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Proteomic Analysis Identifies *In vivo* Candidate Matrix Metalloproteinase-9 Substrates in the Left Ventricle Post-Myocardial Infarction

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Abstract

MMP-9 deletion has been shown to improve remodeling of the left ventricle (LV) post-myocardial infarction (MI), but the mechanisms to explain this improvement have not been fully elucidated. MMP-9 has a broad range of *in vitro* substrates, but relevant *in vivo* substrates are incompletely defined. Accordingly, we evaluated the infarct regions of wild-type (wt) and MMP-9 null (null) mice using a proteomic strategy. Wt and null groups showed similar infarct sizes (48 ± 3 in wt and $45 \pm 3\%$ in null), indicating that both groups received an equal injury stimulus. LV infarct tissue was homogenized and analyzed by two-dimensional gel electrophoresis and mass spectrometry. Of 31 spot intensity differences, the intensities of 9 spots were higher and 22 spots were lower in null mice compared to wt (all $p < 0.05$). Several extracellular matrix (ECM) proteins were identified in these spots by mass spectrometry, including fibronectin, tenascin-C, thrombospondin-1, and laminin. Fibronectin was observed on the gels at a lower than expected molecular weight in the wt group, which suggested substrate cleavage, and the lower molecular weight spot was observed at lower intensity in the MMP-9 null group, which suggested cleavage by MMP-9. Immunoblotting confirmed the presence of fibronectin cleavage products in the wt samples and lower levels in the absence of MMP-9. In conclusion, examining infarct tissue from wt and MMP-9 null mice by proteomic analysis provides a powerful and unique method to identify *in vivo* candidate MMP substrates.

Keywords

cardiac remodeling; MMP-9; extracellular matrix; mice; proteomics; myocardial infarction

1 Introduction

Following myocardial infarction (MI), the left ventricle (LV) undergoes a robust remodeling response that involves the accumulation of extracellular matrix (ECM) proteins. Excessive accumulation of ECM correlates directly with morbidity and mortality rates.[4] ECM turnover is regulated by matrix metalloproteinases (MMPs), but global MMP inhibition strategies that block multiple MMP family members have not been efficacious in the

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treatment of inflammatory and degenerative processes in humans.[5] This is due, in part, to a lack of complete understanding of MMP biology. With 25 MMPs identified, global inhibition strategies block both negative and positive MMP functions. MMP-9, in particular, is elevated post-MI, and MMP-9 gene deletion protects against ventricular rupture and decreases the extent of ventricular dilation.[6,7] MMP-9, therefore, is a lead therapeutic target in the post-MI setting. At the same time, however, MMP-9 also serves beneficial roles in the post-MI setting, including the regulation of neovascularization.[8] Before MMP-9 specific strategies can be applied clinically, a more complete catalogue of MMP-9 substrates is needed to fully understand the net effect of MMP-9 inhibition in the post-MI LV. We hypothesized that MMP-9 deletion improves ventricular remodeling through direct ECM protein alterations that can be evaluated using proteomics.

Accordingly, we used 2-dimensional gel electrophoresis (2-DE) to look for differences in protein levels in the infarct region of the LV of 7 day post-MI wild-type (wt) and MMP-9 null (null) mice and mass spectrometry to identify proteins present in these spots. The identified proteins include known ECM and non-ECM substrates of MMP-9 and multiple potential candidate substrates that have not been previously associated with MMP-9 or post-MI remodeling.[9]

2 Methods

2.1 Mice

Male C57/BL6J wild type (n=12) and MMP-9 null (n=10) mice that were 4.5 months of age were used for this study. All animal procedures were conducted according to the “Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 85–23, revised 1996) and were approved by the UTHSCSA Institutional Animal Care and Use Committee. MI was induced by surgical ligation of the coronary artery, as described previously.[8] Tissue was collected on the seventh day after MI. The mice were anesthetized with 5% isoflurane, the coronary vasculature was flushed with 0.9M saline, and the hearts were excised. The hearts were stained with 1% 2,3,5-triphenyltetrazolium chloride (Sigma) and photographed for infarct size. The LV and right ventricle were separated and weighed individually, and the infarct region was isolated, snap frozen in liquid nitrogen, and stored at -80°C .

2.2 Protein extraction

Proteins were extracted from the infarct region of the LV by homogenizing the tissue sample in proteomic extraction buffer (Sigma Reagent 4; 7 M urea, 2 M thiourea, 40 mM Trizma[®] base and the detergent 1% C7BzO) and 1x Complete Protease Inhibitor Cocktail (Roche). Protein concentrations were determined using the Bradford assay. Due to the high urea content in Reagent 4, the insoluble protein extracts were diluted 1:40 with water prior to the Bradford assay. The proteins in each sample (10 μg) were separated by one-dimensional SDS gel electrophoresis and stained with Coomassie blue to verify protein concentration and loading accuracy (Figure 1A). Densitometry of the entire lane was 7783 ± 384 for the wt infarct group and 7312 ± 407 for the MMP-9 null group ($p=0.4$) indicating that tissue handling was not different between the two groups (Figure 1B).

2.3 Two-dimensional gel electrophoresis (2-DE)

Protein samples (500 μg) were reduced with 2.5% tributylphosphine in 1x Complete Protease Inhibitor Cocktail for 1 h at room temperature. Iodoacetamide was added to a final concentration of 3%. The samples were incubated at room temperature for 1 h and then centrifuged at $425 \times g$ for 5 min to pellet debris. The supernatants were acetone precipitated and centrifuged at $20,817 \times g$ for 10 min. The pellets were air-dried for 30 min and resuspended in 200 μl of Reagent 4 and 1x Complete Protease Inhibitor Cocktail. After

incubating the pellet at 30°C for 30 min, the mixture was applied to an 11-cm pH 3–10 IPG strip (Proteome Systems), rehydrated overnight at room temperature, and focused for 75,000 Vh. The IPG strips were then equilibrated in 5 ml of Equilibration Buffer (Proteome Systems) for 20 min. Pre-cast gels (Criterion™ XT, 4–12%, Bis-Tris, 11 cm, 1 mm; Bio-Rad) were used for the second-dimension. The gels were run at 200 V (25–50 mA/gel), fixed in 25% methanol/10% acetic acid for 30 min at room temperature, and stained overnight with the Blue Gel Stain Kit (Proteome Systems) which is a Coomassie blue stain. The gels were scanned using a Kodak Image Station 4000MM camera interfaced with Molecular Imaging Software, version 4.0 (Eastman Kodak Company). Images were saved as 16-bit tiff files. The images were analyzed using the Progenesis PG200 software package (Nonlinear Dynamics). Statistical significance of differences in spot intensities was assessed by an unpaired Student's t-test. Spots that exhibited significant differences between the 2 groups ($p < 0.05$) were selected for identification by mass spectrometry.

2.4 Identification of spots by mass spectrometry

Coomassie-stained gel spots were manually excised using a One Touch™ 1.5 mm 2-DE spot picker (The Gel Company) and digested *in situ* with trypsin (Promega modified) in 40 mM NH_4HCO_3 at 37°C for 4 h prior to analysis by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS), using a Thermo Fisher LTQ linear ion trap mass spectrometer fitted with a New Objective PicoView 550 nanospray interface. On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC micro HPLC: column, PicoFrit™ (New Objective; 75 μm i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MSB5, 5 μm , 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 $\mu\text{l}/\text{min}$. MS conditions were: ESI voltage, 2.9 kV; isolation window for MS/MS, 3; relative collision energy, 35%; scan strategy, survey scan followed by acquisition of data dependent collision-induced dissociation (CID) spectra of the seven most intense ions in the survey scan above a set threshold. The uninterpreted CID spectra were searched against the NCBI nr_20070216 database by means of Mascot (Matrix Science). Trypsin was specified as the proteolytic enzyme, and up to two missed cleavages were allowed. Peak lists were created using extract_msn.exe. Precursor and fragment ion mass tolerances were both ± 0.8 Da. Methionine oxidation and cysteine carbamidomethylation were considered as variable modifications for all searches. Cross correlation of the Mascot results with X! Tandem and determination of protein identity probabilities were accomplished by Scaffold™ (Proteome Software).

2.5 Immunoblotting

To confirm changes in specific proteins, immunoblotting was performed on the infarct samples using antibodies against the following proteins: adiponectin (gift from Dr. Lily Q. Dong), serpin 1d (abcam, cat# ab14226), brain abundant membrane attached signal protein 1 (BASP-1) (abcam, cat# ab25732), desmin (Sigma, cat# D8281), fibronectin (Chemicon, cat# Ab1954), galectin-3 (R&D Systems, cat# AF1197), peroxiredoxin 1 (abcam, cat# ab15571), peroxiredoxin 3 (Sigma, cat# P-1247), prohibitin-2 (ProteinTech Group, cat# 12295-1-AP), SPARC (R&D Systems, cat# AF942), tenascin-C (abcam, cat# ab6346) and thrombospondin-1 (abcam, cat# ab2962). Equal quantities of total protein (10 μg) were loaded on one 26-well 4–12% Criterion Bis-Tris gel (Bio-Rad). Immunoblotting was performed as previously described.[10] Molecular Imaging Software (Kodak) was used for densitometry.

2.6 Statistical analyses

Data are reported as mean $\pm$ SEM. All samples were analyzed individually and were not pooled at any point of the study. Statistical significance of the 2-DE normalized volumes

and the immunoblot intensities (arbitrary units) were assessed using an unpaired Student's t-test. A $p < 0.05$ was considered significant.

3 Results

3.1 Morphometric analysis

Necropsy values and infarct sizes for wt and null mice are shown in Table 1. Consistent with an equal injury given to both groups, wt and null mice had similar infarct sizes, LV masses, LV to body weight ratios, and lung masses at 7 days after MI.

3.2 Differentially expressed proteins resolved by 2-DE analysis and identified by mass spectrometry

Representative 2-DE images for wt and MMP-9 null are shown in Figure 2A. Thirty-one protein spots exhibited statistically significant differences in normalized spot volumes between the wt and the MMP-9 null infarct samples (all $p < 0.05$). In the null group, the intensity was higher in nine protein spots and lower in 22 protein spots (Table 2). The mass spectrometry analyses successfully identified proteins in each spot examined, with multiple proteins frequently found per spot (an average of 7.3 proteins per spot). Details of the protein identifications are included in supplementary Table 1).

Of the proteins identified, we focused on potential MMP-9 substrates. Eighty-five of the identified proteins were selected either because migration on the gel indicated an apparent molecular weight that was lower than expected or based on a previous association with MMP-9 in the literature (Table 2). These proteins were categorized as intracellular, extracellular or membrane proteins (Figure 2B). The list of proteins identified included voltage dependent anion channel protein 1 and 2 and several cathepsins, which have not been previously associated with post-MI remodeling. Haptoglobin and hemoglobin were found in multiple spots; elevations of these proteins in plasma have been proposed as circulating biomarkers of LV remodeling.[11] In line with findings of Cieniewski-Bernard and colleagues who studied post-MI rat LV, [12] alpha-crystallin b chain, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), heat shock proteins, and peroxiredoxin were identified as differentially expressed in the infarct samples. Anticoagulant proteins (annexins) and cell proliferation inhibitors (prohibitins) were also detected in multiple spots, as had been previously reported in the mouse MI model.[13]

Interestingly, the quantities of multiple ECM components (fibronectin, laminin, tenascin-C) and matricellular proteins (secreted protein acidic and rich in cysteine and thrombospondin-1) exhibited statistically significant differences in the MMP-9 null mice compared to wt infarcts. This is the first time multiple ECM proteins have been identified by 2-DE and the first time that putative MMP-9 substrates have been identified from *in vivo* LV extracts by mass spectrometry after 2-DE. Intermediate filaments (vimentin and desmin), plasma proteins (adiponectin and fibrinogen), and a protein specifically involved in synaptic plasticity [brain abundant membrane attached signal protein 1 (BASP-1)] were also identified.

3.3 Immunoblot analysis

As an alternative method to determine whether the known MMP-9 substrates and identified candidate substrates were present at different levels in the post-MI LV of the null group, extracts of the infarct region of wt and null mice were analyzed by immunoblotting. We selected 12 target proteins based on antibody availability and one of 2 criteria: a) the protein was previously identified as *in vitro* MMP-9 substrate; b) the protein was localized extracellularly.

Of the 12 proteins analyzed by immunoblotting, four completely agreed with the 2-DE gel results (Figure 3). Immunoblots using antibodies specific for desmin, serpin-1, tenascin-C, and thrombospondin-1 matched the 2-DE gel results in terms of intensity and apparent molecular weight. Three proteins showed different results for the immunoblot compared to 2-DE gels (Figure 4). The intensity difference seen by the immunoblot for BASP-1 agreed with the 2-DE results (both were lower in the null LV compared to wt); however, BASP-1 was observed at a higher apparent molecular weight of 150 kDa on the immunoblot, compared to a size of 55 kDa seen in the 2-DE gel. The intensity differences found by immunoblot analysis for galectin-3 and prohibitin-2 were in the opposite direction compared to 2-DE, but both analyses observed proteins at the same molecular weight. This suggests the possibility that other protein components in the spot contributed to the higher intensity seen in the null group by 2-DE gel analysis. Both galectin-3 and prohibitin-2 exhibited lower immunoblot intensity of the full-length protein in null mice compared to wt, indicating that MMP-9 deletion altered the expression of these proteins.

The well-documented *in vitro* cleavage of fibronectin by MMP-9 was detected in our *in vivo* samples, and the immunoblots results agreed with the 2-DE analysis (Figure 5). The immunoblot analysis using anti-fibronectin antibody detected protein bands at 277 kDa, as well as some lower molecular weight fragments at 70, 120, and 166 kDa. Immunoblot analysis for adiponectin, peroxiredoxin 1 and 3, and laminin did not show significant differences between groups, although these proteins were identified in differentially expressed spots.

4 Discussion

The primary goal of this study was to identify potential *in vivo* MMP-9 substrates that exhibit differences in quantity in mouse left ventricle infarct regions following myocardial infarction when comparing MMP-9 null animals to wt. There were three key findings in this study. First, differences in protein quantities of known MMP-9 substrates were detected by our 2-DE gel analysis, which validates our approach. Second, ECM proteins were resolved by 2-DE and differences were detected, which is the first time ECM proteins have been identified in the LV infarct proteome. Third, the global proteomic effect of MMP-9 deletion was catalogued for the infarct region, which provides direction for MMP-9 targeted therapeutic strategies. We found differences in levels of previously known *in vitro* MMP-9 substrates (fibronectin, tenascin-C, galectin-3, and serpin-1) as well as in proteins that may be indirectly regulated by MMP-9 (BASP-1, desmin, and prohibitin-2);[14–17] and proteins that may be negatively regulated by MMP-9 (thrombospondin-1).[18]

Since MMP-9 expression is strongly induced post-MI and absence of MMP-9 attenuates the degree of LV remodeling,[8] MMP-9 inhibition has been proposed as a possible therapy for the post-MI patient. However, as suggested by this study, deletion of MMP-9 strongly alters a wide array of proteins that have positive and negative effects on remodeling. At 7 days post-MI, metabolic changes and inflammatory responses are fulminant.[19] Not surprisingly, the majority of the intracellular proteins detected in our study were metabolic proteins. The cell proliferation inhibitor, prohibitin-2, and the macrophage chemotaxis inflammatory protein, galectin-3, [20] were at lower levels in the null mice, based on immunoblot analysis. Galectin-3 is a known MMP-9 substrate with identified effects on both macrophage phagocytosis and fibroblast activation.[21–24] The detection of BASP-1 in the LV infarct tissue was not surprising as Ohsawa and colleagues recently showed that BASP-1 staining in mouse tissues paralleled active caspase 3 staining raising the possibility that BASP-1 in the post-MI LV correlates with cardiac apoptotic cell death.[25]

The detection of ECM proteins and adhesion molecules was a key element of this study, as the high abundance of mitochondrial proteins often masks the detection of other protein families when whole left ventricles are analyzed. There remains, however, the possibility that every protein was not resolved in the 2-DE gels. By focusing on the infarct region only, we enriched for ECM and were able to focus on the often undetected extracellular matrix. Fibronectin is an ECM glycoprotein that is produced by multiple cell types (including macrophages) and is a known *in vitro* substrate for MMP-9. In the wt LV infarct extracts, full-length fibronectin as well as fibronectin fragments were at lower levels in the MMP-9 null group compared to wt, in agreement with the results of Marom et al.[15] The fact that full length fibronectin was higher in the wt than the null supports previous studies that show fibronectin matricryptic fragments in the post-MI LV can trigger a feedback mechanism to induce fibronectin expression.[26]

Tenascin-C is a known MMP-9 ECM substrate that often co-localizes with MMPs at sites of active remodeling and has been previously shown to upregulate MMP-9 expression. The full-length form of tenascin-C was also found at lower levels in the null group, indicating that MMP-9 deletion may also modulate the quantity of tenascin.[27] Strikingly, a 22 kDa fragment that maps to the *N*-terminus of thrombospondin-1 was found to be higher in the null mice. This integrin-binding fragment has been previously shown to stimulate angiogenesis.[28] This finding may explain the beneficial effects on angiogenesis observed with MMP-9 deletion post-MI.[8]

In agreement with Richard Schulze and colleagues, in our study we identified cytoplasmic and nuclear protein differences between the wt and null that further indicate that MMP-9 can cleave or regulate proteins found within the intracellular space.[1] Multiple forms of the intermediate filament, desmin, were found in our 2-DE and immunoblot analyses, which suggest a role of MMP-9 in cytoskeletal adaptations.[2] In addition, prohibitin-2, a mitochondrial resident protein that can modulate mitochondrial morphology and translocate to the nucleus was also identified in our study. Together, these data support the concept that MMP-9 can indirectly influence transcriptional regulation.[3]

In conclusion, we have identified multiple proteins that were present at significantly different levels in the left ventricle infarcts of post-MI MMP-9 null mice compared to post-MI wild-type mice. Among these are proteins previously known to be *in vitro* MMP-9 substrates, as well as several proteins that are potentially novel MMP-9 substrates or proteins regulated by MMP-9. Fibronectin was validated as an *in vivo* MMP-9 substrate in the post-MI setting. Additionally, detection of higher intensity levels of the thrombospondin-1 *N*-terminal fragment in MMP-9 null mice after MI may provide key information regarding the mechanism by which MMP-9 inhibition facilitates angiogenesis. Being able to resolve and evaluate ECM proteins using proteomics provides a powerful way to further our understanding of the global influence of MMP-9 on the post-MI LV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Coomassie blue stained gel showing qualitative similarities among the individual extracts (A), and densitometry of the stained lanes indicates equal loading (B).

Figure 2. Representative 2-DE gels of the wt and MMP-9 null post-MI groups (A), and of the candidate MMP-9 substrates identified (B), showing that 61% are classified as intracellular, 5% as membrane, and 34% as extracellular proteins.

Figure 3.

Putative MMP-9 Substrates. Immunoblot analysis of LV infarct homogenates for desmin (A), serpin 1d (B), tenascin-C (C), and thrombospondin-1 (D) matched the 2-DE analysis in molecular weight and intensity differences. Lower levels of desmin (92 kDa), full length serpin 1d (55 kDa) and full-length tenascin-C (277 kDa) were detected in the MMP-9 null, compared to wt levels. Thrombospondin-1 fragment (22 kDa) was found at higher levels in the MMP-9 null LV extracts.

Figure 4.

Proteins indirectly influenced by MMP-9 deletion. Immunoblot analysis of LV infarct extracts for BASP-1 (A), galectin-3 (B), and prohibitin-2 (C) yielded results that differed from the 2-DE analysis. Immunoblot intensity staining of the LV infarct homogenates using an anti-BASP-1 antibody demonstrated lower intensity in BASP-1 in the MMP-9 null; however, the protein band identified by the antibody corresponded to a 150 kDa vs a 55 kDa detected in the 2-DE gels. Immunoblots for galectin-3 and prohibitin-2 agreed with the molecular weight of the 2-DE analysis, but lower protein intensity was detected in the immunoblots, suggesting other proteins identified in the 2-DE spots contributed to the intensity differences found in the 2-DE gels.

Figure 5.

Fibronectin is a putative MMP-9 substrate. Spot 74 from the 2-DE gels indicated a significant lower intensity in fibronectin levels in the MMP-9 null LV extracts when compared to the wt (A and B). Mass spectrometry analysis of the spot, identified fibronectin as one of the proteins with lower intensity in the MMP-9 null LV infarct region (C). Immunoblot analysis of the LV infarct homogenates verified the lower intensity of fibronectin in the MMP-9 null at 166 kDa which agreed with the 2-DE analysis (D). In addition, the polyclonal antibody also indicated differences in full-length fibronectin (277 kDa) and fibronectin fragments (120 kDa and 70 kDa). A commassie blue stained gel indicates equal protein loading among the wt and MMP-9 null LV protein extracts (E), and the densitometry of the fibronectin full-length band and the fragments shows a significantly lower intensity of the fibronectin bands at the indicated molecular weights in the MMP-9 null LV infarct when compared with the wt ($p < 0.05$).

Table 1

Necropsy Data

	Wild Type (n=12)	MMP-9 null (n=10)
Age (months)	5.0±0.2	4.4±0.3
Weight (g)	28.7±0.7	28.1±0.7
LV mass (mg)	127±4	116±7
LV/BW (mg/g)	4.4±0.1	4.1±0.1
Lung mass (mg)	186±10	188±15
MI (%)	48±3	45±3

All parameters p=n.s.

Table 2

Candidate *in vivo* MMP-9 Substrates Identified

Spot #	Normalized Spot Volume		p	Protein Identified	Total spectra ^a	pI (Obsr.)	MW (kDa)	
	Wild-type	MMP-9 Null					Obsr.	Calc.
3	1748±194	3321±501	0.003			9.5	12	
				Fatty acid binding protein 4	23			15
				Hemoglobin	15			16
				Profilin 1	23			15
6	862±68	1417±172	0.005			9.4	34	
				Fibrinogen	13			61
				Prohibitin-2	26			34
				Voltage dependent anion channel (VDAC) 1 or 2	27/6			33
8	585±77	363±37	0.013			6.1	13	
				Fatty acid binding protein 5	18			15
24	956±134	660±62	0.040			4.3	43	
				Haptoglobin	4			39
				Retinolocalbin	7			38
25	1801±168	1252±66	0.005			6.7	20	
				Crystallin B chain, alpha	12			20
				Peroxisomal protein 1	9			22
				Transgelin	3			23
28	2533±266	3621±206	0.019			5.9	11	
				Fatty acid binding protein 3	25			15
				Hemoglobin	5			16
31	842±88	1196±152	0.045			9.5	34	
				Galectin-3 ^b	6			34
				Serine protease inhibitor	6			46
				VDAC 1 or 2	30/7			32
37	83±6	59±5	0.007			5.7	88	

Spot #	Normalized Spot Volume		p	Protein Identified	Total spectra ^a	pI (Obsr.)	MW (kDa)	
	Wild-type	MMP-9 Null					Obsr.	Calc.
				Fibrinogen	6			49
				Gelsolin	12			81
47	825±68	606±25	0.003	Annexin A1	24	6.6	38	
				Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)				39
					17			36
51	7513±724	10132±488	0.016			7.9	15	
				Myoglobin	72			17
55	1005±46	755±75	0.003			5.3	31	
				Annexin A4	21			36
				Apolipoprotein A-IV	4			45
				Apolipoprotein E	24			36
				Apolipoprotein J	4			52
				Cathepsin B	4			37
				Cathepsin D	4			45
				Cathepsin Z	6			34
				Chloride intracellular channel 1	11			27
				Collagen, type I, alpha 2 ^b	5			130
				Collagen, type III, alpha ^b	9			136
				Desmin ^b	3			92
				Pregnancy zone protein	3			166
				Prohibitin ^b	24			34
				Thrombospondin 1 ^b	7			170
59	944±53	719±32	0.002			6.9	38	
				Annexin A2	10			39
				GAPDH	19			36
61	627±48	478±47	0.045			5.6	63	

Spot #	Normalized Spot Volume		p	Protein Identified	Total spectra ^a	pI (Obsr.)	MW (kDa)	
	Wild-type	MMP-9 Null					Obsr.	Calc.
				Hemopexin	5			51
64	1681±118	1298±95	0.020			4.6	43	
				Desmin ^b	14			92
				Secreted protein acidic and rich in cysteine	7			34
				Vimentin	80			52
66	1467±65	1161±67	0.004			4.6	193	
				Laminin gamma 1	15			177
67	1170±66	929±39	0.005			4.0	43	
				Calumenin isoform 2	17			37
				Haptoglobin	8			39
69	452±29	361±14	0.010			5.7	51	
				Enolase	42			50
				Fibrinogen	8			55
70	255±18	320±26	0.031			5.6	22	
				Apolipoprotein A-1	15			31
				Heat shock protein beta 1	16			23
				Peroxiredoxin 1	5			22
				Peroxiredoxin 3	8			28
				Peroxiredoxin-6	9			25
				Rab11B	21			24
				Thrombospondin 1 ^b	6			170
71	169±17	211±11	0.049			7.4	53	
				Enolase, beta	36			47
73	256±16	208±10	0.025			6.2	64	
				Alpha-1-syntrophin	5			62
74	13245±683	10764±727	0.032			5.4	166	
				Fibronectin ^b	7			277
75	1477±84	1224±62	0.038			4.5	26	

Spot #	Normalized Spot Volume		p	Protein Identified	Total spectra ^a	pI (Obsr.)	MW (kDa)	
	Wild-type	MMP-9 Null					Obsr.	Calc.
78	328±12	275±13	0.005	14-3-3 protein zeta isoform	4	6.1	50	28
				Enolase, alpha	16			47
79	2175±75	1841±70	0.004	Thymidylate kinase family LPS-inducible member	3	4.7	97	50
				Fibrinogen	4			49
				Heat shock protein 90	6			92
				Valosin-containing protein	20			89
80	6566±314	7699±395	0.027			7.2	15	
				Myoglobin	68			17
81	2771±71	2376±87	0.002			4.7	92	
				Heat shock protein 1	4			85
				Heat shock protein 90	26			115
82	3915±156	3359±88	0.013			4.0	55	
				brain abundant, membrane attached specific protein 1	6			
				Calreticulin	32			150
				Serpinald protein	4			48
83	1953±87	1692±53	0.023			4.8	101	55
				Desmin ^b	13			92
				Heat shock protein 90	33			92
				Valosin-containing protein	52			89
84	4936±149	4296±303	0.038			5.1	277	
				Desmin ^b	3			92
				Fibronectin ^b	17			277
				Tenascin	4			277
85	319±11	283±9	0.021			6.9	34	
				VDAC	20			32

Spot #	Normalized Spot Volume		p	Protein Identified	Total spectra ^a	pI (Observed)	MW (kDa)	
	Wild-type	MMP-9 Null					Observed	Calculated
86	261±6	290±8	0.011			4.9	29	
				Adiponectin	5			27
				Annexin A5	6			36
				Apolipoprotein J	3			52
				Cathepsin B	22			37
				Cathepsin D	6			45
				Cathepsin Z	5			34
				Chloride intracellular channel 1	24			27
				Collagen a1(I) ^b	2			137
				14-3-3 epsilon isoform	3			29

^a total number of peptides assigned with a confidence level of ≥ 95%; all proteins listed were ≥ 99.9% confidence based on analysis by Scaffold; only proteins with at least two high-confidence assigned peptides are listed

^b examined further by immunoblotting