down <i>d</i>
dynamin 1-like, isoform CRA_b	up <i>c</i>	down <i>d</i>
EMILIN-1	down <i>c</i>	no change
fibronectin 1, isoform CRA_d	up <i>c</i>	down <i>d</i>
laminin gamma 1, isoform CRA_b	no change	down <i>d</i>
sorbin and SH3 domain-containing protein 2	no change	down <i>d</i>

Age-dependent changes (middle-age × old)		
Protein	Wild Type	MMP-9 null
sorbin and SH3 domain-containing protein 2 isoform X62	no change	down ^d
tenascin-X	no change	up ^d

^a p<0.05 WT middle-age versus WT old;

^b p<0.05 MMP-9 null middle-age versus MMP-9 null old;

^c p<0.05 WT middle-age versus MMP-9 null middle-age;

^d p<0.05 WT old versus MMP-9 null old. Proteins highlighted in bold displayed both age- and genotype-dependence.