Effects of cell aging on proteomics and post-translational modifications of UMSCs

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Abstract

Background

Mesenchymal stem cells (MSCs) are thought to be critical for successful regenerative medicine and immunosuppressive therapy. However, long term culturing of MSCs is known to impair cellular function and induces aging of MSCs.

Methods

To investigate the cellular mechanisms involved in the aging of MSC cultures, we analyzed human umbilical cord mesenchymal stem cells (hUMSCs) exposed to different culture conditions (glucose concentrations were 5.5 mM/25 mM/40 mM) for 7/30 days using tandem mass tag (TMT) labeling quantitative proteomics, analyzing posttranslational protein modifications and applying bioinformatics methods. Differentially expressed proteins (DEPs) were clustered and functional annotated by Gene Ontology (GO) enrichment analysis. Pathway enrichment analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation tool. To identify the changes in N-glycans properties during culturing of hMSCs for either 7 or 30 days under three different glucose concentrations, we analyzed the mesenchymal stem cell glycome using a mass spectrometry-based N-glycan profiling method.

Results

Here, we found that the morphology of hUMSCs during a long-term culturing period changed significantly compared with 7 days conditions. More specifically, we fund that a total of 66 proteins (fold change >1.50 and P-value <0.05 as criteria) were differentially expressed in long term culture hUMSCs, with 49 proteins upregulated and 17 proteins downregulated. Our GO analysis showed that aging exerts a side effect on
UMSCs by affecting variable molecular functions and biological processes, such as lysosome organization, exopeptidase activity, hydrolase activity, glycosaminoglycan binding and post-translational protein modification. KEGG analysis demonstrated that differential abundance proteins were significantly enriched in lysosomes, renin-angiotensin system, other glycan degradation and autophagy. The results of glycosylation analysis showed that cell N-glycans is associated with aging.

**Conclusion**
In conclusion, our comprehensive analysis revealed that the difference in differentially expressed proteins and post-translational protein modification levels between aging group vs control, which could provide a possible basis for aging and cell quality control study.

**Keywords**
mesenchymal stem cells; aging; quantitative proteomics; post-translational modifications

**Introduction**
Stem cell therapy possesses considerable potential for the clinical treatment of numerous diseases currently untreatable[1]. MSCs are non-haematopoietic, multipotent progenitor cells, which exhibit clonogenic and self-renewing capabilities and differentiate into a wide range of different cell types. These MSCs possess numerous advantages over stem cell therapy, including the ability for nontumorigenic differentiation, their prevalent homing to injured tissues, their immunodulatory and immunosuppressive properties, paracrine support as well as wound-healing effects, and their wide distribution throughout the entire adult organism[2]. Therefore, MSCs have attracted considerable interest as a suitable tool for disease treatment, and in particular, hUMSCs. These cells constitute an ideal and practical source of MSCs as they are abundant, readily to obtain, painless harvesting procurement, along with precluding any ethical concerns[3]. MSC-based regenerative therapies are currently used in clinical treatment of various diseases, including diabetic vasculopathy, graft-vs.-host disease (GvHD), autoimmune diseases and ischemic diseases. In addition, MSCs are used as vehicles for delivering anti-cancer drugs.

When cells proliferate to a certain extent in vitro, the cells become growth arrested, senesce and even death with cell to cell contact inhibition[4]. Under aging stress inducing conditions, it was shown that significant decline in cell number and function which cause stem cell regenerative capacity damage. Along with the prolongation of the culture time, MSC progressively lose activity on account of aging, which strongly affects stem cell applications[5].

The important predisposing factors that affect cellular senescence include changes in the internal and external cellular environment, increased cell replication and
information exchange between neighboring cells. One hallmark of aging constitutes the progressive impairment in protein homeostasis\cite{6,7}. Protein homeostasis is required for preserving cellular stability and functionality, including genetic replication, catalysis of metabolic reactions, and the immune response\cite{8}. It is now well established that misregulation of protein maintenance, affects cell’s capacity to regenerate, with decline of the regeneration capacity being directly linked to the accumulation of time-dependent damage\cite{9}. Loss of proteostasis impairments is one of the sign for aging, which involves accumulation of damaged organelles and unwanted protein aggregates\cite{6}. Autophagy-lysosomal system and the ubiquitin-proteasome system are critical for aging, and directly act on cellular protein homeostasis\cite{10,11}. To study the molecular mechanisms behind aging, method using proteomics combined with bioinformatics is currently used to analyze age-related MSCs. Recent advancement of proteomic technique enables the set of proteins responsible for a particular cell phenotype to be illuminated, which have been repeatedly used to help researchers to analyze the system biology and exploring the active factors in MSC\cite{12,13}. As proteins mediate most intracellular activity and communication between cells, mass spectrometry proteomics approaches are a promising tool to elucidate differential cell states and changes in signaling pathways\cite{14}.

Protein post-translational modification directly affects protein function. Studies on aberrant regulation of glycosylation and the occurrence of various diseases have been carried out extensively, including cancer, autoimmune disease and inflammation\cite{15}. Glycosylation is closely related to aging. Glycosylation is one of the most abundant post-translational modifications (PTMs) of proteins\cite{16}. IgG glycome composition alters gradually with age\cite{17}. Another study showed that during the early onset of Alzheimer’s Disease, a considerable number of proteins change their glycosylation patterns, indicating that pathogenesis is partially characterized by glycosylation\cite{18}. Glycosylation of proteins constitutes one of the quality control indexes of protein synthesis. N-linked glycosylation also act as reporters of the folded state and age of the glycoprotein\cite{19}. A structural characterization of the cell N-glycans is critical to gain a better understanding of glycan functions in both inter- and intracellular processes. Protein glycosylation may play an important role in the aging process of UMSCs.

The importance of proteins and their glycosylation of in stem cell aging has been exposed to view. To study the response of aging to MSC and mine of interested protein, we apply TMT combined with liquid chromatography with tandem mass spectrometry (LC-MS/MS). In the present study, we subjected hUMSCs cultured in different culture conditions to a protein expression and post-translational modifications analysis. And then we classified the functions of the identified proteins, characterized signaling pathways as well as protein-protein interaction (PPI) networks that are differentially expressed at the protein level in MSCs exposed to 30 days culture conditions compared to 7 days expansion conditions. These findings of critical proteins were expected to provide a theoretical basis for cell quality control analysis. The results would provide
us more information on the protein and post-translational modifications level responsible senescence of MSC.

**Methods**

**Culture of human umbilical cord mesenchymal stem cells**

Human umbilical cord mesenchymal stem cells were purchased from Ever Union Biotechnology Company. Mesenchymal tissues were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (HyClone, US) in a humidified 5% CO₂ atmosphere at 37°C. Once cells reached 70–80% confluence, the adherent cells were trypsinized using 0.25% trypsin (Thermo Scientific, US) and further propagated in larger flasks. UMSCs in passages 3–5 were used for further experiments.

To understand the effects of aging on MSCs, MSCs were cultured for 7 or 30 days, respectively. In addition, cells were washed and cultured at three different glucose concentrations: 5.5 mM glucose medium (1 g/L), 25 mM glucose medium (4.5 g/L) and 40 mM glucose medium (7.2 g/L).

**N-glycan release and isolation from cell glycoproteins**

Approx. 4x10⁶ cells were first reduced with DL-Dithiothreitol (Sigma-Aldrich, US) solution for 60 min at 37°C followed with iodoacetamide (Sigma-Aldrich, US) in the dark at room temperature for 1 to 2 hours. The glycoproteins were further incubated with trypsin at a mass ratio of 1:50 (trypsin: protein) at 37°C overnight. The digested samples were purified using the Oasis hydrophilic-lipophilic balanced (Waters, Milford, MA) 5% acetic acid / propan-1-ol system (J.T. Baker, US). The N-glycans were separated from the mixture using the Sep-Pak® C18 (Waters, Milford, MA) propanol-1-ol / 5% acetic acid system.

**Permethylation of N-glycans and N-glycan detection by matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight multistage mass spectrometry (MALDI-TOF-MS²)**

Dried N-glycans were permethylated according to a previously reported method[20–22] and then further purified using a Sep-Pak® C18 acetonitrile (ACN)/water system (J.T. Baker, US). Permethylated N-glycans (1.5 μL) were mixed with 2,5-dihydroxybenzoic acid (ProteoChem, US) solution (10 mg/mL, containing 50%ACN and 0.1% trifluoroacetic acid) in a 1:1 ratio onto the μ-focus MALDI plate target (900 μm, 384 circles) until dry at room temperature. The N-glycan samples were analyzed using an Axima MALDI Resonance mass spectrometer with a QIT-TOF configuration with the following settings: 200 shots per sample, Mid 850+, power 90,250 resolution and the positive-ionization mode. Data were analyzed using the databases (CFG, Carbbank, GlycomeDB and Glycosciences) to predict the relative structures of N-glycans using GlycoWorkBench.

**Sample preparation for proteomics and Tandem Mass Tags labeling**

At least one hundred micrograms of proteins were reduced with 5 µL of the 200 mM tris(2-carboxyethyl)phosphine (Thermo Scientific, US) for 1 hour at 55 °C.
Subsequently, alkylation was with a final concentration of 375 mM iodoacetamide for 30 min at room temperature protected from light. Proteins were precipitated by adding pre-chilled (-20°C) acetone (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) to precipitate for at least 4 hours. Next, proteins were dissolved with 0.5 μg/μL trypsin at 37°C overnight. MSC peptides were reconstituted in 0.5 M triethyl ammonium bicarbonate (Thermo Scientific, US) and labeled with TMT 6-plex reagent according to the manufacturer’s instructions. Labeled peptides were then pooled equally, before being desalted using hydrophilic-lipophilic balanced (HLB) C18 cartridge columns, and finally concentrated via vacuum centrifugation.

**Proteomics on Thermo Scientific nanoLC-Q EXACTIVE (QE)**

Nanospray LC-MS/MS analysis were performed using an EASY-nLC1000 HPLC system coupled to a Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer. Samples were resolved in 20 μL 0.1% formic acid (solvent A), before being loaded onto a gradient elution through a C18-reversed-phase analytical column (75 μm x 20 cm, particle size: 3 μm). The QE parameter settings were described as follows: 300 nL/min flow rate was maintained throughout the entire process; Solvent A was 0.1% formic acid dissolved in water; and Solvent B was acetonitrile mixed with 0.1% formic acid. After equilibration, a linear gradient was started as follows: 0–8 min, 96%A, 4%B; 8–58 min, 92%A, 8%B; 58–70 min, 68%A, 32%B; 70–71 min: 10%A, 90%B;71–78 min: 10%A, 90%B. Mass spectrometry data were acquired using a data-dependent Top20 method dynamically choosing the most abundant precursor ions (1 MS scan followed by 20 MS/MS scans) from the scan range (300–1600 m/z) for full scan. The dynamic exclusion duration was set to 40.0 s. The electrospray voltage applied was set to 2.0 kV and the heated capillary temperature was maintained at 320 °C.

**Database search and bioinformatics analysis**

The MS/MS spectra were processed using proteome Discoverer software program, version 1.4. The raw MS/MS data was processed using SEQUEST HT search engine which was set up to search the Uniprot-Proteome-Human(20180920) database. The SEQUEST HT software settings were as follows: (1) Trypsin was specified as cleavage enzyme, allowing up to two missed cleavage sites; (2) alkylation of cysteine as static modification, and oxidation of methionine of protein as dynamic modifications; (3) a parent ion tolerance of 10 ppm for peptide ions and 20 mDa for fragment ion mass tolerance, respectively; (4) false discovery rate (FDR) was adjusted to ≤ 1% for protein identification and the peptide-spectrum matches. Only proteins with more than 1.20-fold or less than 0.83-fold change and a p-value < 0.05 in two comparable groups were considered as differentially expressed proteins (DEPs).

Metascape analysis (http://metascape.org/) was used to analyze Gene Ontology annotation of DEPs. Furthermore, Kyoto Encyclopedia of Genes and Genomes database description was performed by using Metascape. As a result, two-sample two-tailed Student’s t-test with a p-value < 0.01 was considered as significantly enriched. The protein–protein interaction network was examined using the online Search Tool for
the Retrieval of Interacting Genes/Proteins (STRING) algorithm (https://string-db.org).

**Results**

**The morphology of the hUMSCs at long-term culture conditions**
After 7 days of cultivation, the adherent cells retained a typical fibroblastic spindle shape, exhibited plastic-adherent growth, and proliferated quickly in vitro. The MSCs showed highly characteristic traits of aging in long-term cell culture. The cells were not clearly spaced. A small number of cells were found to be irregular in shape and flatten progressively with a rapid decline in attachment of MSCs.

![Fig. 1 The morphology of UMSCs at long-term culture conditions.](image)

UMSCs retained a typical fibroblastic spindle shape for 7 days. After 30 days of culturing in 5.5 mM glucose medium (1 g/L), 25 mM glucose medium (4.5 g/L) and 40 mM glucose medium (7.2 g/L), MSCs showed highly characteristic traits of aging.

**Protein identification and quantification of differentially expressed proteins**
To assess the dynamic changes of proteins and to identify the cellular mechanisms responsible for aging in hUMSCs, we quantified the proteins obtained from six experimental groups of human umbilical cord mesenchymal stem cells, which were cultured in 5.5/25/40 mM glucose medium for either 7 or 30 days, using a high-throughput quantitative proteomics by the 6-labeled TMT peptide labeling coupled with LC-MS/MS techniques. The process diagram of our quantitative proteomic study is shown in Fig. 2.
Fig. 2 Quantitative proteomics workflow for hUMSCs in aging condition. In the 5.5 mM glucose groups for 7 days/30 days, 167 proteins were differentially expressed based on Student’s t-test with “fold change >1.50 or <0.67 and P-value <0.05” as thresholds. Overall, 106 and 61 proteins were upregulated and downregulated in 1947 quantified proteins, respectively. In the comparisons 25 mM glucose groups and 40 mM glucose groups for 7/30 days, 122 (67 upregulated, 55 downregulated) and 113 (87 upregulated, 26 downregulated) differentially expressed proteins were changed, respectively. Interestingly, we found that changes in protein expression were highest when cells were incubated at low glucose concentrations.

Table 1 Statistical analysis of significant differentially expressed protein of MSC under six culture conditions

| Sample comparable group | Numbers of proteins | Differentially Expressed Proteins | Up-regulated Differentially Expressed Proteins | Down-regulated Differentially Expressed Proteins |
|-------------------------|---------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------|
| 5.5 mM (7 days vs 30 days) | 1947                | 167                             | 106                                           | 61                                            |
| 25 mM (7 days vs 30 days)  | 1947                | 122                             | 67                                            | 55                                            |
| 40 mM (7 days vs 30 days)  | 1947                | 113                             | 87                                            | 26                                            |

vs_ : preceding the reference sample, followed by the sample, same as below

Proteins which met the criteria (p value <0.05 and fold change >1.5) were designated as DEPs. The information about DEPs was present in the compared groups of 5.5 mM (7 days vs 30 days), 25 mM (7 days vs 30 days) and 40 mM (7 days vs 30 days).

In particular, to reveal the common and specifically DEPs associated with aging-regulated mechanism, we performed Venn diagram analysis. Venn diagram showed comparison of up-regulated proteome in 5.5 mM (7 days vs 30 days), 25 mM (7 days vs 30 days) and 40 mM (7 days vs 30 days) (Figure 3A). As shown in Fig 3A, 49 proteins were differentially expressed in all three compared groups. These proteins were related to lytic vacuole, lysosome organization, secretory granule lumen, carbohydrate
derivative catabolic process, autolysosome and autophagy. Our analysis showed that 106, 67, and 87 proteins exhibited significant changes in expression levels from 7 days to 30 days of culturing in 5.5 mM, 25 mM, and 40 mM glucose, respectively. Venn diagram analysis of down-regulated proteome was performed within 7 days vs 30 days (Figure 3B). A grand total of 17 differentially expressed proteins were clustered in all three groups, suggesting that these DEPs participate in the regulation of cytoskeletal organization, including processes such as cadherin binding and microtubule cytoskeleton organization.

Fig. 3 (A) Up-regulated DEPs were displayed by the Venn diagram in the compared groups of 5.5 mM (7 days vs 30 days), 25 mM (7 days vs 30 days) and 40 mM (7 days vs 30 days). (B) Down-regulated DEPs were analyzed by the Venn diagram in the compared groups of 5.5 mM (7 days vs 30 days), 25 mM (7 days vs 30 days) and 40 mM (7 days vs 30 days).

**N-glycan profiling of cell membrane glycoproteins and cell glycoproteins**

To identify the changes in N-glycans properties during culturing of hMSCs for either 7 or 30 days under three different glucose concentration, we analyzed the mesenchymal stem cell glycome using a MS-based N-glycan profiling method. As shown in Fig.4, hMSCs exhibit a characteristic and complex protein N-glycosylation profile.

![Mass spectra of N-glycan peaks in cell glycoproteins.](image)

Fig. 4 Composition and putative structure of N-glycans identified during culturing of hMSCs for
either 7 or 30 days hMSCs in under three different glucose concentration
Mass spectra of N-glycan peaks obtained from whole cell glycoprotein samples. Symbol key: NN-acetylglucosamine (blue square), mannose (green circle), galactose (yellow circle), fucose (red triangle), SAN-Acetylneuramic acid (purple diamond), GAN-glycolyneuraminic acid (light green diamond).

Our analysis showed that for 7 days samples, the N-glycan peaks derived from cell glycoproteins primarily represented sugars of the high mannose type. In contrast, for 30 days samples the total glycomes are mainly comprised of the complex type N-glycan Fuc1Hex5HexNAc4. Our analysis also showed that protein post-translational modification features of MSCs for 7 days and 30 days were consistently different in biological replicates, indicating that stem cells for short-term and long-term culture exhibit glycosylation features that correlate with culture time. Our results strongly suggest that while glucose concentration affects changes in N-glycan patterns only negligibly, culture time asserts a considerable effect on N-glycan patterns. On the basis of these results, we hypothesized that cell culturing affects protein post-translational modification. Thus, analysis of glycosylation changes should provide a suitable tool to evaluate the aging process of MSC in cell culture.

Bioinformatics analysis reveal aging regulated UMSCs
To investigate aging regulated hUMSCs, we performed Gene ontology enrichment analysis for the differential proteins was submitted to the bioinformatics tool Metascape, which involved analysis of molecular function (MF), biological process (BP) and cellular component (CC). The result of 7 days/30 days samples showed that up-regulated differentially expressed proteins were enriched in cell components such as lytic vacuole, primary lysosome, ficolin-1-rich granule, Golgi lumen and early endosome. And on the basis of various molecular functions, they primarily played important roles in lysosome organization, carbohydrate derivative catabolic process, glycosphingolipid metabolic process, lysosomal transport, autophagy and post-translational protein modification. Further, they were mainly enriched in biological processes, for instance, exopeptidase activity, hydrolase activity, glycosaminoglycan binding, extracellular matrix structural constituent, integrin binding, protein homodimerization activity were enriched (p<0.01). The down-regulated differential abundance proteins were found to be involved in positive regulation of cytoskeleton organization, cadherin binding, xenobiotic metabolic process and microtubule cytoskeleton organization in the 7 days versus 30 days group (p<0.01).
**Fig. 5** Bioinformatics analysis for differentially regulated proteins of MSC in six culture conditions. Diagram showing the GO analysis of (A) up-regulated differentially expressed proteins of 7 days versus 30 days group (B) down-regulated differentially expressed proteins of 7 days versus 30 days group using Metascape analysis. (C) KEGG pathway analysis of the differentially up-regulated proteins of UMSCs involved in aging.

To further investigate the biological relevance of the observed up-regulated differentially expressed proteins in response to aging-induced alterations, we utilized KEGG pathway annotation tool. As shown in Fig.5C, the optimized differentially expressed proteins after culture for 7 days/30 days groups were largely enriched in 5 pathways ($p < 0.01$), including lysosome, renin-angiotensin system, other glycan degradation and autophagy.

**Protein interaction network of differentially expressed proteins in response to aging-induced alterations**

To identify the protein-protein interaction networks of aging of hUMSCs, we constructed a protein interaction map using the STRING algorithm. The up-regulated PPI network was constructed based on DEPs by 46 nodes, 80 edges, an average node degree of 3.48 with local clustering coefficient 0.529 and PPI enrichment $p$-value <0.01. Only high interaction scores (score > 0.7) were chosen.
As shown in Fig.6, these results indicate that the strong enrichment of the protein-protein interaction network was associated with lysosome, renin-angiotensin system, other glycan degradation and autophagy. In accordance with the cellular components, PPI were mainly involved in lysosome, vacuolar lumen and secretory vesicle; concerning the molecular function, hydrolase activity and exopeptidase activity were all enriched in the PPI network; for biological processes, secretion, regulated exocytosis, neutrophil degranulation and secretion by cell were commonly found in compared groups. An interaction with a combined score > 0.4 was considered as statistical significance.

Also, overlapping down-regulated aging-related DEPs (17 DEPs) were identified (61 DEPs, 55 DEPs and 26 DEPs in 5.5 mM (7 days/30 days), 25 mM (7 days/30 days) or 40 mM (7 days/30 days) group, respectively), followed by further molecular network analysis. Notably, the down-regulated aging-related PPI network was involved in regulation of cellular component size, positive regulation of protein complex disassembly, cytoskeleton organization, actin filament fragmentation and Fc gamma R-mediated phagocytosis by 15 nodes and 12 edges.

**Discussion**

The process of aging is a complex phenomenon in which a series of cellular and molecular changes appear to be involved. In summary, we showed that aging of long-term cell culture affects cell morphology and suppresses cell proliferation. Additionally, we further adopted efficient quantitative proteomics and post-translational protein modification methods for aging-induced cellular responses.

Our results showed that proteins associated with autophagy - lysosome are upregulated in UMSCs, under long-term culture conditions. In our results, the PPI showed that Cathepsin D (CTSD), Beta-galactosidase (GLB1), Cathepsin B (CTSB), Lysosome-associated membrane glycoprotein 1 (LAMP1), and Lysosome-associated membrane glycoprotein 2 (LAMP2) implicated in lysosome, which might be related to cellular processes, transport and catabolism. These findings are in agreement with several previous studies. Confirming past studies, the autophagy-lysosomal system was declined with aging[10,11]. CTSD, CTSB, LAMP1, and LAMP2 are also autophagy-related protein. Aging-related pathologic processes can trigger cell damage via different pathways, involving a variety of enzymatic reactions, including proteolytic enzymes from lysosome into the extracellular matrix (ECM).[23] For instance, CTSD and CTSB, lysosomal protease involved in lysosomal proteins degradation, are up-regulated at the protein level in UMSc[24,25] in response to oxidative stress, and the activity of CTSB increases with age[26,27], which is considered to play a major role in aging. GLB1 is a lysosomal enzyme which is the origin of senescence associated-β-gal activity (SA-β-gal) [28]. LAMP family participated in many physiological and pathological processes including phagocytosis, autophagy and aging. LAMP1 and LAMP2 proteins line the
lysosome membrane, and protect it against the action of the hydrolytic enzymes[29].

We also found that hexosaminidase subunit beta (HEXB) and plasma alpha-L-fucosidase (FUCA2) were significantly upregulated in the PPI with carbohyd rase as the core of the network. Previous studies have showed that elevated expression of HEXB, localized to lysosomes, might enhance the degradation of GM2-gangliosides, a variety of other molecules containing terminal N-acetyl hexosamines, affect signaling and progressively expose different saccharides with increased protein age[30]. Here, we found that FUCA2 was in the center of the protein-protein interaction network between 7 days and 30 days groups. Alpha-L-fucosidase (AFU) is responsible for hydrolyzing the alpha-1,6-linked fucose joined to the reducing-end N-acetylg glucosamine of the carbohydrate moieties of glycoproteins in all mammalian cells. Many studies reported that FUC has been proposed as a serum marker for the diagnosis of hepatocellular carcinoma (HCC)[31,32]. Altered expression of fucosylated glycans has also been observed in several pathological processes, including atherosclerosis, sperm maturation in mammals, and cancer[33]. In our study, FUCA2 was upregulated in 7 days and 30 days groups (49 overlapping DEPs), which played a vital role in a variety of biological processes, such as regulated exocytosis, neutrophil mediated immunity, secretion by cell and neutrophil degranulation. One previous study showed that FUCA2 and HEXB involved in the modification of glycans are up-regulated with age[34], accompanied by alterations in the glycosylation of membrane components. These changes in turn would result in the alteration in cellular signaling. Subsequent work could focus on the HEXB and FUCA2 carbohydrate enzymes for further in-depth research on cell aging.

Due to the prominent cell-surface localization and lineage-specific signatures of glycans, glycans in glycolipids and glycoproteins are optimally positioned to help MSC communicate with its environment through either niche interactions or signaling modulations[35], including both cell-to-cell contacts[36,37] and interactions with extracellular matrix components[38]. Furthermore, components of the glycocalyx on the cell are involved in various biological phenomena including cell adhesion, cell differentiation, and proliferation[39]. Dynamic analysis of the MSC’s glycome in different culture time state will provide useful information about characteristics of their cell and understand important roles in the self-renewal and differentiation of stem cells. Glycan expression profiles have been predicted to be extensively utilized, provide insight into the glycobiology of hUMSC and critically contribute to future studies exploring the role of stem cell glycans.

In conclusion, multiple aging-related altered molecular characterizations, large-scale quantitative protein, protein posttranslational modification profiling. The data obtained using TMT-based quantitative proteomics and MALDI-TOF-MS protein posttranslational modifications revealed more information about the important functions and metabolic pathways involved in aging, thereby provide a more complete picture of the underlying mechanism. These findings offered a basis for in-depth understand molecular mechanism of aging-related MSCs.
**Conclusion**
Here we showed 49 up-regulated differentially expressed proteins and 17 down-regulated differentially expressed proteins were identified commonly from 7 days vs 30 days. DEPs were used to analyzed by Gene ontology, Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses and protein-protein interactions. Most of the up-regulated DEPs were involved in exopeptidase activity, hydrolase activity, lytic vacuole, lysosome organization, renin-angiotensin system, other glycan degradation and autophagy, while down-regulated DEPs participated in positive regulation of cytoskeleton organization, cadherin binding and xenobiotic metabolic process. In addition, aging may affect protein and post-translational protein modification.

**Availability of data and materials**
All data generated or analysed during this study are included in this published article.

**Abbreviations**
MSCs: Mesenchymal stem cells
hUMSCs: human umbilical cord mesenchymal stem cells
TMT: tandem mass tag
DEPs: Differentially expressed proteins
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
GvHD: graft-vs.-host disease
PTMs: post-translational modifications
LC-MS/MS: liquid chromatography with tandem mass spectrometry
PPI: protein-protein interaction
DMEM: Dulbecco’s modified Eagle’s medium
MALDI-TOF-MS*: matrix-assisted laser desorption/ionization quadrupole ion trap
time-of-flight multistage mass spectrometry
ACN: acetonitrile
HLB: hydrophilic-lipophilic balanced
QE: Q EXACTIVE
FDR: false discovery rate
DEPs: differentially expressed proteins
STRING: Search Tool for the Retrieval of Interacting Genes/Proteins
MF: molecular function
BP: biological process
CC: cellular component
CTSD: Cathepsin D
GLB1: Beta-galactosidase
CTSB: Cathepsin B
LAMP1: Lysosome-associated membrane glycoprotein 1
LAMP2: Lysosome-associated membrane glycoprotein 2
ECM: extracellular matrix
SA-β-gal: senescence associated-β-gal activity
HEXB: hexosaminidase subunit beta
FUCA2: plasma alpha-L-fucosidase
AFU: Alpha-L-fucosidase
HCC: hepatocellular carcinoma

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JHC, LC and YL conceived and designed this study; JYW performed the experiments, analyzed the data, drafted and critically revised the manuscript; YL, KLZ and JHC revised the manuscript; KLZ, QS and JYZ provided suggestions and discussion; YL, LC and JHC contributed to funding acquisition; All authors read and approved the final manuscript.

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