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Milk Fat Globule Protein-Epidermal Growth Factor-8: a Pivotal Relay Element within the Angiotensin II and Monocyte Chemoattractant Protein-1 Signaling Cascade Mediating Vascular Smooth Muscle Cells Invasion

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Abstract

Advancing age induces aortic wall thickening that results from the concerted effects of numerous signaling proteins, many of which have yet to be identified. To search for novel proteins associated with aortic wall thickening, we have performed a comprehensive quantitative proteomic study to analyze aortic proteins from young (8 mo) and old (30 mo) rats and identified 50 proteins that significantly change in abundance with aging. One novel protein, the milk fat globule protein

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epidermal growth factor 8 (MFG-E8), increases 2.3-fold in abundance in old aorta. Transcription and translation analysis demonstrated that aortic MFG-E8 mRNA and protein levels increase with aging in several mammalian species including humans. Dual immunolabeling shows that MFG-E8 colocalizes with both angiotensin II (Ang II) and monocyte chemoattractant protein-1 (MCP-1) within vascular smooth muscle cells (VSMCs) of the thickened aged aortic wall. Exposure of early passage VMSCs from young aorta to Ang II markedly increases MFG-E8 and enhances invasive capacity to levels observed in VSMCs from old rats. Treatment of VSMCs with MFG-E8 increases MCP-1 expression and VSMCs invasion that are inhibited by the MCP-1 receptor blocker, vCCI. Silencing MFG-E8 RNA substantially reduces MFG-E8 expression and VSMCs invasion capacity. The data indicate that arterial MFG-E8 significantly increases with aging and is a pivotal relay element within the Ang II/MCP-1/VSMC invasion signaling cascade. Thus, targeting of MFG-E8 within this signaling axis pathway is a potential novel therapy for the prevention and treatment of the age-associated vascular diseases such as atherosclerosis.

Keywords

MFG-E8; Angiotensin II; Monocyte chemoattractant protein-1; Vascular smooth muscle cells; Aging

Introduction

Proinflammatory processes and associated elevated invasion capacity of VSMCs are increased within the diffuse thickening of the arterial wall that evolves with advancing age.[1-4] In humans, this age-associated arterial remodeling is an independent risk factor for the epidemic of quintessential human cardiovascular diseases, i.e., atherosclerosis hypertension, and stroke.[1, 3, 5, 6] The age-associated arterial wall thickening and other aspects of arterial remodeling is evolutionarily conserved in various mammalian species, including rodents, nonhuman primates and humans.[1,3,7-14] The thickened arterial intima is formed due to VSMCs invasion and proliferation, and is not limited to secretion within the sub-endothelial space.[2, 3, 7-14] A growing body of evidence indicates that VSMCs within the arterial media begin to express and activate proteases such as matrix metalloprotease type II (MMP2) and calpain-1, which enable cytoskeletal remodeling, degradation of (1) basement membranes that surround VSMCs; (2) adjacent matrix; (3) elastic lamina. Thus VSMCs invasion into the sub-endothelial space is driven, at least in part by angiotensin II (Ang II).[7-14] VSMCs invasion is also facilitated by an intimal-medial concentration gradient of monocyte chemoattractant protein-1 (MCP-1), and platelet-derived growth factor-BB (PDGF-BB).[14] Transcription, translation, and activation of MMP2, calpain-1, MCP1, and PDGF-BB are linked to an age-associated increase in local arterial Ang II signaling via the AT1 receptor.[7-14]

However, numerous yet identified proteins that have crucial roles in the coordinated VSMCs invasion process likely change in abundance or become post-translationally-modified with aging. A complete understanding of mechanisms involved in the increased VSMCs invasive capacity with aging requires identification of the proteome changes within functional classes and characterization of interconnections between known pathways as well as indicating previously unknown key regulators. To this end, we have performed a comprehensive quantitative proteomic study using both two-dimensional gel electrophoresis (2DE)-based and mass spectrometry (MS)-based [isobaric tag for relative and absolute quantification (iTRAQ)] approaches to analyze aortic proteins obtained from young (8 mo) and old (30 mo) rats (schematic diagram of proteomic experimental design, Online Figure I). Furthermore, we employed 2DE in combination with Pro-Q Diamond Phosphoprotein Gel Stain and deglycosylation experiments to characterize selected post-translational modifications (PTMs) of proteins. Utilizing these approaches in combination with qRT-PCR, and Western blotting, immunostaining, and VSMCs functional assays, we have discovered (1) that at least 50 proteins

change abundance in the aorta associated with aging, (2) that MFG-E8 (aka lactadherin) markedly increases with aging, not only in rat aorta but also in non-human primate and human aorta, and (3) that MFG-E8 is a novel link between proinflammatory molecules Ang II and MCP-1 signaling and promotes the invasive capacity of VSMCs.

Materials and Methods

For details on the materials and methods used in the present study (including arterial specimens, isolation of aorta and preparation of aortic proteins, 2D silver stained and DIGE, in-gel tryptic digestion and peptide desalting, mass spectrometry analysis and protein identification, detection of phosphoproteins and glycoproteins, iTRAQ analysis, Ingenuity pathway analysis, real time PCR analysis, Western blotting analysis, immunohistochemistry and immunofluorence, VSMCs isolation and culture, MFG-E8 siRNA silencing, VSMCs invasion assay, and statistical analysis), please see the online data supplement, available at http://circres.ahajournals.org.

Results

Quantification and identification of the aortic proteome by 2DE and iTRAQ MS based analysis

Using 2DE (silver stained and DIGE gels), we have obtained 2-D gel maps of 286 identified non-redundant proteins from rat aorta and observed 18 proteins (Table 1) that significantly change abundance (±1.5-fold) with aging (annotated protein gel maps for young and old aorta are shown in Online Figures II & III; corresponding to identifications listed in Online Table 2). Using iTRAQ, 880 proteins were quantified and between both methods, 50 proteins were shown to have significantly different abundance associated with aging (±1.5-fold) (Online Table 3). The results of the two methods were complimentary, increasing the confidence of the results. Totally 923 non-redundant proteins were detected by two methods. The majority of the proteins detected by 2DE (243, 85%) are also quantified by iTRAQ. About 5% (50) of the proteins identified have significantly different abundance with aging (Table 1). Of the proteins found to differ with aging, 5 had PTMs. Acidic calponin 3 was found to be a phosphoprotein (Online Figure IV) while MFG-E8 (Figure 1A & B), alpha-1-inhibitor III (data not shown), kininogen 1 (data not shown), and periostin (Online Figures V & VI) were detected as N-linked glycoproteins.

Bioinformatic functional analysis of differentially abundant age-associated arterial proteins

To identify potential roles of differentially abundant proteins with aging, proteins were grouped into various functional classes based on Swiss-Prot protein function and gene ontology, and if needed, literature search (Table 1),[15,16] with proteins being clustered into a number of cellular pathways, including apoptosis/cell cycle/proliferation, cytoskeleton/invasion, extracellular matrix/cell adhesion, metabolism, etc (Table 1). Notably, the majority of those proteins were related to the aging process or to the Ang II signaling cascade in other organs i.e. prostate, skeletal muscle, skin etc. (see online supplemental discussion), but many had not been previously linked specifically to arterial aging.[7-14] Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com) shows that the primary pathway was cellular movement where 14 of these proteins could be linked together (Online Figure VIIA). The proteins include MFG-E8, apolipoprotein E, insulin-like growth factor binding protein 7, MMP-2, biglycan, calponin 1, clusterin, collagen type I alpha 1 chain, collagen type 1 alpha 2 chain, fibronectin (FN), periostin, serine protease HTRA1, TGF-β3 and vitronectin. The proteins increase in abundance in the old aorta. Together with Ang II, MCP1, NF-κb, PDGF and other molecules, the proteins form a network of cellular movement, a salient feature of invasion (Online Figure VIIA). Interestingly, MFG-E8, a protein that markedly increased 2.3-fold in abundance with aging, is shown to be directly linked to protein kinase AKT [17,18] and extracellular signal-regulated

kinase ½ (ERK1/2)[18] in this network (Online Figure VIIA). MFG-E8 is a multi-functional glycoprotein originally found in milk and mammary epithelial cells.[18-27] It is of particular interest because its internal fragment, known as medin, is an essential component of amyloid plaque deposition in aged human arteries.[25, 26] Its involvement in this cellular movement network indicates it may play an important role in regulating VSMCs invasive capability. Specifically, links between MFG-E8 and key molecules in vascular remodeling, such as Ang II and MCP-1, have not been established. We focused upon MFG-E8 to determine how it specifically relates to the cell invasion signaling axis.

Transcriptome, immunolocalization and post-translational modification analysis of MFG-E8

Using Quantitative Reverse Transcription PCR (qRT-PCR), MFG-E8 mRNA levels were increased 2.7-fold in older compared to younger rat aorta (Figure 2A, $p<0.05$). Western blotting analyses of 1DE (data not shown) and 2DE (Figure 2B) confirmed the increased abundance of MFG-E8 in older vs. younger aorta. In addition to changes in the abundance of MFG-E8 with aging, three arterial MFG-E8 spots with similar molecular weight (MW) were observed in the 2DE of the sample from old aorta (Figure 2B). Importantly, treatment of the tissue samples with PNGase F, which specifically removes N-linked carbohydrates, shifted the triplicate spots to a lower MW, as anticipated for PTM by deglycosylation (Figure 2D). The newly appeared spots were identified as MFG-E8 by mass spectrometry. Immunofluorescence staining demonstrated that the intensity of glycosylated MFG-E8 increases within the old compared to the young aortic wall, and that MFG-E8 staining colocalizes with that of alpha smooth muscle actin (α-SMA), a marker of VSMCs (Figure 2C).

Age-associated increase in MFG-E8 expression is conserved across species

To determine whether the age-associated increase in MFG-E8 expression is conserved across various mammalian species, aortic tissues from nonhuman primates and humans were utilized for Western blotting and immunostaining studies. In nonhuman primates, Western blot analysis shows that the abundance of aortic MFG-E8 increases ~9.0 fold with aging, with the molecular weight indicating it is glycosylated (Figure 3A). Immunolabelling analysis indicates that MFG-E8 staining (brown color) markedly increases in both the intima and the media aortic wall in older versus younger monkeys (Figure 3B). Similarly, in humans, Western blotting analysis and dual immunofluorescence staining of MFG-E8 indicate an age-associated increase ~6.5 fold of the glycosylated form of MFG-E8 (Figure 3C& D), predominantly located in the inner media and intimae and also colocalized with α-SMA, as in the rat and monkey (Figures 3C& 3B).

Exogenous administration of angiotension II to young VSMCs mimics aging by elevating MFG-E8 production

Although bioinformatic functional analysis indicates that MFG-E8 is involved in a signaling network of cell movement (Online Figure VIIA), it fails to place it specifically downstream of the Ang II signaling cascade, a central feature of arterial aging.[7-14] Dual immunofluorescence demonstrates that both Ang II and MFG-E8 expression within VSMCs are closely associated and that both markedly increase within the rat aortic wall, particularly in the thickened aged intima (Figure 4A). Its negative control is provided in Online Figure VIII.

We used early passage VSMCs to detect a potential functional link between Ang II and MFG-E8. Both glycosylated (upper bands) and unglycosylated forms (lower bands) of MFG-E8 protein (Figure 4B, top) were detected, and both increased with aging in early passage VSMCs (Figure 4B, bottom).[27] Treatment of young VSMCs with Ang II induced MFG-E8 production, predominantly in the native form (unglycosylated) in a dose-dependent manner, up to the levels of old untreated VSMCs (Figure 4B, bottom). Importantly, elevated MFG-E8, including glycosylated and unglycosylated forms, in old cells is significantly reduced (~40%) by

treatment with [Sar1, Gly8]-Angiotensin II acetate hydrate (SG), an AT-1 receptor blocker (Online Figure IX). This novel finding indicates that MFG-E8 is a downstream molecule of Ang II initiated signaling.

MFG-E8 stimulates functional MCP-1 production in aging VSMCs and consequently enhanced invasion

Prior studies indicate that chronic infusion of Ang II to young rats increases MCP-1 expression; and that the acute exposure of early passage VSMCs from young rat aortae potentiates their invasive capacity, in part, via enhanced MCP-1 expression.[10, 28-29] We probed for a relationship between MFG-E8 and MCP-1. Dual immunohistostaining of old aotae demonstrated that MFG-E8 colocalization with MCP-1, preferentially in the thickened intima (Figure 5A, and in Online Figure X) and immunocytostaining of old VSMC indicates that MFG-E8 also colocalizes with MCP-1, preferentially in the perinuclear region (Figure 5B). MFG-E8 treatment of early passage VSMCs increased the functional dimer product of MCP-1 (Figure 5C).[10, 30] Conversely, MFG-E8 silencing (>70%) substantially reduces MCP-1 expression and its dimerization within VSMCs, but does not effect MCP-1 mRNA levels (Online Figure XIA, B). Furthermore, the Ang II-induced increase in MCP-1 expression is markedly inhibited by MFG-E8 silencing (Online Figure XIC). Notably, the results in Figures 5 and 6 suggest that Ang II is upstream of MFG-E8, and that MCP-1 is downstream of MFG-E8 within the Ang II signaling cascade that potentiates VSMCs invasion. These data establish novel interactions among Ang II, MCP-1 and MFG-E8 (Online Figure VIIB).

Next, we determined whether MFG-E8 is required for enhanced VSMCs invasion, and whether it acts in this respect via MCP-1. Boyden chamber analysis (Figure 6A, left panel) shows that, in control, VSMCs invasive capability significantly was increased in old compared to young cells. MFG-E8 siRNA successfully knocked down MFG-E8 protein expression in both young and old VSMCs (Figure 6A, right panel), and reduced the invasive capability of both young and old VSMCs, eliminating age differences in invasive capacity that were present in control (Figure 6A, left panel). Exposure of VSMCs to Ang II increased the invasive capability of VSMCs in an age-dependent fashion, and this increase was substantially reduced by MFG-E8 silencing (Figure 6B). The addition of exogenous MFG-E8 also increaseed VSMCs invasive capability in an age-dependent fashion (Figure 6C), and these effects were substantially reduced by treatment with a specific MCP-1 receptor blocker, vCCI (Figure 6C).

Discussion

In this study, we present for the first time a proteome profile of arterial aging in FXBN rats, a well-characterized rodent model with similarities to the human anatomic and physiologic phenotype. Using a combination of 2DE and iTRAQ, we identified and characterized 923 proteins within the aortic wall, of which 50 significantly change abundance with aging, some of which also have PTMs such as phosphorylation and glycosylation. The proteins with significant abundance changes (Table 1) observed in this study play significant roles in a number of cellular pathways including cellular movement, cell proliferation and apoptosis, energy metabolism etc, and may contribute significantly to vascular remodeling with aging. Some of these proteins have been related to aging in other organs i.e. prostate, skeletal muscle, and skin etc (for expanded discussion of the proteins, see online supplements). Only a few proteins have been specifically linked to arterial aging, such as MMP2, or TGF-β.[7-14] These proteins, including Ang II, MCP-1, MFG-E8, MMP2, and TGF-β etc, are involved in a network of cellular movement (Online Figure VIIA).

Furthermore, the present results establish molecular links between Ang II, MFG-E8, and MCP-1 in aged VSMCs. It has been previously established that Ang II and AT1 expression and signaling increase within the arterial wall with aging.[3, 8, 9, 12, 14] Exogenously infused

Ang II to young animals produces the structural molecules of the arterial wall resembling old untreated animals,[9, 12] while chronic blockade of Ang II signaling in animals reverses and retards these age-associated structural molecular changes.[31-33] Ang II can promote expression of MCP-1,[10, 28-29] a potent chemokine that is an element of the AKT-associated proinflamatory network and plays an important role in chemotaxis and atherosclerosis.[34] Interestingly, MFG-E8 stood out in our proteomic analyses as it had not previously been linked directly with AngII/MCP-1, VSMCs or aging although it has been shown to induce cell specific apoptosis, metastasis and angiogenesis.[18-24] Previous studies have shown that MFG-E8 is abundantly expressed in metabolically active tissues, i.e. atherosclerotic plaques [25-26,35] and tumors[24]. We have shown that MFG-E8 is N-linked glycosylated and increases in abundance in aortae of old vs young rats (Table 1). This age-related increase in transcription and translation of MFG-E8 is preserved across species (rat, nonhuman primates, human), suggesting it may have a central role in arterial aging. As such we investigated the interplay between MFG-E8 and Ang II, and for the first time, we uncovered the important finding that Ang II induces MFG-E8 in VSMCs isolated from rat aortae. In fact, MFG-E8 is required for the Ang II to increase MCP-1. Ang II was previously shown to co-express with MCP-1.[10, 28-29] Furthermore, we show that Ang II or MCP-1 also colocalizes with MFG-E8 in the arterial wall, particularly in the aged thickened intimae. These finding suggest that MCP-1 not only receives Ang II signals, but also that this cellular information is relayed via MFG-E8. Of note, treatment of isolated VSMCs with MFG-E8 induces the biologically active dimer of MCP-1 in a dose dependent manner. This action of MFG-E8 is profound, since the active MCP-1 homodimer is a potent chemoattractant of smooth muscle cells, if produced locally.[30]

In addition, MFG-E8 enhanced the Ang II induced invasion potential of the isolated VSMCs (Figure 6). We observed that the invasiveness of old VSMCs is greater than young cells, and that this effect depends upon Ang II signaling (Figure 6). However, silencing MFG-E8, a downstream molecule of Ang II signaling similarly reduces the invasive capacity of both young and old cells; while exogenous MFG-E8 treatment of young VSMCs increases their invasiveness, up to the levels of old untreated cells. Interestingly, a CCR2 blocker, vCCI, can interrupt MFG-E8/MCP-1 signaling, substantially inhibiting this effect. Thus, MFG-E8 is a novel link between AngII/MCP-1 signaling and VSMCs invasive capacity within the aortic wall.

Taken together, our study demonstrates a comprehensive non-biased proteomic approach to better understand the biology of age-associated thickening of the aorta and has identified a novel AngII/MFG-E8/MCP-1signaling axis (Online Figure VIIB) that links the cellular movement signaling cascades with the age associated increase in smooth muscle cell invasive capability. The abundance changes of other proteins observed in this proteomic study suggest that coordinated actions occur through this signaling axis. Notably, recent findings show that MFG-E8 is expressed in advanced human atherosclerotic plaques and MGF-E8 deficiency results in the accumulation of apoptotic cells and accelerates atherosclerosis in mice[36]. Previously, we have performed immunostaining for inflammatory cells, including monocyte/macrophage and lymphocyte markers, and assessed apoptosis by Tunnel staining in the vasculature of young and old animals. Results have shown that these cells are not detected in both the intima and the media, rarely observed in the adventia.[11] Obviously the regulation of these proteins is complex, however based on the evidence above and that provided in this manuscript, it is likely that the accumulation of MFG-E8 in the arterial wall plays a critical role in arterial aging. Our data show MFG-E8 accumulates in the aorta with aging and promotes VSMCs invasion, an important step in vascular thickening and arthrosclerosis. Further studies will show whether MFG-E8 can be a potential molecular target for the intervention to prevent / retard human arterial aging, arthrosclerosis and other vascular diseases which involve inflammation and smooth muscle cells invasion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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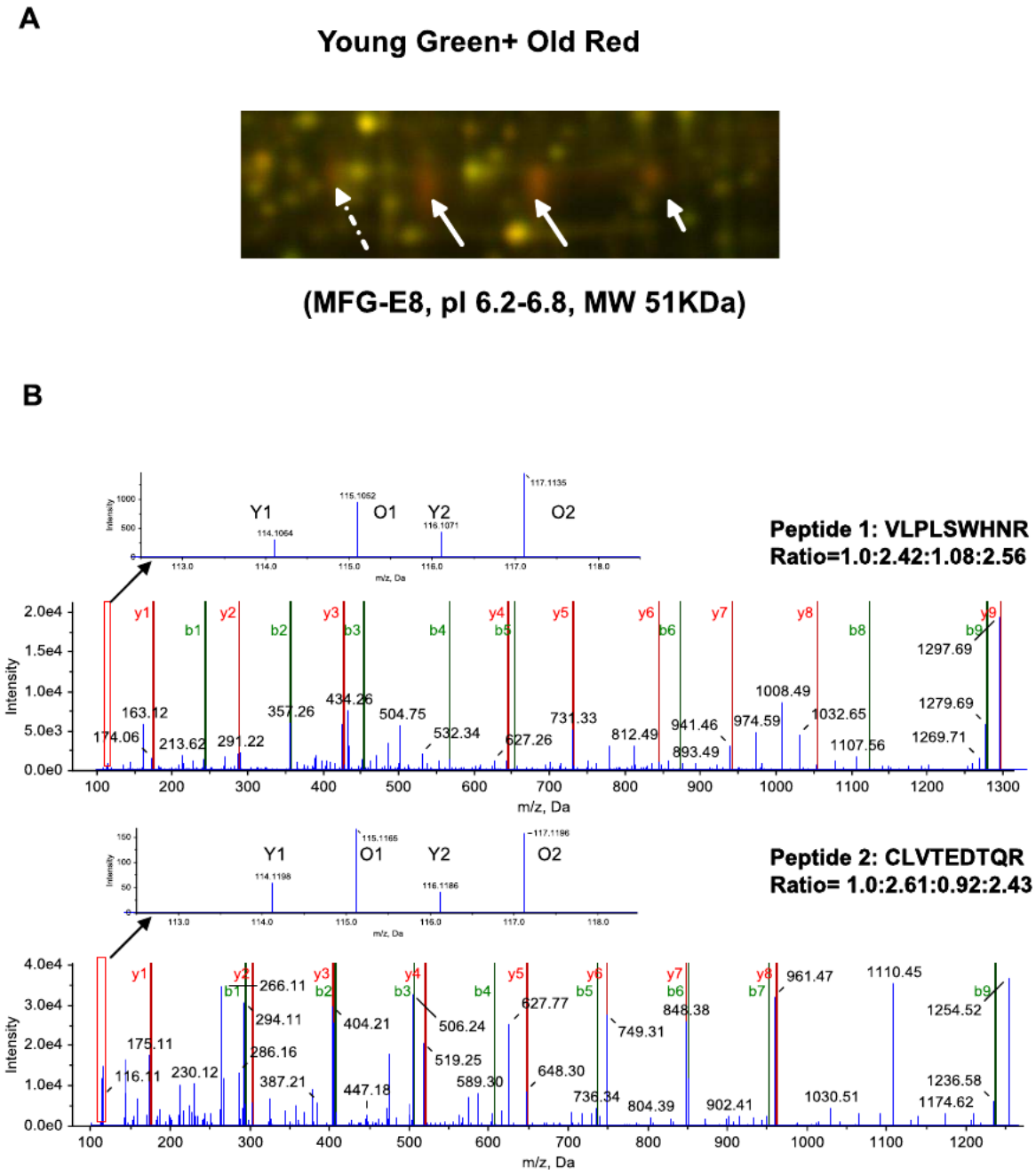

Figure 1. Quantification and characterization of MFG-E8 by 2DIDE and iTRAQ in aging rat aorta

A, Enlarged region of 2D DIGE gel map showing that MFG-E8 is more abundant in aged aorta than young adult samples (2.53±0.15, n=8). Three solid white arrows point to gel spots identified as MFG-E8. Identifications were done using LC MS/MS. **B**, iTRAQ MS spectra showing that MFG-E8 is more abundant in aged than young adult aorta. Representative MS spectra of two peptides VLPLSWHNR (409-417) and CLVTEDTQR (79-87) indicate the abundance of MFG-E8 is ~2.5 fold increased.

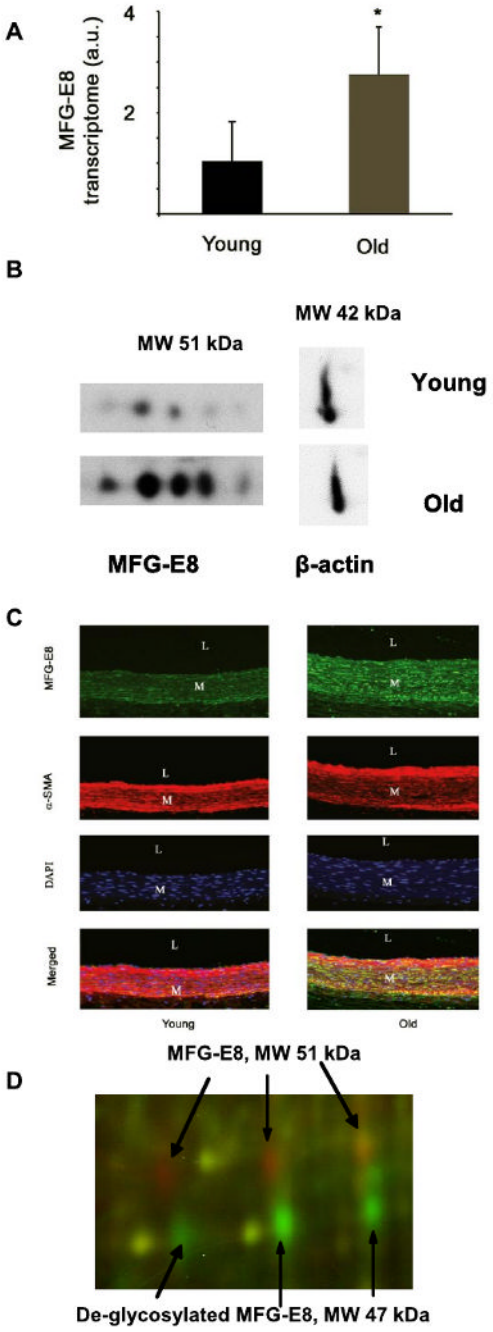

Figure 2. Validation and location of the MFG-E8 expression within the rat aortic wall

A, Average MFG-E8 transcriptome (n=4). **B**, 2DE western blot of MFG-E8. **C**, Immunofluorescence staining for MFG-E8 (green) and a-SMA (red). Counterstaining nuclei with a DAPI dye (blue). Merged image shows staining overlap (yellow) (ordinal magnification ×200) **D**, Enlarged region of DIGE 2DE showing PNGase F-treated MFG-E8 compared to an untreated endogenous sample (red) from the old rat aorta. PNGase F treatment (green) results in the removal of N-linked carbohydrates, shifting the spot position to a lower molecular weight.

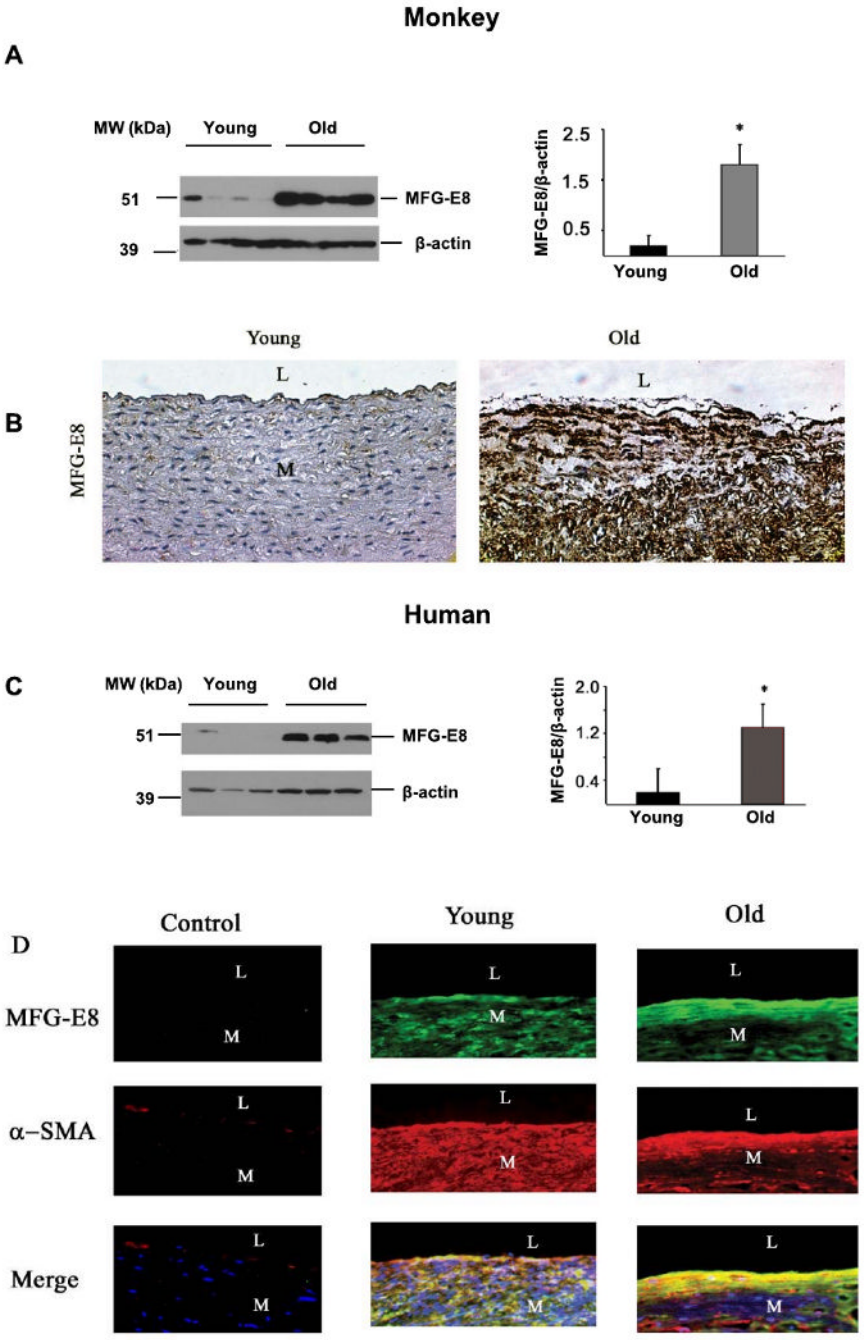

Figure 3. The age-associated arterial MGF-E8 increase is conserved in mammalian species including humans

A, Representative Western blots of monkey aortic MFG-E8 (left panel) and average data (right panel, n = 4). *p<0.05, old versus young. **B**, Immunohistostaining for MFG-E8 (brown color) within the monkey thoracic aortic wall (original magnification ×100). L=lumen; M=media. **C**, Representative Western blots of human aortic MFG-E8 (left panel) and average data (right panel, n = 5). *p <0.05, old versus young. **D**, Double immunofluorescence staining for MFG-E8 (green) and a-SMA (red) and merged image (yellow) within the human thoracic aortic wall (original magnification ×100). L=lumen; M=media.

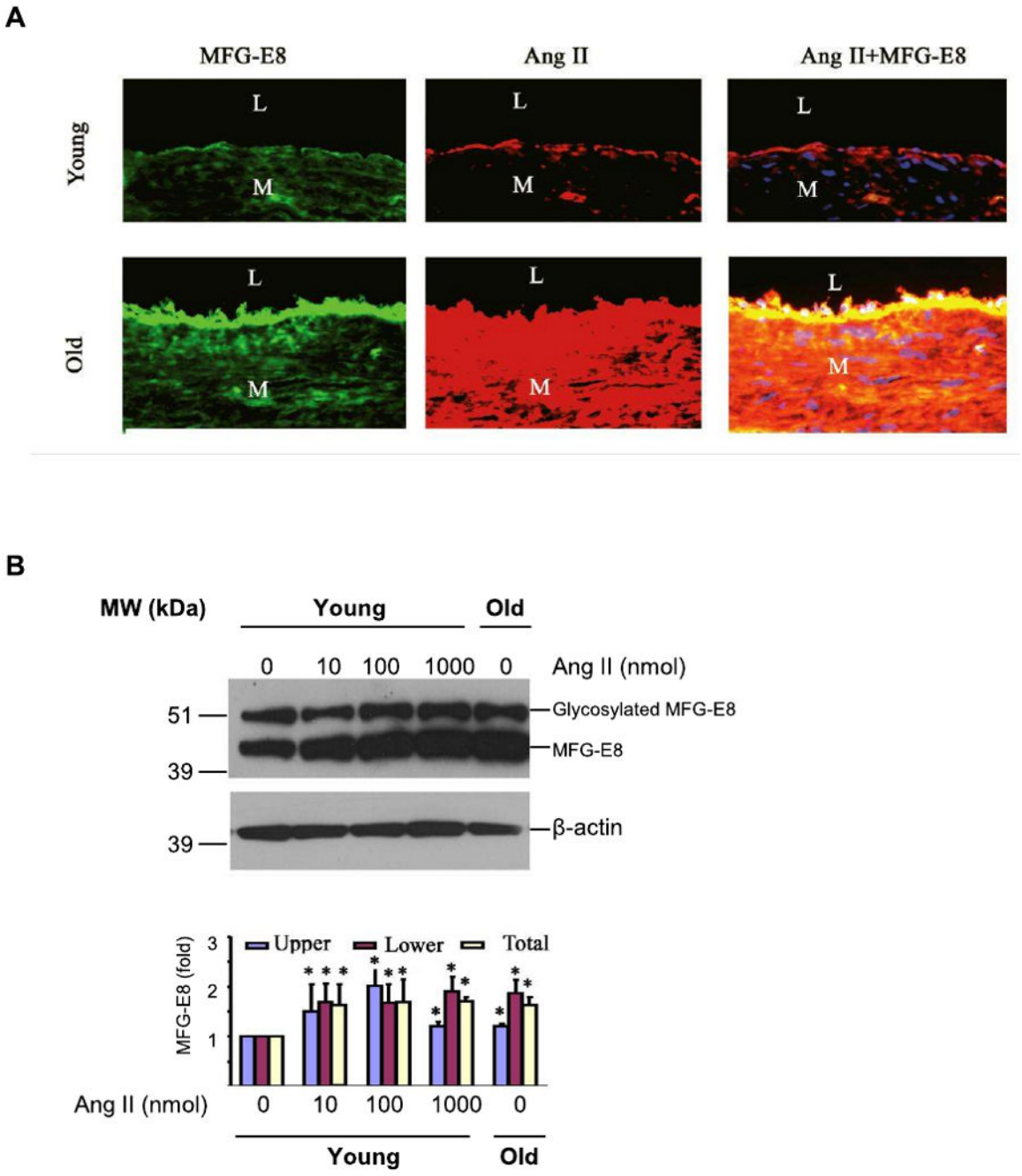

Figure 4. Interplay between Ang II and MFG-E8 in vivo and in vitro

A, Immunofluorescence staining for MFG-E8 (green) and Ang II (red) and merged image (yellow) in rat aortic wall. Nuclei counterstaining with DAPI(Blue). (original magnification × 400). L=lumen; M=media. **B**, Representative 1DE western blots of MFG-E8 in smooth muscle cells isolated from young or old rat aorta with or without Ang II treatment with indicated concentration levels (Upper panels). The MFG-E8 immunoreactivity doublet is the N-linked glycosylated form (upper bands) and unglycosylated (lower bands) as shown by deglycosylation of the sample with PNGase F resulting in enhancement of the lower MW band. Average data (lower panel). *p<0.05 old untreated and young treated cells vs. young control cells.

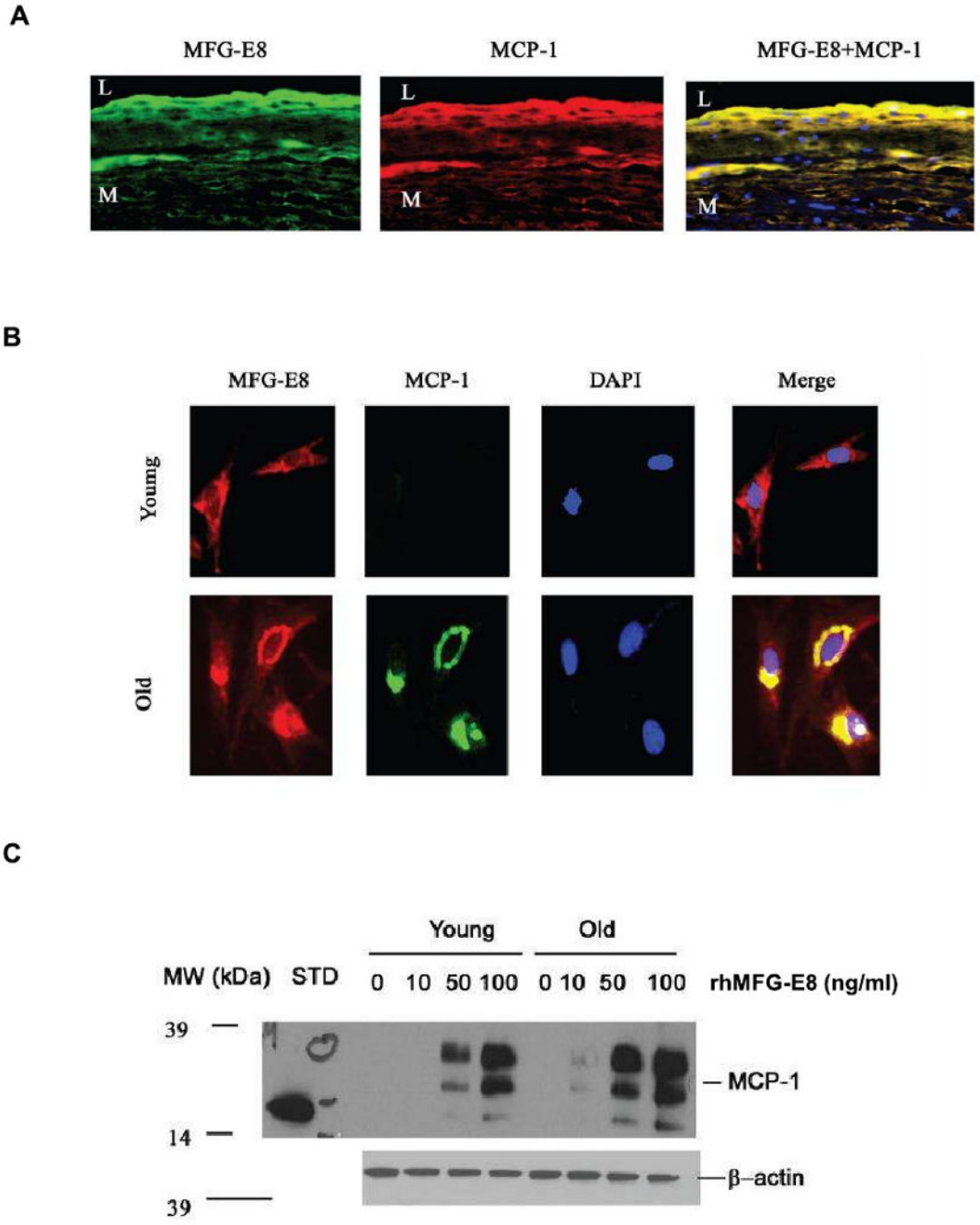

Figure 5. Interaction between MFG-E8 and MCP-1 in vivo and in vitro

A, Immunofluorescence staining for MFG-E8 (green) and MCP-1 (red) and merged image (yellow) in old human aorta. Nuclei counterstaining with DAPI (blue) (original magnification ×200). L=lumen; M=media. B, immunofluencence photomicrographs (×400) for MFG-E8 (red color), MCP-1 (green color), nuclei (blue color), and co localization of MFG-E8 and MCP-1 (yellow color, right panels). **C**, Representative Western blots show that the functional dimer of MCP-1 increases in VSMC treated with MFG-E8 in both young and old cells in a dose-dependent manner.

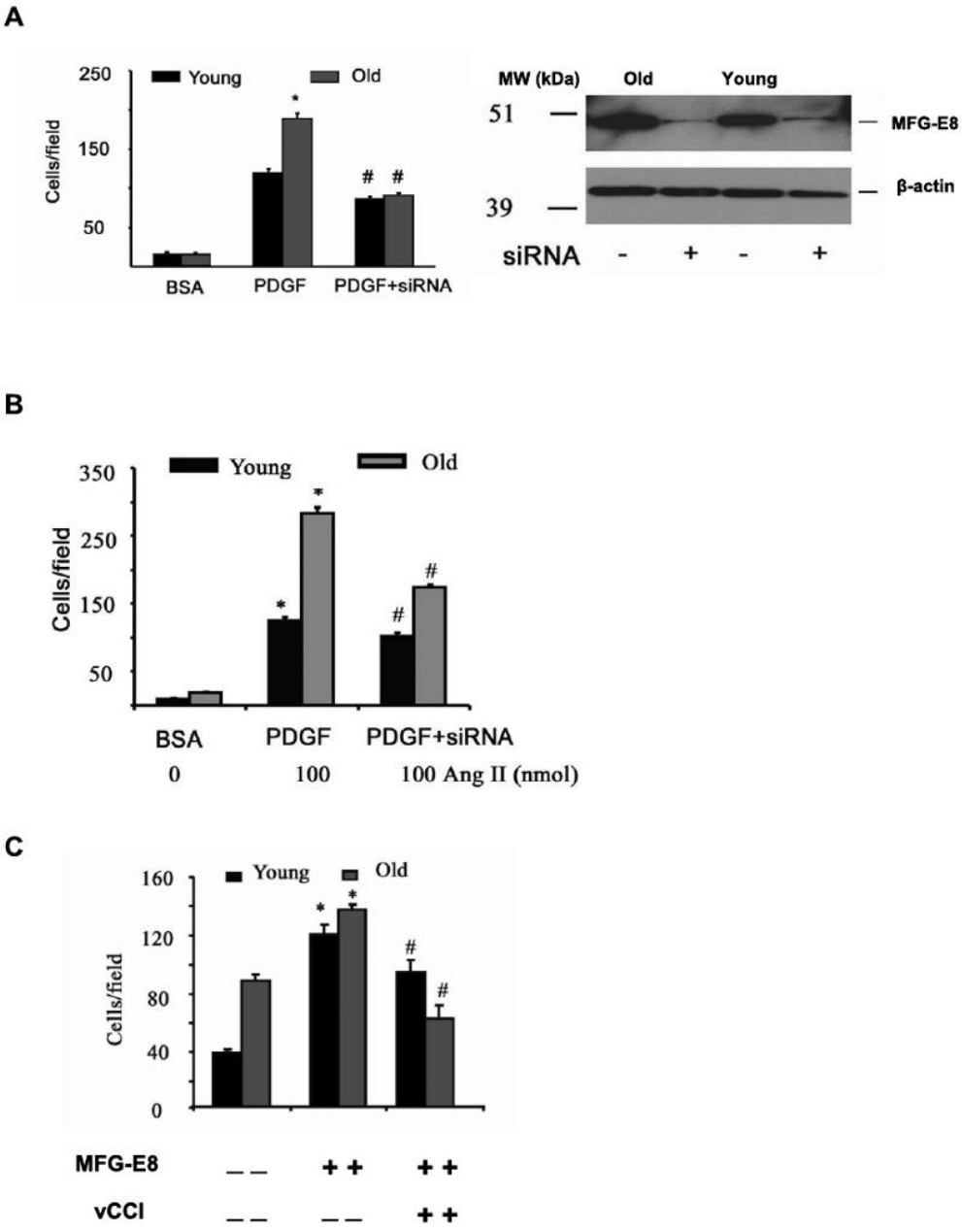

Figure 6. VSMCs invasion assay

A, Average invasion analysis of VSMC with and without siRNA of MFG-E8 (left panel).*p<0.05, old versus young cells chemoattracted by PDGF (10ng/ml); #p<0.05, young and old cells with MFG-E8 silencing versus sham silencing, respectively. Representatve Western blots show that the over majority of MFG-E8 protein is knocked down via siRNA approach (right panel). **B**, Average invasion analysis of VSMC with and without siRNA of MFG-E8 and Ang II treatment. *p<0.05, old versus young cells with treated with Ang II (100 nmol) and chemoattracted by PDGF(10 ng/ml); # p<0.05, young and old cells with MFG-E8 silencing versus sham silencing, respectively. **C**, Average invasion analysis of VSMC treated with and without MFG-E8 plus vCCI. *p<0.05 young and old cells treated with MFG-E8 (50ng/

ml) versus untreated controls respectively; #p<0.05, young and old cells treated with MFG-E8 plus VCCi versus MFG-E8 treated, respectively.

Table 1

Differentially abundant proteins identified by 2-D DIGE and iTRAQ

Protein class	Protein name	Accession[1]	iTRAQ[2] O/Y	DIGE[3] O/Y	PTM[4]
Apoptosis/cell cycle/proliferation					
	MFG-E8	MFGM_RAT	2.31±0.21	2.53±0.15	Glycosylation
	Serine protease HTRA1	HTRA1_MOUSE	2.03±0.17	ND	
	Integral membrane protein 2B	ITM2B_RAT	2.28±0.63	ND	
	Cysteine and glycine-rich protein 2	CSRP2_RAT	1.56±0.08	ND	
	Platelet factor 4 (CXCL4)	PLF4_RAT	3.09±1.5	ND	
	Insulin-like growth factor-binding protein 7 (IGFBP-7)	IBP7_MOUSE	1.40±0.2	ND	
Cell Metabolism					
	Creatine kinase M-type	KCRM_RAT	0.58±0.02	ND	
	Glycerol 3-Pdehydrogenase 1	GPDA_RAT	0.58±0.10	0.52±0.08	
	Cytochrome c oxidase subunit 5B	COX5B_RAT	0.66±0.02	ND	
	Glutamine synthetase	GLNA_RAT	0.58±0.10	ND	
	2-oxoglutarate dehydrogenase E1 component	ODO1_RAT	0.67±0.11	ND	
	2,4-dienoyl-CoA reductase	DECR_RAT	0.63±0.06	ND	
	3-ketoacyl-CoA thiolase	THIM_RAT	0.66±0.07	0.60±0.07	
	Hydroxyacyl-coenzyme A dehydrogenase	HCDH_RAT	0.61±0.12	ND	
	Trifunctional enzyme subunit beta	ECHA_RAT	0.66±0.07	ND	
	Long-chain-fatty-acid--CoA ligase 1	ACSL1_RAT	0.63±0.12	0.62±0.07	
	Isocitrate dehydrogenase [NADP]	IDHP_RAT	0.75±0.05	0.65±0.05	
	Pyruvate carboxylase	PYC_RAT	0.93±0.02	0.56±0.07	
Cytoskeleton/invasion/migration					
	RalA-binding protein 1	RBP1_RAT	1.49±0.02	ND	
	Actin-related protein 2/3 complex subunit 4	ARPC4_MOUSE	1.54±0.07	ND	
	STE20-like serine/threonine-protein kinase	SLK_RAT	0.59±0.03	ND	
	Calponin-1	CNN1_RAT	1.42±0.06	1.69±0.06	
	Calponin-3	CNN3_RAT	1.45±0.07	1.75±0.06	Phosphorylation
Extracellular matrix/cell adhension					
	Transforming growth factor beta-3	TGFB3_RAT	2.22±0.21	ND	
	Clusterin	CLUS_RAT	1.89±0.26	ND	
	72 kDa type IV collagenase (MMP-2)	MMP2_RAT	1.40±0.11	ND	
	Biglycan	PGS1_RAT	1.56±0.06	ND	
	Collagen alpha-1(I) chain [5]	CO1A1_RAT	0.39±0.11	0.35±0.05	
	Collagen alpha-2(I) chain [5]	CO1A2_RAT	0.54±0.14	0.45±0.06	
	Fibronectin 1	FINC_RAT	1.68±0.10	2.1±0.19	
	SPARC-like protein 1	SPRL1_RAT	1.47±0.14	ND	
	Vitronectin	VTNC_MOUSE	1.91±0.09	ND	
	Apolipoprotein A-I	APOA1_RAT	0.68±0.13	0.60±0.04	
	Kininogen 1	KNT1_RAT	1.67±0.22	1.86±0.19	Glycosylation
	Periostin (OSF-2)	POSTN_MOUSE	2.43±0.13	2.46±0.19	Glycosylation
	Apolipoprotein E	APOE _RAT	1.55±0.29	ND	
	Fetuin-A Alpha-2-HS-glycoprotein	FETUA_RAT	0.61±0.20	ND	
	Fetuin-B	FETUB_RAT	0.66±0.05	ND	
Miscellaneous					
	Adenylate cyclase type 5	ADCY5_RAT	1.54±0.08	ND	
	Alpha-1-antiproteinase	A1AT_RAT	0.66±0.09	0.58±0.09	
	Alpha-1-inhibitor III	A1I3_RAT	0.67±0.14	0.60±0.08	Glycosylation
	Carbonic anhydrase 3	CAH3_RAT	0.63±0.23	ND	
	carboxylesterase 1	EST2_RAT	0.49±0.03	ND	
	Contrapsin-like protease inhibitor 1	SPA3K_RAT	0.66±0.05	ND	
	Contrapsin-like protease inhibitor 3	SPA3L_RAT	0.62±0.10	ND	
	Ferritin light chain	FRIL1_RAT	1.56±0.12	1.91±0.09	
	Major urinary protein	MUP_RAT	0.46±0.04	ND	
	Solute carrier family 2, facilitated glucose transporter member 4	GTR4_RAT	2.01±0.02	ND	
	Transthyretin	TTHY_RAT	0.59±0.18	0.63±0.12	
	Tripartite motif-containing protein 47	TRI47_MOUSE	1.97±0.09	ND	

[1] Accession is from SwissProt data base

[2] Changes in abundance with p value less than 0.05 and over 1.5 fold on 4 animals in each group using iTRAQ; ND not detected by this method

[3] Changes in abundance with p value less than 0.05 and over 1.5 fold on 8 animals in each group using 2DE;, ND not detected by this method

[4] Potential PTM based on PNGase F and phospho-staining experiments (see online supplements)

[5] Soluble form of collagen decreased where as the insoluble form that is known to increase with aging will not be able to get observed by 2DE or iTRAQ