Urinary Proteome Changes During Short-term Growth and Development in Rats

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Abstract

How short-term can urinary proteomes reflect changes in growth and development in animal organisms? When conducting urinary biomarker studies using animal models during periods of rapid growth, should the impact of short-term development on urinary proteins be considered? In this study, urine samples were collected from 10 Wistar rats aged 6-8 weeks at intervals of 3 and 6 days, and analyzed using label-free quantitative proteomics technology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results demonstrated that the urinary proteome can sensitively reflect short-term growth and developmental changes in rats. Specifically, comparing the urinary proteomes of Day0 and Day6, 195 differential proteins were identified after screening (FC$ $1.5 or $ $0.67, P<0.05). Randomized grouping validation revealed an average of 17.99 randomly generated differential proteins, indicating that at least 90.77% of the differential proteins were not produced by random chance. These results confirm that the differential proteins identified through temporal comparisons were not randomly generated. GO and KEGG pathway analyses of differential proteins enriched numerous biological processes and signaling pathways related to growth and development, providing evidence that the urinary proteome can reflect short-term growth and development in rats, offering a means for in-depth and detailed studies of growth and development, and highlighting a confounding factor that warrants careful consideration in animal experiments using 6-8-week-old rats for model construction. This study demonstrates that the urinary proteome can detect differences in urinary proteins of 6-8-week-old rats with intervals as short as 3-6 days, expanding the sensitivity boundaries of urinary proteomics and revealing the sensitive and fine-grained characterization capability of the urinary proteome for organismal changes.

Full Text

Urinary Proteome Changes During Short-Term Growth and Development in Rats

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Abstract

How acutely can the urinary proteome reflect short-term developmental changes in an organism? When conducting urinary biomarker studies using rapidly growing animal models, is it necessary to consider the influence of short-term development on urinary proteins? This study addressed these questions by collecting urine samples from ten 6-8-week-old Wistar rats at intervals of 3 and 6 days, followed by analysis using label-free quantitative proteomics with high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results demonstrate that the urinary proteome can sensitively capture the physiological changes associated with short-term growth and development in rats. Comparing the urinary proteomes of Day 0 and Day 6, we identified 195 differential proteins after applying screening criteria (fold change \$ \$1.5 or \$ \$0.67, $P<0.05$). Random grouping validation revealed that the average number of randomly generated differential proteins was 17.99, indicating that at least 90.77% of the identified differential proteins were not produced by chance. This confirms that the differential proteins identified across time points reflect genuine biological changes rather than random variation. Gene Ontology (GO) and KEGG pathway analyses of these differential proteins enriched numerous biological processes and signaling pathways related to growth and development, providing strong evidence that the urinary proteome can reflect short-term developmental changes in rats. These findings offer a powerful tool for in-depth and meticulous studies of growth and development while highlighting an important confounding factor that must be considered in animal experiments using 6-8-week-old rats for model construction. This study demonstrates that the urinary proteome can detect differences in urinary proteins of 6-8-week-old rats over intervals as short as 3-6 days, thereby expanding the sensitivity boundaries of urinary proteomics and revealing its capacity for sensitive and precise characterization of physiological changes.

Keywords: urine; proteomics; short-term growth and development

1.1 Influence of Rat Growth and Development on Urinary Proteome

Current research employing urinary proteomics to investigate rat growth and development remains scarce. In 2023, Pan Xuanzhen et al. [?] pioneered the use of urinary proteome technology to track developmental changes across a cohort of rats at ten critical time points spanning from childhood, adolescence, and early adulthood through middle age and into senescence near death, thereby filling a significant gap in developmental research. They identified organ-specific changes and age-related pathway alterations covering nearly all developmental stages, providing the first demonstration that urine can reveal comprehensive aspects of organismal growth and development and offering new perspectives for monitoring patient conditions, clinical prognosis, and future aging research [?].

1.2 Urinary Biomarkers

Biomarkers are objective indicators that reflect normal and pathological physiological processes [?] and can predict, monitor, and diagnose multifactorial diseases at various stages in clinical settings [?]. Compared with blood-based biomarkers that are more widely used, the potential of urinary biomarkers remains underexplored, particularly for early disease diagnosis and state prediction. Because blood is regulated by homeostatic mechanisms, disease-induced changes in the blood proteome are metabolically cleared and do not manifest as obvious alterations during early disease stages. In contrast, urine is produced by glomerular filtration of plasma and is not subject to homeostatic regulation, making it highly sensitive to physiological changes. Even minute disease-related changes can be observed in urine, with studies showing that urinary changes appear far earlier than blood changes, pathological sections, or even clinical symptoms, enabling applications in early diagnosis. Since the urinary proteome is susceptible to various factors such as diet, medication, and daily activities, employing simple and controllable systems is key to obtaining accurate results. Animal models are particularly suitable because their genetic and environmental factors can be artificially controlled to minimize extraneous influences. For example: (1) Zhang et al. [?] found that 29 urinary proteins changed in a transgenic mouse model of Alzheimer's disease before amyloid plaque deposition in the brain, with 24 of these proteins previously reported as AD-related or candidate biomarkers; (2) Wu et al. [?] identified 10 altered urinary proteins in a Walker 256 subcutaneous tumor rat model before the tumor became palpable; (3) Zhang Y et al. [?] detected 15 differential proteins in urine from a chronic pancreatitis rat model during week 2, before pathological changes appeared, with 5 previously linked to pancreatitis; (4) Ni et al. [?] observed urinary protein changes in a C6 glioma rat model before MRI-detectable symptoms; (5) Zhang et al. [?] identified 40 differential urinary proteins in a thioacetamide-induced liver fibrosis rat model before pathological changes, with 15 previously reported as fibrosis-related; (6) Yin et al. [?] found that urinary glucose levels showed frequent disturbances before blood glucose elevation in obese type 2 diabetic rats, indicating potential for early diabetes detection; and (7) Huang et al. [?] identified previously reported chronic obstructive pulmonary disease (COPD) biomarkers in rats after only two weeks of exposure to traditional cigarette smoke. Comparative studies have further shown that when tumor cells grow in different organs—including subcutaneous [?], liver [?], bone [?], lung [?], and brain [?]—the urinary protein changes differ, demonstrating urine's potential to distinguish the same tumor cells growing in different anatomical sites. Moreover, urine collection is non-invasive and readily obtainable [?], making it an excellent source of biomarkers and highlighting animal models as a crucial approach in urinary proteomics research.

However, animal growth and development during experimental periods represent a significant and unavoidable influencing factor. Can the urinary proteome reflect short-term developmental changes in rats? Must we consider the impact of short-term development on urinary proteins when conducting biomarker studies using rapidly growing animals? This question challenges the sensitivity boundaries of urinary proteomics. Therefore, this study selected rapidly developing 6-8-week-old Wistar rats to investigate the dynamic changes in urinary proteome during growth and development within a one-week timeframe (Figure 1 [Figure 1: see original paper]), revealing the fine granularity of urinary proteome in reflecting developmental changes and exploring the long-overlooked factor of "growth and development" as a confounding element in animal model experiments.

2.1 Urine Sample Collection

This study utilized ten healthy male Wistar rats aged 6-8 weeks (180-200 g) of SPF grade, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (license number: SYXK(Beijing)2021-0011). All rats were housed under standard conditions (temperature (22$±$1)°C, humidity 65%-70%). Following a three-day acclimation period, experiments commenced. All procedures were reviewed and approved by the Animal Ethics Committee of the College of Life Sciences, Beijing Normal University (approval number: CLS-AWEC-B-2022-003). Behavioral changes were monitored throughout the experiment, with body weight recorded every three days.

After the three-day acclimation, all ten rats were placed in metabolic cages to collect 12-hour urine samples, designated as Day 0 samples. Additional 12-hour urine samples were collected on Day 3 and Day 6. During urine collection, rats were fasted and deprived of water. All collected urine samples were stored at -80°C.

2.2 Urine Sample Processing

Urinary Protein Extraction and Quantification: Urine samples collected at three time points were centrifuged at 12,000×g for 40 min at 4°C. The supernatant was transferred to new Eppendorf tubes, and three volumes of pre-chilled absolute ethanol were added. After thorough mixing, proteins were precipitated overnight at -20°C. The following day, samples were centrifuged at 12,000×g for 30 min at 4°C, and the supernatant was discarded. Protein pellets were resuspended in lysis buffer (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L dithiothreitol, 50 mmol/L Tris) and centrifuged at 12,000×g for 30 min at 4°C. The supernatant was transferred to new tubes, and protein concentration was measured using the Bradford method.

Urinary Protein Digestion: One hundred micrograms of urinary protein were loaded onto a 10 kDa ultrafiltration membrane (Pall, Port Washington, NY, USA) placed in an Eppendorf tube, and 25 mmol/L NH_4HCO_3 solution was added to a final volume of 200 L. Twenty mM dithiothreitol (DTT, Sigma) was added, vortexed, and heated in a metal bath at 97°C for 5 min before cooling to room temperature. Fifty mM iodoacetamide (IAA, Sigma) was added, mixed, and incubated in the dark at room temperature for 40 min. Membrane washing was then performed: (1) 200 L UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) was added and centrifuged at 14,000×g for 5 min at 18°C, repeated twice; (2) sample loading: the processed sample was added and centrifuged at 14,000×g for 40 min at 18°C; (3) 200 L UA solution was added and centrifuged at 14,000×g for 40 min at 18°C, repeated twice; (4) 25 mmol/L NH_4HCO_3 solution was added and centrifuged at 14,000×g for 40 min at 18°C, repeated twice; and (5) trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for overnight digestion at 37°C. The following day, peptides were collected by centrifugation at 13,000×g for 30 min at 4°C, desalted using HLB columns (Waters, Milford, MA), dried in a vacuum concentrator, and stored at -80°C.

2.3 LC-MS/MS Tandem Mass Spectrometry Analysis

Digested samples were dissolved in 0.1% formic acid, and peptide concentration was quantified using a BCA kit and diluted to 0.5 g/ L. A pooled peptide sample was prepared by combining 4 L from each sample and fractionated using a high pH reverse-phase peptide separation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Ten fractions were collected by centrifugation, dried in a vacuum concentrator, and reconstituted in 0.1% formic acid. iRT reagent (Biognosys, Switzerland) was added at a 10:1 sample-to-iRT ratio to calibrate peptide retention times. For analysis, 1 g of peptides from each sample was analyzed using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA) coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

For spectral library generation, the ten fractions were analyzed in Data Dependent Acquisition (DDA) mode. Mass spectrometry data were acquired in high-sensitivity mode. Full MS scans were acquired at a resolution of 60,000 over a range of 350-1500 m/z. Individual samples were analyzed in Data Independent Acquisition (DIA) mode using a 36-window DIA method. A single DIA analysis of pooled peptides was performed after every ten samples as a quality control measure.

2.4 Database Searching and Label-Free DIA Quantification

Raw data (RAW files) acquired from LC-MS/MS were imported into Proteome Discoverer (version 2.1, Thermo Scientific) and searched against the Swiss-Prot rat database (released May 2019, containing 8,086 sequences) with iRT sequences appended. Search results were then imported into Spectronaut Pulsar (Biognosys AG, Switzerland) for processing and analysis. Peptide abundance was calculated by summing the peak areas of respective fragment ions in MS2 spectra, and protein intensity was determined by summing the abundances of its constituent peptides.

2.5 Data Analysis

Each sample was analyzed in two technical replicates, and average values were used for statistical analysis. Samples from different time points were compared to identify differential proteins using two sets of criteria: relaxed (fold change (FC) \$ \$1.5 or \$ 0.67, $two - tailedunpairedt - testP < 0.05)andstrict(FC$ \$2 or \$ \$0.5, P<0.01). Random grouping validation was performed to verify the reliability of identified differential proteins and confirm that observed differences resulted from actual developmental changes rather than random variation.

Functional enrichment analysis of differential proteins was conducted using the Wukong platform (https://www.omicsolution.org/wkomic/main/), UniProt (https://www.uniprot.org/), and OmicsBean database (http://www.omicsbean.cn/). Protein-protein interaction (PPI) networks were analyzed using STRING (https://cn.string-db.org/), and literature searches were performed in PubMed (https://pubmed.ncbi.nlm.nih.gov) to characterize protein functions.

3.1 Rat Growth and Development Characteristics

Body weight was monitored throughout the experimental period, with measurements recorded every three days (Figure 2 [Figure 2: see original paper]). Behavioral observations confirmed that rats remained active with normal food and water intake, showing no obvious abnormalities.

3.2 Analysis of Urinary Proteome Changes in Rat Short-Term Growth and Development Model

(1) Urinary Protein Identification

Urine samples were collected from ten 6-8-week-old male Wistar rats on Day 0, Day 3, and Day 6. LC-MS/MS analysis of peptides from 30 urine samples identified a total of 844 proteins (\$ \$2 unique peptides, protein-level FDR<1%). The quantitative protein intensity spanned five orders of magnitude across the 30 samples from three time points (Figure 3 [Figure 3: see original paper]).

(2) Differential Protein Identification Across Time Points

Comparing urinary proteins from different developmental time points using relaxed criteria (FC$ $1.5 or $ $0.67, two-tailed unpaired t-test P<0.05) identified 37 differential proteins between Day 0 and Day 3 (Table 1), 75 between Day 3 and Day 6 (Table S1), and 195 between Day 0 and Day 6 (Table S2).

To further assess the significance of differential proteins identified across time points, stricter criteria were applied (FC$ $2 or $ $0.5, P<0.01). This revealed 1 differential protein between Day 0 and Day 3, 13 between Day 3 and Day 6, and 46 between Day 0 and Day 6 (Table 2). Notably, 8 differential proteins were repeatedly identified in both the Day 3 vs Day 6 and Day 0 vs Day 6 comparisons, all showing consistent expression trends. UniProt analysis revealed that 5 of the down-regulated proteins were associated with the aging pathway, including broad substrate specificity ATP-binding cassette transporter ABCG2, gamma-glutamyltransferase 1, glutathione synthetase, alpha-crystallin B chain, and glutamate-cysteine ligase regulatory subunit. Eleven differential proteins were associated with developmental pathways, including upregulated Crk-like protein, which participates in acetylcholine receptor signaling and morphogenesis of multiple organs.

(3) Random Grouping Validation

To determine the likelihood that identified differential proteins arose by chance, random grouping validation was performed on proteins identified from Day 0 (n=10) and Day 3 (n=10) samples using relaxed criteria (FC$ $1.5 or $ 0.67, $P < 0.05).Among 92,378 possible combinations, the average number of differential proteins generated randomly was 1$ or $ $0.5, P<0.01), the average number of random differential proteins was 0.79 (6.08% false discovery rate), indicating at least 93.92% were genuine. For Day 0 vs Day 6 samples, relaxed criteria produced an average of 17.99 random differential proteins (9.23% false discovery rate), confirming that at least 90.77% were not random, while strict criteria yielded 0.88 random proteins (1.91% false discovery rate), indicating at least 98.09% were genuine.

(4) Functional Analysis of Differentially Expressed Proteins

Protein-protein interaction (PPI) network analysis of differential proteins identified between Day 0 and Day 6 (FC$ $1.5 or $ $0.67, P<0.05) was performed using STRING (Figure 4 [Figure 4: see original paper]). To determine the biological relevance of these protein networks, OmicsBean (http://www.omicsbean.cn/) was used for Gene Ontology (GO) and pathway analysis. Representative biological processes, cellular components, molecular functions, and pathways are shown in Figure 5 [Figure 5: see original paper].

Comparisons between Day 0 and Day 3 enriched biological processes including growth regulation, positive regulation of growth, regulation of developmental growth, regulation of multi-organism processes, and positive regulation of multi-organism processes. Day 3 vs Day 6 comparisons enriched processes such as inner ear development, ear development, glutamate metabolic process, and positive regulation of cell proliferation via VEGF-activated platelet-derived growth factor receptor signaling pathway. Day 0 vs Day 6 comparisons enriched single-organism developmental process, developmental process, multicellular organism development, anatomical structure development, aging, tissue development, and system development, with some processes involving up to 40% of differential proteins, indicating high confidence. Cellular component analysis across all three time point comparisons consistently identified extracellular exosomes, extracellular vesicles, extracellular organelles, extracellular region parts, extracellular non-membrane-bound organelles, vesicles, and membrane-bound vesicles. Enriched molecular functions included protein binding, catalytic activity, protein complex binding, and macromolecular complex binding. Representative enriched signaling pathways that appeared repeatedly across different time groups included RAP1 signaling, axon guidance, protein processing in the endoplasmic reticulum, gap junctions, glutathione metabolism, and other metabolic pathways.

4. Discussion

This study employed mass spectrometry-based label-free quantitative proteomics to analyze six days of growth and development in ten rapidly developing 6-8-week-old Wistar rats. Comparing urinary proteins between Day 0 and Day 3 identified 37 differential proteins using relaxed criteria (FC\$ \$1.5 or $\$ 0.67, P < 0.05), withrandomgroupingvalidationconfirming 66.68$ \$2 or \$ \$0.5, P<0.01) identified only 1 differential protein. Day 3 vs Day 6 comparisons identified 75 differential proteins (relaxed criteria) with 82.27% credibility, and 13 differential proteins (strict criteria) with 93.92% credibility. Day 0 vs Day 6 comparisons identified 195 differential proteins (relaxed criteria) with 90.77% credibility, and 46 differential proteins (strict criteria) with 98.09% credibility. These results demonstrate that differential proteins identified across Day 0, Day 3, and Day 6 are reliable, with credibility increasing over longer time intervals and under stricter screening criteria. Having excluded random generation, we conclude these differential proteins are genuinely associated with short-term rat growth and development.

Under strict criteria (FC\$ \$2 or $\$ 0.5, P < 0.01), 8 differentialproteinswererepeatedlyidentifiedinbothDay3vs$
$a, reportedasastress-relatedgene, playsimportantrolesinsynapseandfilopodiaformation, whicharecritical$
$richalpha-2-glycoprotein1hasbeenshowntoregulateangiogenesis, epithelial-$
$mesenchymaltransition(EMT), andapoptosisthroughthetransforminggrowthfactor-$
$beta(TGF- $\$) signaling pathway, influencing tumor development [?]. In Day 0 vs Day 6 comparisons under strict criteria, we identified 3-hydroxybutyrate dehydrogenase 2, with research indicating that rats can regulate expression of mitochondrial D-3-hydroxybutyrate dehydrogenase to modulate different developmental and physiological stages [?]. Alpha-lactalbumin can improve energy balance and metabolism [?] and promote growth and maturation of the small intestine in suckling rats [?]. Adenosylhomocysteinase has been identified as a biomarker for frailty and may play regulatory roles in aging and age-related diseases [?]. N-myc downstream-regulated gene 1 (NDRG1) is an intracellular protein induced under various stress and cell growth regulatory conditions, upregulated by differentiation signals and suppressing tumor metastasis in vari-

ous cancer cell lines [?]. Win et al. demonstrated that \$ \$1 integrin signaling is required during specific transitional windows in developing β-cells to maintain islet mass and vascularization [?], while Masuzaki et al. identified integrin \$ $1asakeydeterminantofliverstructureandacriticalregulatorofTGF-$ \$ secretion [?]. Barzegar Behrooz et al. reviewed evidence that CD133 (prominin-1) can increase angiogenesis and promote neural cell growth and differentiation by activating Wnt signaling [?]. These differential urinary proteins likely play key roles in rat growth and development, demonstrating the exquisite sensitivity of urinary proteomics in reflecting dynamic changes over just six days while excluding random generation.

Functional enrichment identified important biological processes related to disease progression, including growth regulation, positive regulation of growth, regulation of developmental growth, regulation of multi-organism processes, positive regulation of multi-organism processes, inner ear development, ear development, glutamate metabolic process, positive regulation of cell proliferation via VEGF-activated platelet-derived growth factor receptor signaling pathway, single-organism developmental process, developmental process, multicellular organism development, anatomical structure development, aging, tissue development, and system development. Additionally, we enriched the RAP1 signaling pathway. Chrzanowska-Wodnicka et al. demonstrated that the small GTPase Rap1 participates in fundamental cellular functions essential for functional vascular development and formation in mice [?]. Other repeatedly identified pathways across multiple time points included axon guidance, protein processing in the endoplasmic reticulum, gap junctions, glutathione metabolism, and other metabolic pathways.

These results demonstrate that the urinary proteome can sensitively reflect dynamic physiological changes during normal short-term growth and development in rats. We propose that the rate of physiological change may vary across different developmental stages. Our findings in rapidly developing 6-8-week-old male Wistar rats provide a powerful tool for detailed growth and development studies while highlighting a critical confounding factor for future urinary proteomics animal experiments. Researchers should identify an optimal temporal balance between periods of vigorous growth and age-related baseline pathologies to minimize interference from developmental and aging-related changes.

5. Conclusion

This study demonstrates that the urinary proteome can reflect remarkably short-term developmental changes in rats. Therefore, when conducting biomarker studies using rapidly growing animal models, it is essential to consider the impact of short-term development on urinary proteins.

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Table S1: Differential Protein Information for Day3 vs Day6 (FC$ $1.5 or $ $0.67, P<0.05)

Accession	Protein names	Trend	Fold change	P value
P07314	Glutathione hydrolase 1 proenzyme		2.89E-04	
Q63041	Alpha-1-macroglobulin		8.50E-04	

Accession	Protein names	Trend	Fold change	P value
Q812E9	Neuronal membrane glycoprotein M6-a		8.91E-04	
D3ZUQ1	Lipase		1.21E-03	
P14668	Annexin A5		1.22E-03	
G3V847	Sodium-dependent neutral amino acid transporter B(0)AT3		1.22E-03	
M0R7L1	Lipase		1.95E-03	
A0A0A0MXT4	Solute carrier organic anion transporter family member		2.12E-03	
A0A0G2JSJ2	Cytidine/uridine monophosphate kinase 1		2.40E-03	
A0A0G2KB26	Matrix remodeling-associated protein 8		2.42E-03	
Q9JJ19	Na(+)/H(+) exchange regulatory cofactor NHE-RF1		2.53E-03	
Q80W57	Broad substrate specificity ATP-binding cassette transporter ABCG2		2.75E-03	
Q641Z6	EH domain-containing protein 1		3.81E-03	
D4AA31	Prolylcarboxypeptidase		4.13E-03	
D3ZE16	Alpha-L-iduronidase		4.22E-03	
F7F389	Complement C9		4.71E-03	
Q5M876	N-acyl-aromatic-L-amino acid amidohydrolase		4.74E-03	

Accession	Protein names	Trend	Fold change	P value
Q5I0E1	Leucine-rich alpha-2-glycoprotein 1		5.04E-03	
Q9JJ40	Na(+)/H(+) exchange regulatory cofactor NHE-RF3		5.28E-03	
A0A0G2K595	Solute carrier family 23 member 1		6.11E-03	
D3ZLA3	Copine 3		6.91E-03	
G3V7D0	Matrix metallopeptidase 8		7.13E-03	
A0A140TAG4	NPHS1 adhesion molecule, nephrin		7.25E-03	
A0A0G2QC04	Plastin 1		9.17E-03	
D3ZH39	receptor protein-tyrosine kinase		9.70E-03	
D4A400	Lactoperoxidase		1.04E-02	
Q5M8C3	Serine		1.11E-02	
A0A0G2JZ18	Pentraxin 4		1.13E-02	
F1MAD3	Polycystin 1, transient receptor potential channel interacting		1.15E-02	
P61459	Pterin-4-alpha-carbinolamine dehydratase		1.18E-02	
G3V6A0	Platelet-derived growth factor receptor alpha		1.23E-02	
Q6Q0N1	Cytosolic non-specific dipeptidase		1.25E-02	
P48508	Glutamate–cysteine ligase regulatory subunit		1.37E-02	
P53790	Sodium/glucose cotransporter 1		1.39E-02	

Accession	Protein names	Trend	Fold change	P value
G3V8X5	Solute carrier family 5		1.47E-02	
A0A0G2JWD0	Prominin 1		1.55E-02	
P80254	D-dopachrome decarboxylase		1.60E-02	
A0A0A0MXX5	3-hydroxybutyrate dehydrogenase 2		1.69E-02	
F1M978	Inositol-1-monophosphatase		1.74E-02	
A0A0G2JT43	Solute carrier family 2, facilitated glucose transporter member 5		1.86E-02	
G3V7L4	Cadherin 16		2.03E-02	
F1LQI1	Hydroxyacyl glutathione hydrolase		2.26E-02	
P50137	Transketolase		2.39E-02	
P97546	Neuroplastin		2.43E-02	
Q5U362	Annexin		2.47E-02	
A0A0G2JSV5	3-hydroxyanthranilate 3,4-dioxygenase		2.48E-02	
Q6MG71	Choline transporter-like protein 4		2.55E-02	
Q9WUW9	Sulfotransferase 1C2A		2.69E-02	
P09606	Glutamine synthetase		2.75E-02	
P04904	Glutathione S-transferase alpha-3		2.92E-02	
D4A4U3	Magnesium-dependent phosphatase 1		3.13E-02	
F1LVR0	IgLON family member 5		3.36E-02	
P01681	Ig kappa chain V region S211		3.45E-02	
E9PU23	Delta-like protein		3.45E-02	

Accession	Protein names	Trend	Fold change	P value
B0BNJ1	LOC683667 protein		3.61E-02	
D3ZV91	trans-L-3-hydroxyproline dehydratase		3.61E-02	
G3V6D9	Na(+)/H(+) exchange regulatory cofactor NHE-RF		3.64E-02	
A1L1J8	RAB5B, member RAS oncogene family		3.66E-02	
Q64602	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial		3.76E-02	
P51607	N-acylglucosamine 2-epimerase		3.80E-02	
P02770	Albumin		3.81E-02	
D3ZXY4	Aldehyde dehydrogenase 8 family, member A1		3.84E-02	
Q6AXM6	Intercellular adhesion molecule 2		3.86E-02	
A0A0G2JSQ7	Kallikrein 1-related peptidase B3		3.93E-02	
P38918	Aflatoxin B1 aldehyde reductase member 3		4.10E-02	
Q3KRC4	G-protein coupled receptor family C group 5 member C		4.10E-02	
Q5FVI6	V-type proton ATPase subunit C 1		4.24E-02	

Accession	Protein names	Trend	Fold change	P value
Q4V7D9	Acid sphingomyelinase-like phosphodiesterase		4.37E-02	
Q499Q4	Phosphoglucomutase 1		4.40E-02	
Q05030	Platelet-derived growth factor receptor beta		4.40E-02	
D4A8C5	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase		4.41E-02	
Q5I0D7	Xaa-Pro dipeptidase		4.64E-02	
G3V8L3	Lamin A, isoform CRA_b		4.72E-02	
P16975	SPARC		4.85E-02	
P32755	4-hydroxyphenylpyruvate dioxygenase		4.85E-02	

Table S2: Differential Protein Information for Day0 vs Day6 (FC$ $1.5 or $ $0.67, P<0.05)

Accession	Protein names	Trend	Fold change	P value
Q80WD0	Reticulon-4 receptor-like 1		3.67E-06	
A0A0G2JY48	receptor protein-tyrosine kinase		5.51E-06	
Q5M876	N-acyl-aromatic-L-amino acid amidohydrolase		8.75E-06	
D4A269	Histidine triad nucleotide binding protein 1		8.89E-06	
P29534	Vascular cell adhesion protein 1		1.19E-05	

Accession	Protein names	Trend	Fold change	P value
A0A0A0MXX5	3-hydroxybutyrate dehydrogenase 2		1.24E-05	
F1M4Q3	Hemicentin 1		1.82E-05	
F1M6Q3	Collagen type IV alpha 2 chain		1.97E-05	
D4A4U3	Magnesium-dependent phosphatase 1		2.02E-05	
P00714	Alpha-lactalbumin		2.23E-05	
Q5U355	Integrin alpha FG-GAP repeat containing 1		4.11E-05	
A0A0G2KB26	Matrix remodeling-associated protein 8		4.16E-05	
Q641Z6	EH domain-containing protein 1		5.69E-05	
Q3KRC4	G-protein coupled receptor family C group 5 member C		6.12E-05	
A0A0G2JSV5	3-hydroxyanthranilate 3,4-dioxygenase		7.46E-05	
P80254	D-dopachrome decarboxylase		9.14E-05	
Q6MG71	Choline transporter-like protein 4		9.90E-05	
A0A0G2K8I5	Protocadherin 19		1.48E-04	
Q812E9	Neuronal membrane glycoprotein M6-a		1.52E-04	
A0A0G2KA90	Desmocollin 1		1.54E-04	
B0BNG3	Lman2 protein		1.63E-04	
P07314	Glutathione hydrolase 1 proenzyme		1.73E-04	
A0A0G2K013	Actinin alpha 4		1.90E-04	

Accession	Protein names	Trend	Fold change	P value
Q68G31	Phenazine biosynthesis-like domain-containing protein		2.09E-04	
F1LQ08	Carbonic anhydrase		2.30E-04	
F1LRJ9	Methanethiol oxidase		2.32E-04	
Q5FWU4	Hemochromatosis type 2		2.41E-04	
A0A0G2K5X1	Putative lysozyme C-2		2.48E-04	
P46413	Glutathione synthetase		2.61E-04	
A0A0G2JZH0	Calcium binding protein 39		2.73E-04	
F1M498	Gastrokine 3		2.83E-04	
P10760	Adenosylhomocysteinase		2.85E-04	
P24268	Cathepsin D		3.07E-04	
D3ZSL2	Costars family protein ABRACL		3.13E-04	
Q63355	Unconventional myosin-Ic		3.37E-04	
F1MAD3	Polycystin 1, transient receptor potential channel interacting		3.65E-04	
G3V7D0	Matrix metallopeptidase 8		3.70E-04	
P23928	Alpha-crystallin B chain		3.76E-04	
Q9JJ19	Na(+)/H(+) exchange regulatory cofactor NHE-RF1		3.85E-04	
A1L1J8	RAB5B, member RAS oncogene family		4.43E-04	
A0A0G2K8H2	Chondroitin sulfate proteoglycan 4B		6.10E-04	

Accession	Protein names	Trend	Fold change	P value
Q6JE36	N-myc downstream-regulated gene 1 protein		6.23E-04	
Q99MA2	Xaa-Pro aminopeptidase 2		6.28E-04	
P39069	Adenylate kinase isoenzyme 1		6.37E-04	
M0R8R4	Oncoprotein-induced transcript 3 protein		6.72E-04	
G3V7L4	Cadherin 16		7.01E-04	
F1LY81	Integrin beta		7.13E-04	
A0A0G2K0C1	Carboxylic ester hydrolase		7.54E-04	
G3V7C6	Tubulin beta chain		7.59E-04	
D3ZLA3	Copine 3		7.92E-04	
P41740	Atrial natriuretic peptide receptor 3		8.39E-04	
A0A0A0MXT4	Solute carrier organic anion transporter family member		8.67E-04	
A0A0G2JSK5	Integrin beta		8.85E-04	
F1LWS4	Complement factor H-related 2		9.15E-04	
D3ZZR3	Cathepsin S		9.83E-04	
P97615	Thioredoxin, mitochondrial		9.94E-04	
P16617	Phosphoglycerate kinase 1		1.12E-03	
G3V8K8	Protein Z, vitamin K-dependent plasma glycoprotein		1.16E-03	
Q6AYS0	Secreted and transmembrane 1A		1.18E-03	
A0A096MIU6	Fc fragment of IgG receptor IIa		1.19E-03	

Accession	Protein names	Trend	Fold change	P value
Q5M7T6	V-type proton ATPase subunit		1.22E-03	
Q7TQ94	Deaminated glutathione amidase		1.26E-03	
A0A140TAG4	NPHS1 adhesion molecule, nephrin		1.27E-03	
G3V8V1	Granulin precursor		1.31E-03	
F1LQT4	Carboxypeptidase N subunit 2		1.31E-03	
A0A0G2JWD0	Prominin 1		1.48E-03	
D4A400	Lactoperoxidase		1.57E-03	
Q5U2U2	Crk-like protein		1.57E-03	
A0A0G2JXJ3	FAM3 metabolism regulating signaling molecule D		1.57E-03	
F1MA46	Protocadherin 12		1.59E-03	
O35112	CD166 antigen		1.67E-03	
A0A0H2UHQ0	Solute carrier family 3		1.69E-03	
Q5I0E1	Leucine-rich alpha-2-glycoprotein 1		1.77E-03	
P07150	Annexin A1		1.82E-03	
M0R7L1	Lipase		1.83E-03	
Q9R1E9	CCN family member 2		2.04E-03	
P48508	Glutamate–cysteine ligase regulatory subunit		2.25E-03	
O70540	Mucosal addressin cell adhesion molecule 1		2.26E-03	
A0A0G2JZ18	Pentraxin 4		2.37E-03	
P84039	Ectonucleotide pyrophosphatase/phosphodiesterase family member 5		2.42E-03	

Accession	Protein names	Trend	Fold change	P value
A0A0G2KAJ7	Collagen type XII alpha 1 chain		2.49E-03	
P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2		3.25E-03	
Q01177	Plasminogen		3.44E-03	
P97553	Ephrin-A1		3.61E-03	
P08753	Guanine nucleotide-binding protein G(i) subunit alpha-3		3.69E-03	
Q9R0T3	DnaJ homolog subfamily C member 3		3.69E-03	
F1M7V6	Cell adhesion molecule 4		3.90E-03	
Q9WUK5	Inhibin beta C chain		3.96E-03	
Q6AYS4	Plasma alpha-L-fucosidase		4.05E-03	
Q63530	Phosphotriesterase-related protein		4.10E-03	
Q63259	Receptor-type tyrosine-protein phosphatase-like N		4.24E-03	
Q8K4G9	Podocin		4.47E-03	
G3V9A3	RCG31390		4.51E-03	
D3ZC55	Heat shock 70kDa protein 12A		4.79E-03	
A0A0G2K230	Desmocollin 3		4.94E-03	
D3ZAE6	Meprin A subunit alpha		5.31E-03	
RCG49849	A0A0G2JSS8		5.56E-03	
Q64230	Peroxiredoxin-5		5.71E-03	
Q6AYT0	Quinone oxidoreductase		5.78E-03	
Q5M7T9	Threonine synthase-like 2		5.82E-03	
F1LMI3	Cadherin 3		6.03E-03	

Accession	Protein names	Trend	Fold change	P value
F1LTY5	Chymotrypsin-like		6.48E-03	
G3V8J3	Tubulin alpha-1B chain		6.48E-03	
Q6P9V9	A2RUW1		6.57E-03	
A2RUW1	Toll-interacting protein		6.68E-03	
P53790	Sodium/glucose cotransporter 1		7.03E-03	
Q9JJ40	Na(+)/H(+) exchange regulatory cofactor NHE-RF3		7.15E-03	
A0A0G2K872	Cell adhesion molecule 3		7.29E-03	
P97574	Stanniocalcin-1		7.38E-03	
G3V847	Transporter		8.27E-03	
A0A088DKH8	receptor protein serine/threonine kinase		9.01E-03	
A0A0G2K930	RAB7A, member RAS oncogene family		9.02E-03	
A0A0G2K6T8	Brain-specific angiogenesis inhibitor 1-associated protein 2		9.02E-03	
D3ZUD8	Transmembrane 9 superfamily member		9.53E-03	
P38918	Aflatoxin B1 aldehyde reductase member 3		9.65E-03	
O35276	Neuropilin-2		9.74E-03	
P24368	Peptidyl-prolyl cis-trans isomerase B		9.86E-03	
P16975	Basement-membrane protein 40		1.04E-02	

Accession	Protein names	Trend	Fold change	P value
A0A0G2K8S9	NAD(P)(+)–arginine ADP-ribosyltransferase		1.05E-02	
P18418	Calreticulin		1.08E-02	
P54311	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1		1.10E-02	
D3ZQ25	Fibulin-1		1.12E-02	
F1LZ11	Ig-like domain-containing protein		1.13E-02	
P50137	Transketolase		1.15E-02	
P36375	Glandular kallikrein-10		1.17E-02	
A0A0G2K595	Solute carrier family 23 member 1		1.22E-02	
P01681	Ig kappa chain V region S211		1.23E-02	
B0BNJ1	LOC683667 protein		1.25E-02	
P31044	Phosphatidylethanolamine-binding protein 1		1.25E-02	
Q63751	Vomeromodulin		1.33E-02	
M0R8G6	Dihydrolipoyl dehydrogenase, mitochondrial		1.34E-02	
Q6P6R2	Polymeric immunoglobulin receptor		1.38E-02	
D4A4D5	60S acidic ribosomal protein P2		1.40E-02	
Q08834	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A		1.41E-02	
P61459	Pterin-4-alpha-carbinolamine dehydratase		1.45E-02	

Accession	Protein names	Trend	Fold change	P value
F7F707	Enhancer of mRNA-decapping protein 4		1.47E-02	
Q6Q0N1	Cytosolic non-specific dipeptidase		1.47E-02	
M0RC23	CD248 antigen, endosialin		1.56E-02	
D3ZN06	Acid ceramidase		1.56E-02	
Q9JJI3	Alpha-2u globulin		1.60E-02	
P07861	Neprilysin		1.63E-02	
Q64335	Killer cell lectin-like receptor subfamily G member 1		1.66E-02	
P63322	Ras-related protein Ral-A		1.68E-02	
O55145	Fractalkine		1.68E-02	
D3ZV91	trans-L-3-hydroxyproline dehydratase		1.73E-02	
Q498R7	CXXC motif containing zinc binding protein		1.78E-02	
A0A0G2JXZ9	protein-tyrosine-phosphatase		1.79E-02	
G3V8R1	Nucleobindin 2		1.87E-02	
A0A0G2JTX5	Dipeptidyl peptidase 4		1.98E-02	
F1LM16	Serpin family E member 1		2.02E-02	
A0A0H2UHN2	isopentenyl-diphosphate Delta-isomerase		2.04E-02	
Q5I0D7	Xaa-Pro dipeptidase		2.05E-02	
F1LYX9	Desmoglein 2		2.10E-02	
G3V615	C3/C5 convertase		2.16E-02	
G3V8X5	Solute carrier family 5		2.18E-02	

Accession	Protein names	Trend	Fold change	P value
Q80W57	Broad substrate specificity ATP-binding cassette transporter ABCG2		2.25E-02	
Q63797	Proteasome activator complex subunit 1		2.27E-02	
A0A0G2K5X3	C-type lectin domain family 2 member d3		2.37E-02	
Q5I0M3	Complement component factor h-like 1		2.38E-02	
D3ZQN7	Laminin subunit beta 1		2.43E-02	
Q64602	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial		2.52E-02	
D3ZWD6	Complement C8 alpha chain		2.53E-02	
G3V837	CD1d1 molecule		2.67E-02	
D4A740	Interleukin 17 receptor A		2.74E-02	
O55004	Ribonuclease 4		2.81E-02	
P42854	Regenerating islet-derived protein 3-gamma		2.84E-02	
F1LQI1	Hydroxyacyl glutathione hydrolase		2.85E-02	
G3V6W3	Hepatitis A virus cellular receptor 1		2.86E-02	
A0A0G2K588	Latent transforming growth factor beta binding protein 4		2.95E-02	
D4A5I9	Unconventional myosin-VI		3.02E-02	
Q5U362	Annexin		3.02E-02	
Q642B0	Glypican 4		3.03E-02	

Accession	Protein names	Trend	Fold change	P value
M0R7M5	Solute carrier family 2, facilitated glucose transporter member 5		3.07E-02	
A0A0G2JT43	Spondin-1		3.19E-02	
P13221	Aspartate aminotransferase, cytoplasmic		3.23E-02	
P97584	Prostaglandin reductase 1		3.27E-02	
F1LMA7	Collagen-binding factor Endo180		3.28E-02	
P09606	Glutamine synthetase		3.30E-02	
Q5XI77	Annexin		3.41E-02	
D3ZDH4	receptor protein-tyrosine kinase		3.49E-02	
P62804	Histone H4		3.53E-02	
G3V6D9	Na(+)/H(+) exchange regulatory cofactor NHE-RF		3.69E-02	
A0A0G2JSQ7	Kallikrein 1-related peptidase B3		3.92E-02	
P14480	Fibrinogen beta chain		4.03E-02	
D4A911	Transmembrane serine protease 15		4.13E-02	
A0A0G2K0Q7	Myosin light chain kinase		4.17E-02	
P07943	Aldo-keto reductase family 1 member B1		4.33E-02	
P07632	Superoxide dismutase		4.40E-02	
D4A4R7	RCG21015, isoform CRA_a		4.47E-02	
G3V963	RCG47487, isoform CRA_b		4.48E-02	

Author Contributions Statement

Youhe Gao: Conceived the research idea, designed the study, and revised the final manuscript.
Yuqing Liu: Conceived the research idea, designed the study, performed experiments, collected and analyzed data, drafted and revised the manuscript.
Minhui Yang, Haitong Wang, Yuzhen Chen: Performed experiments.

Note: Figure translations are in progress. See original paper for figures.

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