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Proteomic Profiling of Aging in Glomeruli of Mice by using Two-Dimensional Differential Gel Electrophoresis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Glomerular proteins were analyzed by proteomics to screen proteins participating in maturation of glomeruli before senescence and to find key proteins involved in the aging process.

Material/Methods: Glomeruli of C57BL/6 mice at 8 and 20 weeks were separated by kidney perfusion. Proteomic profiles of glomeruli were investigated by using two-dimensional differential gel electrophoresis and MALDI-TOF mass spectrometry.

Results: We identified 22 differentially expressed proteins. Among them, 3 proteins were significantly up-regulated and 19 proteins were significantly down-regulated in mature mice. Out of these 22 proteins, 18% take part in protein transport, protein targeting, and proteolysis; 5% in glycolysis; 14% in transcription; 9% in electron transport; 9% were chaperones; and 9% were hydrolases.

Conclusions: Our results provide insights into the glomerular differentially expressed proteins correlated with renal aging. In this study we found that aging altered the expression of ATP synthase subunit beta. Further studies on this protein might help to understand the mechanism of renal aging.

MeSH Keywords: Aging • Kidney Glomerulus • Proteomics • Two-Dimensional Difference Gel Electrophoresis

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Background

Aging is a complex phenomenon. Aging refers to time-dependent change – including development and maturation – and aging becomes senescence when it produces impairment [1]. The kidney also participates in aging, and the changes in the kidneys with aging are the most dramatic of any organ; these pronounced changes have been identified to be both structural and functional [2,3]. It is known that during kidney development, the definitive kidney, or metanephros, develops from the primary nephric duct [4], and nephrons increase both in number and size during early postnatal life. To meet the increased metabolic demands of the stage of rapid growth, nephrons grow larger in a benign process from birth to adolescence [5]. After the age of 40 in humans (mature adults), there is an increase in the percentage of glomeruli showing sclerosis, concomitant with a reduction in the number of viable glomeruli [6]. This age-dependent loss of functional nephrons is a slow process at a time when renal function remains adequate for survival. However, the loss of 'renal reserve' renders the aged kidney susceptible to failure when other injuries are superimposed [7]. Thus, the physiological status of nephrons at mature age is correlated with risk for kidney disease [8].

The physiological mechanisms of renal maturation and lack of renal reserve, which eventually leads to senescence, remain largely unknown. To understand the molecular basis of benign development of glomeruli, and to investigate proteins participating in the pre- and post-maturation periods, we analyzed the expression of glomerular proteins in C57BL/6 mice, between young (8 weeks) and mature (20 weeks) stages, which closely represents the benign development of human kidneys (Figure 1). By using the Dynabeads perfusion developed by Takemoto et al. [9], we have successfully isolated glomeruli with high purity [10]. By using two-dimensional differential gel electrophoresis (2D-DIGE), glomerular proteins were separated. With the aid of DeCyder™ 2-D Differential Analysis software, significantly differential protein spots due to aging were obtained. Subsequently, differential protein spots have been identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Material and Methods

Animals

Male C57BL/6 mice (8 weeks of age) were purchased from the Laboratory Animal Center, China Medical University. All mice were housed at the Laboratory Animal Center SPF rodent housing facility at China Medical University according to the Chinese National Standard (GB14925-2001). All experiments were approved by a local committee for ethics in animal research.

Reagents

Collagenase A was purchased from Roche (Roche Diagnostics, Germany). The 2-D Clean-Up Kit and Ettan TM 2-D Quant Kit were purchased from GE (GE Healthcare, USA). Dynabeads M-450 Tosylactivated and a magnetic particle concentrator were purchased from Dynal (Dynal A.S., Norway). Cell strainers (100 μm) were purchased from BD (BD, USA).

Immobilized non-linear pH gradient strips, Cyanine dyes Cy2, Cy3, Cy5, and Amersham™ Deep purple total protein stain were purchased from GE. Mass spectrometry grade Trypsin Gold was purchased from Promega (Promega, USA).

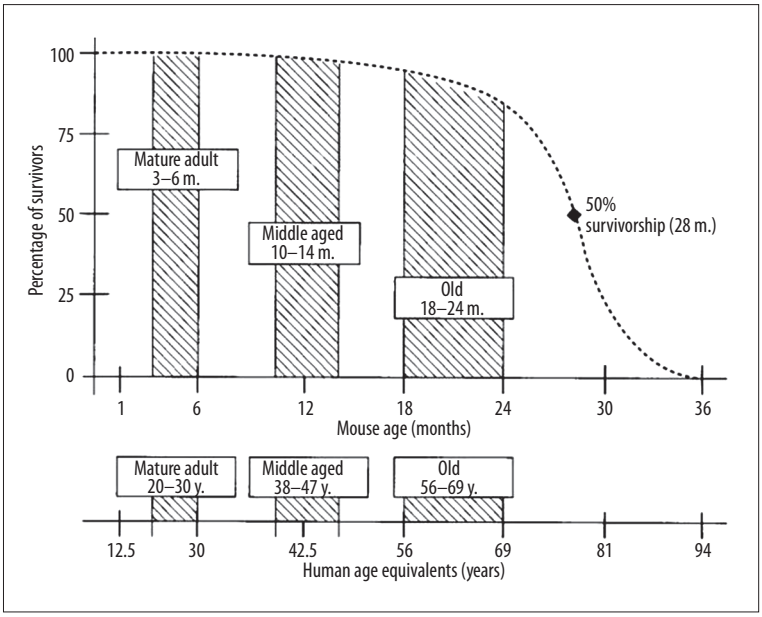

Figure 1. Representative age ranges for mature life history stages in C57BL/6J mice. (Fox JG, Barthold SW, Davisson MT et al.: The mouse in biomedical research, 2nd edition: Diseases, Elsevier, 2007; p. 645)

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Table 1. 2D-DIGE experimental scheme.

Gels	Analytic proteins (150 μg/gel)			Preparative proteins (600 μg/gel)
	Cy3	Cy5	Cy2	
1	C8-1	C20-1	Internal standard	Mixture of all glomerular protein samples
2	C20-2	C8-2	Internal standard	Mixture of all glomerular protein samples
3	C8-3	C20-3	Internal standard	Mixture of all glomerular protein samples

First step, for example, in gel 1, soluble protein samples from the glomeruli of 8- and 20-week C57BL/6 mice were labeled with either Cy3 or Cy5, and an internal standard (the equivalent mixture of all glomerular protein samples) was labeled with Cy2 on the same 2-D gel. Second step, Analytic proteins and preparative proteins were pooled for each gel for next 2-dimensional differential gel electrophoresis. "C8" represents 8-week C57BL/6 mice, and "C20" represents 20-week C57BL/6 mice.

Isolation of mouse glomeruli and sample preparation

Glomeruli of mice were isolated from the 8- and 20-week old C57BL/6 mice, with 25 cases (50 kidneys) in each age group. Briefly, kidneys were perfused with ice-cold PBS via abdominal or thoracic aorta to remove any remaining blood from the blood vessels. Next, Dynabeads in a concentration of 4×10^6/ml PBS were perfused into the kidney at a constant rate of 7.4 ml/min/g kidney. Kidneys were removed, minced into pieces, and digested in collagenase A (1 mg/ml) for 30 min at 37°C with gentle agitation. The digested tissue was pressed through a 100-μm cell strainer, followed by intermittent flushing using ice-cold PBS. The filtered suspension was centrifuged at 200×g for 5 min under a stable temperature of 4°C. After the supernatant was discarded, the pellet was dissolved in 2 ml of PBS, and transferred to a 2-ml tube. Glomeruli that contained Dynabeads were separated from renal tubules by a magnetic particle concentrator. To remove the Dynabeads, the extracted glomeruli were lysed in a 2-DE lysis buffer (7M urea, 2M thiourea, 4% [w/v] CHAPS, 2% [v/v] immobilized pH gradient [IPG] buffer, 40 mM dithiothreitol [DTT]), and sonicated (30 Hz, 4×5 s pulses on ice). The lysates were subsequently centrifuged at 12500×g for 10 min at a temperature of 4°C. The glomeruli protein was purified according to the instructions of the 2-D Clean-Up Kit, and protein concentration was determined using the Ettan TM 2-D Quant Kit. The samples were stored at –70°C. With the exception of the collagenase digestion, the entire procedure was performed on ice.

Fluorescence labeling (minimal labeling) with CyDyes

Protein samples were labeled with CyDyes (dissolved in N,N-dimethylformamide) according to the manufacturer's instructions (GE Healthcare). Typically, 50 μg each of soluble protein samples from glomeruli of 8-week C57BL/6 mice and 20-week C57BL/6 mice were labeled with 400 pmol of either Cy3 or Cy5, respectively. An internal standard (50 μg), which is the equivalent mixture of all glomerular protein samples included in the study, was simultaneously labeled with 400 pmol of Cy2 on the same 2-D gel (Table 1). Labeling reactions were performed on ice in the dark for 30 min. Subsequently, the reaction was quenched with 1 μl of 10 mM lysine for 10 min under the same conditions.

Two-dimensional differential gel electrophoresis (2D-DIGE)

Cy3-, Cy5-, and Cy2-labeled samples (total 150 μg) and preparative samples for later protein identification (600 μg) were pooled (Table 1). An equal volume of 2×sample buffer (7 M urea, 2 M thiourea, 4%[w/v] CHAPS, 2%[v/v] IPG buffer, 40 mM DTT) and 1×hydrated fluid (7 M urea, 2 M thiourea, 2% [w/v] CHAPS, 2%[v/v] IPG buffer, 20 mM DTT) were added to the mixture to make a total volume of 450 μl. Subsequently, isoelectric focusing in Ettan IPGphor (GE Healthcare) at 20°C (74510 Vh) was performed with 24-cm immobilized non-linear pH gradient strips (pH 3–10).

After the first dimension, each strip was equilibrated in 10-ml equilibration solution-1(6 M urea, 75 mM Tris-HCl pH8.8, 29.3% glycerol, 2% sodium dodecyl sulphate [SDS], 0.002% bromphenol blue, 100 mg DTT) and 10-ml equilibration solution-2 (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromphenol blue, 250 mg iodoacetamide) for 15 min at room temperature. After equilibration, the strips were individually overlaid on 12.5% polyacrylamide gels and immobilized with 0.5% agarose in a buffer of 1×Laemmli SDS buffer (25 mM Tris, 192 mM glycine, 0.1% [W/V] SDS) run at 2 W/gel using the Ettan-DALTsix system (GE Healthcare).

Image analysis and statistics

After completion of 2-dimensional electrophoresis, the CyDyes-labeled images were scanned using a Typhoon TRIO scanner (GE Healthcare). Images were analyzed by using DeCyder™ 2-D Differential Analysis Software 6.5 (GE Healthcare). The DeCyder differential in-gel analysis (DIA) module was used for pairwise

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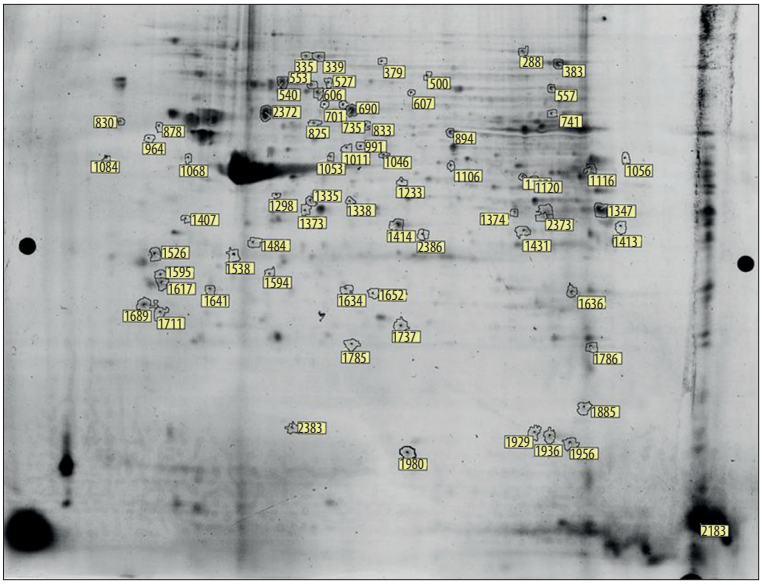

Figure 2. Two-dimensional electrophoresis image of differentially expressed glomerular proteins during maturation. Glomerular proteins were separated by 2-dimensional gel electrophoresis (2-DE). The gel was stained with Deep Purple dye. Twenty-two differentially expressed proteins were labeled on the map. Marked spots represent differentially expressed proteins between young (8 weeks) and mature (20 weeks) C57BL/6 mice. The marked spot numbers refer to "Master No." in Tables 2 and 3.

comparisons of each 8-week and 20-week sample to the internal standard present in each gel and for simultaneous comparison of 8-week/20-week abundance. The DeCyder biological variation analysis (BVA) module was then used to simultaneously match all 9 protein-spot maps from the 3 gels, using Cy3: Cy2 and Cy5: Cy2 DIA ratios. Statistical analysis of differences between the 2 groups was done using the paired *t* test and the level of statistical significance was set at $p<0.05$. The software automatically generated a list of differentially expressed proteins, including fold change, and these differentially expressed proteins were further identified by mass spectrometry.

Deep purple post-staining

Gels were fixed for 2 h in 7.5% (v/v) acetic acid and 10% (v/v) methanol, and washed twice in 35 mM $NaHCO_3$ and 300 mM Na_2CO_3 for 15 min per cycle. The total protein was stained with deep purple dye for 1 h in the dark. Next, post-stained gels were washed twice in 7.5% (v/v) acetic acid and subsequently imaged by a Typhoon 9400 scanner. Post-stained images were matched with CyDyes-stained images using DeCyder™ 2-D Differential Analysis Software, and the chosen spots were picked by use of an Ettan spot picker (GE Healthcare).

In-gel tryptic digestion

The selected gel particles in 96-well plates were washed twice with deionized water, dehydrated with acetonitrile (ACN), extracted with 25 mM NH_4HCO_3, and dehydrated again with ACN. Subsequently, trypsin (Promega, USA) was added for at least 1 h while on ice, followed by an overnight addition of 25 mM NH_4HCO_3 at 37°C.

Table 2. Up-regulated glomerular proteins (C20/C8).

Master No.	Protein name	Acc.no. SwissProt	Ratio up-regulated	Sequence coverage	pI	MW	Protein score	Ref. (Tryqqvason et al., 2007)#
1526	Tropomyosin alpha-3 chain	P21107	1.6	22%	4.68	32900	97	√
1538	60 kDa heat shock protein, mitochondrial	P63038	1.4	33%	5.91	61088	118	√
1786	Transgelin-2	Q9WVA4	1.2	72%	8.39	22552	192	

"C20/C8" represents 20-week C57BL/6 mice *vs.* 8-week C57BL/6 mice. Protein score is –10*Log(*P*), where *P* is the probability that the observed match is a random event. Protein scores greater than 55 are significant ($P<0.05$). Paired *t* test was analyzed by DeCyder™ 2-D Differential Analysis Software 6.5, and it was set as a significantly statistical difference when *p*-value <0.05. The software automatically generated a list of differentially expressed proteins, including fold change. # Tryqqvason et al. [11] isolated glomeruli from female C57BL/6 mice by Dynabeads perfusion, and attained proteomic profiling by using 2-dimensional gel electrophoresis with separate Coomassie and silver staining and subsequent mass spectrometric identifications. Proteins marked with "√" in table 2 and 3 indicate that these proteins were in accord with the results obtained by Tryqqvason et al. [11].

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Table 3. Down-regulated glomerular proteins (C20/C8).

Master No.	Protein name	Acc.no. SwissProt	Ratio down-regulated	Sequence coverage	pI	MW	Protein score	Ref. (Tryqqvason et al., 2007)#
1068	ATP synthase subunit beta, mitochondrial	P56480	1.76	29%	5.19	56265	139	√
878	Histone-binding protein RBBP4	Q60972	1.76	26%	4.79	47910	92	√
830	Selenium-binding protein 1	P17563	1.56	31%	5.87	53051	138	
1011	26S protease regulatory subunit 7	P46471	1.48	36%	5.72	49016	149	√
2383	L-lactate dehydrogenase B chain	P16125	1.41	26%	5.7	36834	94	√
2386	Annexin A5	P48036	1.37	34%	4.83	35787	92	√
1785	Ferritin light chain 1	P29391	1.36	52%	5.66	20847	102	√
1484	Annexin A4	P97429	1.35	32%	5.43	36252	116	√
1373	F-actin-capping protein subunit alpha-2	P47754	1.33	36%	5.57	33118	92	√
1106	Protein disulfide-isomerase A3	P27773	1.3	30%	5.88	57099	176	√
964	Ribonuclease inhibitor	Q91VI7	1.28	41%	4.69	51495	120	√
1595	14-3-3 protein gamma	P61982	1.27	42%	4.8	28456	107	
1220	Aspartate aminotransferase, cytoplasmic	P05201	1.23	31%	6.68	46488	103	
1413	Heterogeneous nuclear ribonucleoproteins A2/B1	O88569	1.22	42%	8.97	37437	143	√
1634	Calreticulin	P14211	1.22	27%	4.33	48136	114	√
1233	Nucleoside diphosphate-linked moiety X motif 19, mitochondrial	P11930	1.2	40%	6.22	40799	129	√
1641	Rho GDP-dissociation inhibitor 1	Q99PT1	1.19	33%	5.12	23450	88	√
1338	14-3-3 protein epsilon	P62259	1.18	43%	4.63	29326	94	√
1594	Chloride intracellular channel protein 4	Q9QYB1	1.18	37%	5.44	28939	100	√

"C20/C8" represents 20-week C57BL/6 mice *vs.* 8-week C57BL/6 mice. Protein score is –10*Log(*P*), where *P* is the probability that the observed match is a random event. Protein scores greater than 55 are significant ($P<0.05$). Paired *t* test was analyzed by DeCyder™ 2-D Differential Analysis Software 6.5, and it was set as a significantly statistical difference when *p*-value <0.05. The software automatically generated a list of differentially expressed proteins, including fold change. # Tryqqvason et al. [11] isolated glomeruli from female C57BL/6 mice by Dynabeads perfusion, and attained proteomic profiling by using 2-dimensional gel electrophoresis with separate Coomassie and silver staining and subsequent mass spectrometric identifications. Proteins marked with "√" in table 2 and 3 indicate that these proteins were accordant with the results obtained by Tryqqvason et al. [11].

Mass spectrometric analysis for protein identification

Each trypsin-digested sample was spotted on an MTP Anchorchip™ target (Bruker Daltonics, Germany). Once the samples were completely dried, 1.1 µl of matrix (4 mg 4-hydroxy-alpha-cyanocinnamic acid dissolved in ACN: 0.1%TFA [7:3]) was also spotted. Tryptic peptides were identified by MALDI-TOF mass spectrometry (Bruker Daltonics) using peptide fragments of assorted lengths as the internal standard. Detailed analysis of peptide mapping fingerprint data was performed by

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flexAnalysis™ 3.0, and protein identification was performed by searching the Swiss Prot database using the MASCOT engine.

Results

After 2-dimensional differential gel electrophoresis, 2387±129 glomerular protein spots were detected. By using DeCyder™ 2-D Differential Analysis Software, 151 differentially expressed protein spots were extracted for further mass spectrometry analysis. By the individual analysis of MALDI-TOF mass spectrometry and peptide mass fingerprinting, 22 differentially expressed glomerular proteins were identified (Figure 2). Among these proteins, 3 proteins were significantly up-regulated and 19 were significantly down-regulated in mature mice (Tables 2 and 3). The distribution of these proteins in different cellular functions are as follows: protein transport, protein targeting, and proteolysis, 18%; glycolysis, 5%; transcription, 14%; electron transport, 9%; chaperones, 9%; and hydrolase, 9%. Among the glomerular differential proteins attained by us, 82% were in accord with the results attained by Tryqqvason et al. [11] (Tables 2 and 3).

Discussion

Aging is a time-dependent and complex biological phenomenon. There is an approximately 25% decline in the glomerular filtration rate starting at age 40 for humans [12–17]. To understand age-related changes in molecular dynamics, we sought to identify proteins participating in maturation of glomeruli, which is the initial phase of aging that eventually leads to senescence. In the current study, we concentrated our efforts on identifying proteins that were differentially expressed between 8-week and 20-week C57BL/6 male mice.

We identified 22 differentially expressed glomerular proteins in C57BL/6 mice at age 8 months *vs.* 20 months. In a previous study, Tryqqvason et al. [11] isolated glomeruli by Dynabeads perfusion and performed a global analysis of glomerular proteomic profiling in C57BL/6 female mice by using 2-dimensional gel electrophoresis with separate Coomassie and silver staining and subsequent mass spectrometric identifications. About 82% of the 22 differentially expressed glomerular proteins that we identified were also present in their global proteomic list. The identification of the additional mismatched 18% that we identified is possibly due to the increased sensitivity of the fluorescent detection system we used. Alternatively, this could also be because only differentially expressed proteins were detected by us, and some proteins detected only in Tryqqvason's work were in a stable expression during maturation.

An important finding of our study is the identification of altered regulation of ATP synthase subunit beta in our young *vs.* mature mice models. We found that ATP synthase subunit beta was significantly down-regulated, with a differential expression rate of 1.76. Differential kidney expression of the enzyme has been previously identified in conjunction with multiple pathophysiological conditions such as acute kidney injury, diabetes, and age-related diseases [18–20].

The mitochondrial ATP synthase is a reversible enzyme with both hydrolytic and synthetic activities, and plays important roles in the regulation of protein, carbohydrate, and lipid metabolism through modulating energy homeostasis [21,22]. ATP synthase consists of a membrane-bound F_0 portion and a soluble F_1 portion. F_1 consists of 5 subunits (α, β, γ, δ, and ε), which catalyzes the synthesis of ATP from ADP and inorganic phosphate through utilizing the transmembrane proton gradient and membrane potential generated during substrate oxidation. This reaction can be reversed through pumping protons in the opposite direction and results in ATP hydrolysis [23–26]. The mechanism by which this reversible enzyme switches from the ATP synthetic to the ATP hydrolytic activity is still not well understood.

The catalytic sites of ATP synthesis are present on the β subunit, which plays a crucial role in energy metabolism and directly participates in the process of energy production. In our study, the expression level of ATP synthase subunit beta in glomeruli decreased most during the process of maturation. Nephrons increase both in number and size during early postnatal life, which could explain the high expression of ATP synthase subunit beta at 8 weeks of age. At the mature stage, glomeruli were in equilibrium, and energy consumption was lower. For instance, at least 50% of available [ATP] in the liver of older rats would have to be committed to sustain maximal Na^+-K^+-ATPase activity, whereas only 30% would be required for mature rats, suggesting lower ATP requirement at early maturity *vs.* older rats [27]. Therefore, the expression of ATP synthase subunit beta may be regulated to be lower at the early mature stage.

In another interesting study, Chakravarti et al. [28] analyzed the expression of proteins during the stage of senescence in the kidneys of 19–22-week-old and 24-month-old C57BL/6 male mice using 2-dimensional gel electrophoresis followed by LC-MS/MS. In their study, the ATP synthase subunit beta was up-regulated in 24 months *vs.* the 19–22 week group, with a differential expression rate of 1.4–1.9. The higher expression of ATP synthase subunit beta was suggested to play a role in the mechanistic pathway to overcome the decline in activity caused by aging-related oxidative damage of the protein. The results of our study further indicate multiple phases of age-dependent ATP synthase subunit beta regulation, with an initial down-regulation during maturation, followed by an up-regulation during post-maturation aging and senescence.

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Conclusions

Our study identified 22 differentially expressed proteins in mature *vs.* young mice. ATP synthase subunit beta was significantly down-regulated in the mature *vs.* young mice. Further studies might help to reveal any possible role and mechanism of the altered regulation of these proteins in aging.

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Conflict of interest

All the authors declared no competing interests.

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