Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-Based proteomic analysis of mRNA splicing relevant proteins in aging HSPCs

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Abstract

Background: HSPC aging was closely associated with the organism aging, senile diseases and hematopoietic related diseases. Therefore, study on HSPC aging born great significance to further elucidate the mechanisms of aging and to treat hematopoietic disease resulted from HSPC aging. Little attention had been paid to mRNA splicing as a mechanism underlying HSPC senescence.

Results: We used our lab’s patented aging model of HSPCs in vitro to analyze mRNA splicing relevant proteins alterations with iTRAQ based proteomic analysis. We found that not only the notable mRNA splicing genes such as SR, hnRNP, WBP11, Sf3b1, Ptbp1 and U2AF1 but also the scarcely reported mRNA splicing relavant genes such as Rbmxl1, Dhx16, Pcbp2, Pabpc1 were significantly down-regulated. We further verified their genes expressions by qRT-PCR. In addition, we reported the effect of Spliceostatin A (SSA), which inhibits mRNA splicing in vivo and in vitro, on HSPC aging.

Conclusions: It was concluded that mRNA splicing emerged as an important vulnerability of HSPC aging. This study improved our understanding of the role of mRNA splicing in the HSPC aging process.

Background

The mammalian blood system consists of many types of differentiated blood cells such as erythrocytes, T-and B-lymphocytes, myeloid cells. These blood cells are mostly short-lived and need to be replaced depending on hematopoietic progenitor cells (HPCs) and ultimately hematopoietic stem cells (HSCs)[1–2].

Although blood is the definitive self-renewing tissue of the body, it does not escape the detrimental effects of the aging process. Hematopoietic aging is manifested in human populations in the form of an increase in organism aging and myelopro- liferative disease, including leukemias, declining adaptive immunity, and greater propensity to anemia[3–7].The previous study indicated age-related alterations in the human blood system occur in hematopoietic stem and progenitor cells (HSPCs)[8–9]. Therefore, study on HSPC aging bore great significance to further elucidate the mechanisms of aging and to treat hematopoietic disease resulted from HSC aging.

mRNA splicing is the process by which introns are removed from a pre-mRNA and exons are joined to

produce a mature mRNA[10]. A single type of pre-mRNA can be spliced in different ways to generate distinct mRNAs and this process is a major contributor to transcriptomic and proteomic diversity in higher eukaryotes[11].In humans, nearly all multi-exon primary transcripts are alternatively spliced[12–13].

If pre-mRNAs with introns were translated into proteins, truncated and deleterious proteins would be produced since intron sequences harbor a large number of termination codons[14].

In recent years, dysregulations of mRNA splicing are emerging to be discovered as important players in organismal ageing, cellular senescence and ageing-related degenerative diseases. Changes in the activity of splicing factors and key splice variants could impact cellular senescence and the aging phenotype[15–18]. However, the studies on mRNA splicing mostly focused on a designated gene so far, lack of systematic and comprehensive analysis. Morever, mRNA splicing alterations in HSPC aging process were never reported[19],whether mRNA splicing was an important mechanism of HSPC aging was worth exploring. So, in the present study, we analyzed the alterations of mRNA splicing relevant proteins with iTRAQ based proteomic analysis and Realtime PCR in our lab’s patented model of aging HSPCs in vitro, aim to explore the mechanism of mRNA splicing in HSPC aging process. Furthermore, we also observed the effect of Spliceostatin A (SSA), which is a chemically stable, methylated derivative of FR901464, binds to the SF3b complex and inhibits splicing in vivo and in vitro[20],on HSC aging.

Results

1.Global profiling of proteins in HSPC

The global profiling of proteins in young HSPCs and aging HSPCs were evaluated by the Isobaric Tags for Relative and Absolute Quantitation(iTRAQ) labelling technology in combination with LC-MS/MS. A total of 2542 proteins were identified according to the standard of protein identification (Peptide Threshold 1.0% FDR, 1 Unique Peptide) from the four samples(two young group samples and two aging group samples).

2.Differentially expressed proteins(DEPs) between aging HSPCs and young HSPCs

The differentially expressed proteins(DEPs) between aging HSPCs and young HSPCs were selected

according to the following criteria: (unique peptide ≥ 2 & p□0.01) &(Fold Change□0.67 or Fold Change□1.5). 302 out of total 2542 proteins were quantified to be differently expressed proteins, including 120 up-regulated proteins and 182 down-regulated proteins. The DEPs were shown as volcano plot (Fig. 1). Furthermore, based on the hierarchical cluster analysis, we also showed the heatmap with the DEPs(Fig. 2).

3.GO analysis of differentially expressed proteins

To obtain a global functional view of the DEPs, Gene Ontology (GO) functional classification annotation was conducted with all the DEPs by using GO database (http://www. Geneontology. org/). A GO term was considered significant at p-value < 0.05. The DEPs were categorized by three sets of Ontologies: BP(biological process), CC(cellular component), and MF(molecular function). As shown in Fig. 3, the top 5 significantly enriched GO terms in the BP category was cellular metabolic process, metabolic process, organic substance metabolic process, cellular nitrogen compound metabolic process and primary metabolic process. In the CC category, membrane- bounded organelle, extracellular exosome, extracellular vesicle, extracellular organ- elle and organelle were the top 5 significantly enriched terms. For MF, RNA binding, binding, heterocyclic compound binding, organic cyclic compound binding and nucleic acid binding were the top 5 significant terms.

It’s easy to see from GO data that mRNA splicing was significantly reduced in the aging group compared with the young group (p < 0.01; Table S1). As shown in Table 1, the DEPs of mRNA splicing were mainly enriched in mRNA splicing via spliceosome, regulation of mRNA splicing via spliceosome,spliceosomal complex assembly, alternative mRNA splicing via spliceosome, regulation of alternative mRNA splicing via spliceosome,mRNA cis splicing via spliceosome,spliceosomal snRNP assembly,positive regulation of mRNA splicing via spliceosome. The key DEPs in these mRNA splicing pathway were shown as Table 1. It might indicate that there was a close relationship between HSPC aging and vulnerability of mRNA splicing.

4.KEGG pathway analysis of differentially expressed proteins

To further reveal the potential metabolic pathway involved in HSPCs aging process, signaling pathways analysis of the DEPs was conducted by using the Kyoto Encyclopedia of Genes and

Genomes(KEGG) database (http://www. genome.jp/kegg). The result showed that the DEPs were classified into 17 different pathways (Table S2). The top significantly different KEGG pathway was glycolysis, lysosomal, ribosomal synthesis and mRNA splicing(Fig. 4). Glycolysis ($p < 0.01$; Fig.S1) and lysosomal pathway ($p < 0.01$; Fig.S2) was significantly increased whereas ribosomal synthesis ($p < 0.01$; Fig.S3) and mRNA splicing ($p < 0.01$; Fig. 5) was significantly reduced in the aging group compare with the young group. We compared and analyzed the DEPs related to mRNA splicing in KEGG database, the results showed that not only the reported well mRNA splicing genes such as SR, hnRNP, WBP11, Sf3b1, U2AF1, Ptbp1 (Fig. 5) but also the possible mRNA splicing relavant genes such as Rbmxl1, Dhx16, Pcbp2, Pabpc1 were significantly down-regulated(Table 1, 2). This conclusion is in line with the recent study which showed age-related alterations of splicing factors[21]. This data suggested that mRNA splicing was an important vulnerability of HSPC aging.

5.Protein-protein interaction analysis

To further disclose the possible relation between mRNA splicing and HSPC aging, we performed a protein-protein interaction analysis by STRING Database(http:// string-db.org) version 9.0 with the DEPs of mRNA splicing, aging and hemopoiesis. As seen in Fig. 6, the DEPs of mRNA splicing, aging and hemopoiesis formed a network containing 32 nodes(Fig. 6). The most prominent cluster within the network mainly included splicing proteins. The most important interconnected node was aging related protein Npm1, which had a 2.21-fold down-regulation in response to aging and had the protein-protein interactions with splicing and hemopoiesis. The other noticeable connected nodes were hemopoiesis associated protein Rps19, Rrs1, Ada and Sart3, which had protein- protein interactions with mRNA splicing(Fig. 6).

6.qRT-PCR

To validate the accuracy of the quantitative proteomic analysis, 8 mRNA splicing related genes were randomly selected for realtime-PCR analysis. The A260/280 ratio of RNA extracted from HSPCs was 1.8–1.9, indicating high purity of RNA.The result showed that the mRNA levels of mRNA splicing related genes including Rbmxl 1, Dhx16, Wbp11, U2af1, U2af2, Pcbp2, Ptbp1, Pabpc1 verified by qRT-PCR had the similar trends to the protein levels tested by the quantitative proteomic analysis. The

fold changes of transcript abundances are provided in Fig. 7. It suggested the proteomic analysis was accurate.

7. Effect of SSA treatment on the aging of HSPC

Spliceostatin A (SSA) (Fig. 8)is a chemically stable, methylated derivative of FR901464, can inhibits mRNA splicing in vivo and in vitro.To verify the important role of mRNA splicing in the HSPC aging process, we treated HSPCs with SSA and observed the effect of SSA treatment on aging-related manifestations of HSPCs with SA-β-gal staining and Mixed colony-forming unit (CFU-Mix) culture Method.SA-β-gal (Senescence-associated-β-galactosidase) is a hallmark of aging that can yield a blue stain in the cytoplasm of aging cells. We found that the percentage of SA-β-gal stain-positive in SSA group(HSPCs were cultured with the culture medium (STEMCELL Technologies,) supplemented with the addition of 100 ng mL − 1 of SSA for 72 h ,Fig. 9B) was significantly higher than the control group(HSPCs were cultured with the culture medium for 72 h, Fig. 9A)). The mixed colony-forming units of the HSPCs in SSA group were less than the control group(Fig. 10). We speculated that the inhibitor of mRNA splicing result in HSPCs aging, it further proved the hypothesis that mRNA splicing was an important mechanism of HSPC aging.

Discussion

Little attention has been paid to mRNA splicing as a mechanism underlying SC/HSPC senescence. RNA splicing is highly regulated in higher eukaryotic cells, performed by spliceosome, a large nuclear macromolecular complex that contains five small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4, U5 and U6) and more than 150 accessory proteins [21–24]. These accessory proteins include SR protein (serine/arginine-rich protein), hnRNP(heterogeneous nuclear ribonucleoprotein,hnRNP), RNA helicase,
et al[25].SR protein contain at least one RNA recognition motif and an arginine serine-rich domain to bind the pre-mRNA, regulating splicing and alternative splicing in a concentration-dependent manner. hnRNP, instead, influence splicing events by preferentially binding to splicing silencers[26].Studies of mammalian splicing factors are often focused on snRNP or regulatory RNA-binding proteins, such as hnRNP and SR proteins, however, much less is known about the contribution of RNA helicases. About splicing factor of RNA helicase, DExD/H-box proteins are concerned and they are a family of RNA-dependent ATPases (or NTPases), which utilize the energy from ATP

hydrolysis to modulate the structure of RNA or ribonucleoprotein complexes [27].

The present study showed that not only the notable mRNA splicing genes such as SR, hnRNP, snRNPs, RNA helicase but also the mRNA splicing relavant gene such as Wbp11, u2af, Rbmxl1, Pcbp2, Pabpc1, thrap3, Raver1 and snu13 were significantly downregulated in aging HSPC. By combining ITRAQ data and Uniprot database, we focus on 4 notable mRNA splicing genes Wbp11, u2af1, u2af2, Ptbp1 and 4 scarcely reported mRNA splicing relavant genes Rbmxl1,Dhx16, Pcbp2,Pabpc1,which were verified by qRT-PCR.

Pabpc1(Polyadenylate-binding protein 1,PABP-1) is the component of mRNP(mRNA ribonucleoprotein) complex which is at least composed of DHX9, DDX3X, ELAVL1, HNRNPU, IGF2BP1, ILF3, PABPC1, PCBP2, PTBP2, STAU1, STAU2, SYNCRIP and YBX1[28]. It serves as a central regulator of mRNA fate, mRNA utilization and destruction [29–30].Pabpc1 may be involved in cytoplasmic regulatory processes of mRNA metabolism such as pre-mRNA splicing, but need to be confirmed. Pcbp2(Poly rC-binding protein 2), is a Single-stranded nucleic acid binding protein that binds preferentially to oligo dC. It is identified as a component of mRNP complex. Cong Ren,et al showed that Pcbp2 regulates p73-dependent biological function in ROS production and cellular senescence[31].Rbmxl1(RNA binding motif protein, X-linked-like-1)is also be known as heterogeneous nuclear ribonucleoprotein G-like 1. Gene burden analysis revealed three genes associated with extreme aging, including Rbmxl1[32]. These data suggested a possible close link among Pcbp2, Rbmxl1 and aging, thus it is unclear what is the roles of Pcbp2/Rbmxl1 in HSC aging process. About splicing factor of RNA helicase, Dhx16(ATP-dependent RNA helicase DHX16), is an ATPase that activates the spliceosome before the first catalytic step of splicing[33]. Gencheva M, et al, further suggest that mutant DHX16 causes a defective spliceosome to retain unspliced gene transcripts in the nuclei of human cells[34].It indicated DHX16 was involved in mRNA splicing, however, whether Dhx16 affects aging is largely unknown.

The most important interconnected node rotein in String analysis, Nucleophosmin (NPM), also known as B23, numatrin and NO38, is a multi- functional protein. It’s involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors p53/TP53 and ARF[35].Luiza Handschuh’s study indicated that aberrant proportions of particular NPM1 transcripts could be linked to abnormal expression of genes encoding alternative splicing (AS) factors.It indicated NPM1 was involved in mRNA splicing, however, whether NPM1 affects aging was unknown.

It was suggested from this study that Rbmxl1, Dhx16, Pcbp2, Pabpc1and NPM screened by the prestent study may play an important role in the process of HSPC aging. In addition, HSPCs treated with SSA, the inhibitor of mRNA splicing, showed the manifestations of cell senescence, further proved the hypothesis that mRNA splicing was an important mechanism of HSPC aging. This, we had reasoned, would provide the experimental support for researching mRNA splicing in HSC aging.

Conclusion

Important mRNA splicing relavant genes, including SR, hnRNP, WBP11, Sf3b1, U2AF1, Ptbp1, Rbmxl1, Dhx16, Pcbp2, Pabpc1 were compromised by HSPC aging. String network analysis suggested that NPM1 were the most important protein regulation hubs of HSPC aging and mRNA splicing.HSPCs treated with SSA, the inhibitor of mRNA splicing, showed the features of cell senescenc proved the hypothesis that mRNA splicing was an important mechanism of HSPC aging.The identification of these mRNA splicing associated proteins can help us to better understand the molecular mechanisms of mRNA splicing in HSPC aging.

Methods

Animals

Equal numbers of male and female C57BL/6J SPF mice were obtained from Shanghai Sippr-BK Experimental Animal Center [Certificate No. SCXK (Shanghai) 2013- 0016]. The mice were 4 weeks of age and 16–18 g in weight, no gender limitation.

Chemicals and reagents

Red blood cell lysis buffer was purchased from Beyotime Biotechnology Co., Ltd. Anti-c-kit (CD117) MicroBead and Lineage Cell Depletion kits were purchased from Miltenyi Co., Ltd. RNA Extraction and Purification kits, Reverse Transcription and Fluorescence Quantitative PCR kits were purchased from Takara Co., Ltd. Stemspan Stem Cell Media were purchased from Stem cell Co., Ltd. Mouse Colony- Forming Unit (CFU-Mix) Assays Using MethoCult™ were purchased from Stem cell Co., SA-β-Gal staining kit and cell cycle detection kit were purchased from Beyotime Biotechnology Co.

Sample Treatment and Sequencing

1. Isolation and Purification of HSPCs

4 weeks-old mice were both euthanatized by chloral hydrate, and were sacrificed with total bone-marrow cells

isolated from femurs and tibiae. All animal experiments were performed in compliance with the guidelines of the Animal Care and Use Committee of SHUTCM(Shanghai Universitiy of Traditional Chinese Medicine).The cells was suspended in red blood cell lysis buffer(Beyotime Biotechnology Co., Ltd), incubated at room temperature for 5 min, then it was centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The precipitate was washed again, what we get was bone marrow mononuclear cells (MNCs). These cells were suspended in PBS containing EDTA and 0.5% BSA. Then we obtained HSC/HPCs through $Lin^{-}c\text{-}kit^{+}$ immunomagnetic bead sorting with lineage cell depletion kits (Miltenyi Co.) and anti-c-Kit microbead (Miltenyi Co.)[36].

2. Modeling and Identification of the Aging HSPC Model in vitro

1) Young HSPC group: Repeat the procedure obove with 4 week-old mice, $n = 10$

2) Old Model HSPC group: Young group HSPCs were cultured with stem cell culture medium (STEMCELL Technologies) + 10 ng/ml IL3 + 10 ng/ml IL6 + 30 ng/ml SCF and incubated at 37 °C,5% CO_2 for 8 days. The medium was changed every 2 to 3 days. $n = 10$.

The details for identification of the model was in our patented method: a method to establish old HSPC model in vitro.

3. Protein Extraction and Quantification

Lysis buffer (2% SDS, 7M urea, 1x Protease Inhibitor Cocktail) was added into the samples($n = 3$). The lysis was performed by sonication on ice for 3 min and kept on ice for 30 min. After centrifugation at 15000 rpm for 15 min at 4℃, the supernatant was collected and transferred to a new Eppendorf tube. An aliquot of the supernatant was taken and the protein concentration was determined by Bio-Rad protein assay (Bio- Rad, Hercules, CA, USA).

4. Isobaric tags for relative and absolute quantification (iTRAQ)

Protein digestion and iTRAQ labeling were performed according to the related literatures. Briefly, 100 ug of protein from each sample was reduced, alkylated and digested with sequence-grade modified trypsin (Promega, Madison, WI) prior to labeling with one of the individual 8-plex-iTRAQ tags (Applied Biosystems, Framingham, MA). The peptide mixture was fractionated by high pH separation using Ultimate 3000 system (Thermo Fisher scientific, MA, USA) connected to a reverse phase column (XBridge C18 column, 4.6 mm x 250 mm, 5 µm, Waters Corporation, MA, USA). Twelve fractions were separated by nanoLC and analyzed by on-line electrospray tandem

mass spectrometry. The experiments were performed on an Easy-nLC 1000 system (Thermo Fisher Scientific, MA, USA) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) equipped with an online nano-electrospray ion source. PEAKS DB was set up to search the Uniprot-mouse database (ver.201711, 51946 entries) assuming the digestion enzyme Trypsin.

5. Identification of Differentially Abundant Proteins

Protein identification and quantification was performed using the Mascot 2.3.02 search engine against the UniProt database (http://www.uniprot.org). To demonstrate the repeatability of the replicates, protein abundances between various biological replicates were compared.

6. Go, KEGG and STRING Enrichment Analyses

In order to obtain a global functional view of the DEPs, Gene Ontology (GO) func- tional classification annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis were conducted. All the DEPs were subjected to GO analysis(http://www. geneontology.org) and KEGG analysis (http ://www. Gen ome.jp/kegg). GO database is an internationally standardized gene functional classi- fication system that comprehensively describes characteristics of genes and their products. KEGG was used to predict the major metabolic and signal transduction pathways involved in DEPs[37]. Furthermore, to study the potential roles of mRNA splicing in HSPC aging,we built a regulatory network with the DEPs using STRING analysis(http://www.string -db.org/)[38].

7. qRT-PCR

To investigate whether the differences in protein abundances were reflected at the mRNA level and to validate the proteomic data, the quantitative real-time polymerase chain reaction(qRT-PCR) was used to verify the mRNA level of mRNA splicing associated proteins between the young group and the aging group. 4 notable mRNA splicing genes Wbp11, u2af1, u2af2,Ptbp1 and 4 scarcely reported mRNA splicing relavant genes Rbmxl1,Dhx16, Pcbp2,Pabpc1 in HSPC were selected for qRT-PCR analysis to validate the accuracy of the ITRAQ proteomic analysis. Total RNA extraction and reverse transcription were performed according to the manufacturer’s instructions for the kit used (9108/9109; RR047A, Takara). The A260/280 ratio of RNA was dete- cted.The primers were designed and synthesized by sango biotech. All primer sequences are listed in Table 3. The GAPDH (internal control) primers were used. SYBR green dye was used for Real-time quantitative PCR (RR420A, Takara). The

$2^{-\Delta\Delta Ct}$ method was used to calculate mRNA expression levels. $\Delta Ct = Ct_{target\ gene}$ - $Ct_{internal\ control\ gene}$ (where Ct is the cycle number when the fluorescence signal reaches the set threshold). The amplification parameters were: 95ºC for 30 s, (95ºC for 5 s, 60ºC for 34 s) for 40 cycles.The analysis was performed with three biological replicates.

8. Detection of HSPC aging related manifestation after SSA treatment

SA-β-gal staining

HSPCs($\sim$ 1000,000 cells) were collected on 72 h after the treatment with 100 ng $mL-1$ of SSA. The cells were fixed with 4% paraformaldehyde for 15 minutes ,then incubated at 37ºC without CO_2 for 16 h in β-galactosidase staining solution. The number of β-galactosidase positive cells per 400 total cells was counted, $n = 3$.

Mixed colony-forming unit (CFU-Mix) of HSPC culture

HSPCs ($\sim$ 1000,00cells) were collected and subjected to mixed colony-forming unit (CFU-Mix) culture: Cells were diluted, in duplicate, with IMDM + 2% FBS and MethoCult™ GF M3434 medium to a final concentration of 5×10^3 per 35 mm dish. 0.3 mL of the diluted cells were added to 3 mL of MethoCult™ and mixed thoroughly. The final cell mixture was dispensed into each 35 mm dish at a volume of 1.1 mL and incubated at 37ºC in 5% CO_2 for 7 days. Photos were taken using an inverted microscope. Finally, we added 1 mg/ml p-iodonitrotetrazolium violet to 24-well cells so as to take photos 24 hours later. The number of CFU-Mix per 5×10^3 cells represented the pluripotency of the HSPCs,$n = 3$.

Statistical analysis

The experimental data was expressed as a mean and standard deviation. Single factor analysis of variance and one-way ANOVA was performed using SPSS 18.0. The LSD or Tamhane test was used to compare differences between two groups. $p < 0.05$ was considered statistically significant.

Abbreviataions

Isobaric Tags for Relative and Absolute Quantitation iTRAQ

DEPs Differentially Expression Proteins DEPs

hematopoietic stem /progenitor cells HSPC

bone marrow mononuclear cells MNCs

Gene Ontology GO

Kyoto Encyclopedia of Genes and Genomes KEGG

Spliceostatin A SSA

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal experimental ethics committee of Shanghai university of Traditional Chinese Medicine, and were carried out in accordance with the institutional guidelines and ethics.

Consent for publication

All authors of this article have consented for publication.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no conflict of interest exists.

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Author Contributions

NZ conceived and designed the research. LL conducted the experiments. All authors have read and approved the manuscript.

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Not Applicable.

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Tables

Table1 Go Differential Expressions of mRNA splicing Associated proteins in HSPCs(Aging/Young)

Number	Pathway Name	Mismatch Repair Related Genes	P Value
1	**mRNA splicing, via spliceosome**	Hnrnpa2b1, Pabpc1, Sf3b6, Snrpg, Snrpd3, Dhx16, Thrap3, Npm1,Pcbp2, Srsf2, Srsf1, U2af2, Ptbp1, Srsf7, Hnrnpr, Rbmxl1, Rbm5, Wbp11, Raver1, Snu13, U2af1, Dcps, Sart3	1.35E-11
2	**regulation of mRNA splicing, via spliceosome**	Hnrnpa2b1, Thrap3, Npm1, Srsf2, U2af2, Ptbp1, Srsf7, Rbmxl1, Rbm5	2.99E-06
3	**spliceosomal complex assembly**	Snrpg, Snrpd3(snRNP), Srsf1, Rbm5, Sart3, Rbmxl1	7.32E-05
4	**alternative mRNA splicing, via spliceosome**	Thrap3, Srsf2, Srsf1, Ptbp1, Rbmxl1, Rbm5	1.90E-04
5	**regulation of alternative mRNA splicing, via spliceosome**	Thrap3, Srsf2, Ptbp1(hnRNP1), Rbmxl1, Rbm5	6.05E-04
6	**mRNA cis splicing, via spliceosome**	Srsf1, Wbp11, Dcps	6.15E-04
7	**spliceosomal snRNP assembly**	Snrpg, Snrpd3, Sart3	8.56E-3
8	**positive regulation of mRNA splicing, via spliceosome**	Thrap3, Rbmxl1	2.38E-2

Table2 KEGG Differential Expressions of mRNA splicing Associated proteins in HSPCs(Aging/Young)				
Gene name	**Geneic expression level**		**Fold change**	**P value**
	OID Group	**Control Group**		
Snrpg	1996000	3746000	-1.88	4.36E-4
Srsf1	3708000	6015000	-1.62	6.97E-3
Rbmxl1	397100	681500	-1.72	9.16E-4
Srsf5	1036000	1726000	-1.67	4.01E-3
Wbp11	274900	461200	-1.68	5.84E-3
Srsf2	2602000	5057000	-1.94	2.36E-3
Sf3b6	488500	918900	-1.88	7.78E-3
Srsf7	2093000	4353000	-2.08	1.58E-4
Snrpd3	687800	1122000	-1.63	9.84E-3
U2af1	914100	1846000	-2.02	5.88E-4
Snu13	1638000	3252000	-1.99	7.24E-3

Table3 Primer Sequences used for PCR amplification		
Primers	**Genbank Reference/ Sequence**	**Fragment Length**
Actin (forward)	**GTACCACCATGTACCCAGGC**	247 bp
Actin (reverse)	**AACGCAGCTCAGTAACAGTCC**	
Rbmxl 1(forward)	**AGGAGGAAGTGGAGGAACTAGG**	179 bp
Rbmxl 1 (reverse)	**TTCGAACTGGTCCGGAAGGT**	
Wbp11(forward)	**AGGAATCCGAGGGCCTTTAC**	293 bp
Wbp11(reverse)	**CCTTCGGTCGCTGAATGAGG**	
U2af1(forward)	**CCTTTAGCCAGACCATTGCC**	188 bp
U2af1(reverse)	**GTCGCAGACGTTCATCTCCT**	
U2af2(forward)	**AAGCGTAGTCACAGTCGCTC**	155 bp
U2af2 (reverse)	**ATCAATCCACCGTGCTCCTC**	
Pcbp2(forward)	**AGGGGATATGCTCCCCAACT**	103bp
Pcbp2(reverse)	**CTCCAACATGACCACGCAGA**	
Ptbp1(forward)	**CTGCAGTATGCTGACCCTGT**	252bp
Ptbp1(reverse)	**TGCATACGGAGAGGCTGACA**	
Dhx16(forward)	**CGGCCCAAGGATAAGGTTGT**	215bp
Dhx16(reverse)	**ACTTCCACACGCTCCAAGAG**	
Pabpc1(forward)	**CAGGGCAAAGGAGTTCACCA**	275bp
Pabpc1(reverse)	**TAAGTTCCGTCTGCCGTTCC**	

Figures

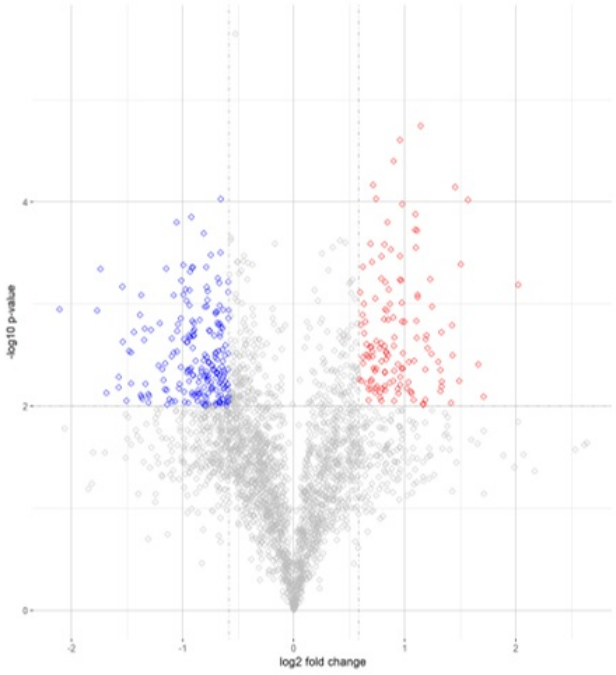


Fig.1 A

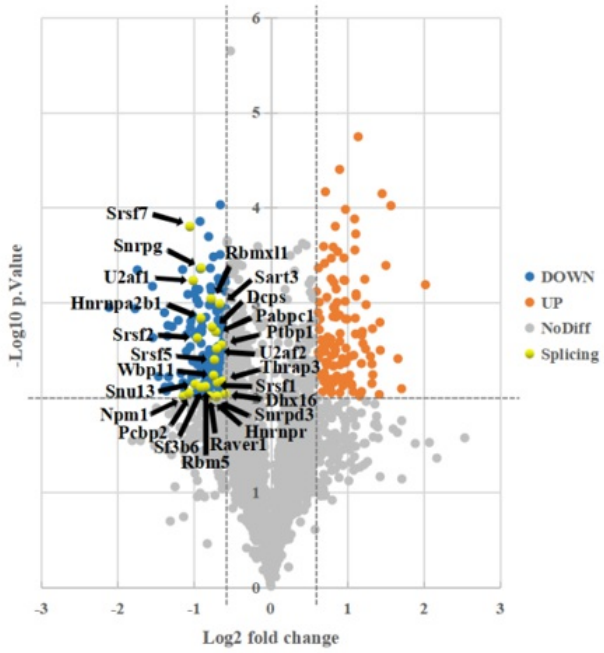
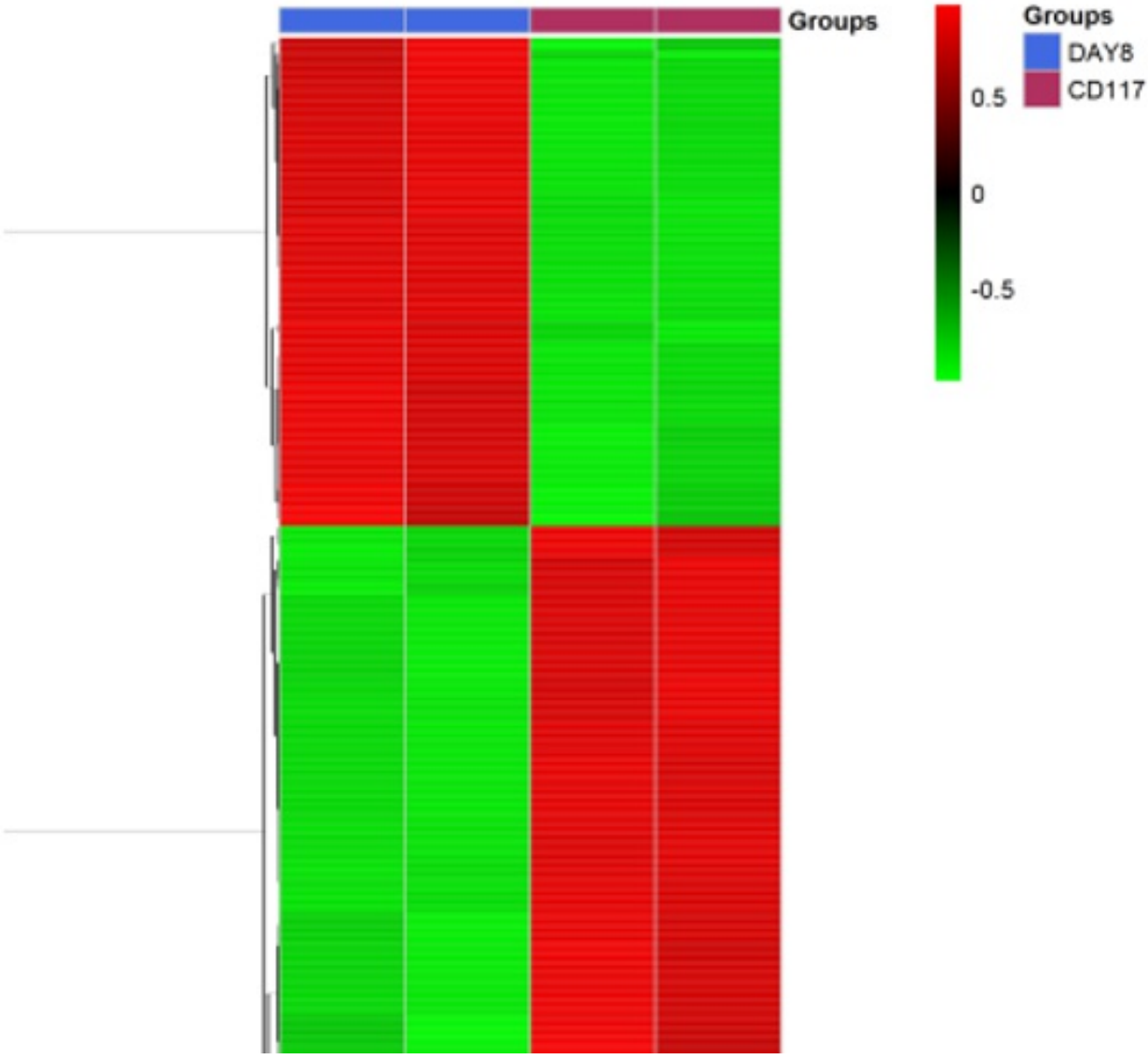


Fig.1 B

Figure 1

The Volcano plot of the DEPs(Differently expressed proteins) (A). Each point represents the difference of protein expression (fold-change) between two groups. Red point represents upregulated protein; blue point represents downregulated protein. Gray point represents no significant differential protein. The X-axis represent -s log2-fold change; The Y-axis corresponds to -log10-P value.(B); mRNA splicing relevant DEPs were marked in the Volcano plot.


Groups
Groups
DAY8
CD117
0.5
0
-0.5

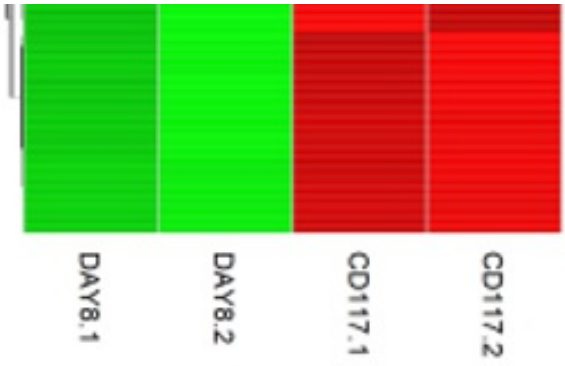


Figure 2

Hierarchical cluster analysis of DEPs Heatmap analysis is an algorithmic approach to find discrete groups with varying degrees of (dis)similarity in a data set represented by a (dis)similarity matrix and is processed with pheatmap package (https://CRAN.Rproject. org/ package= pheatmap). Green signifies down-regulated proteins and red signifies up-regulated proteins in aging group compared with young HSPCs.

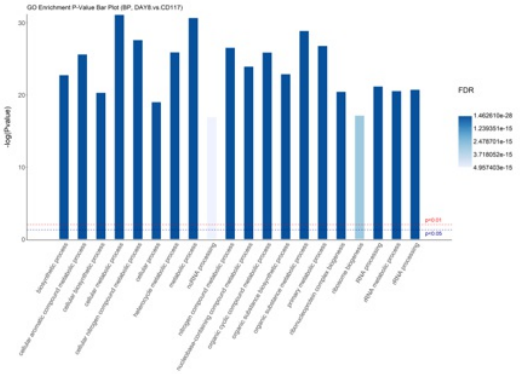


Fig.3A

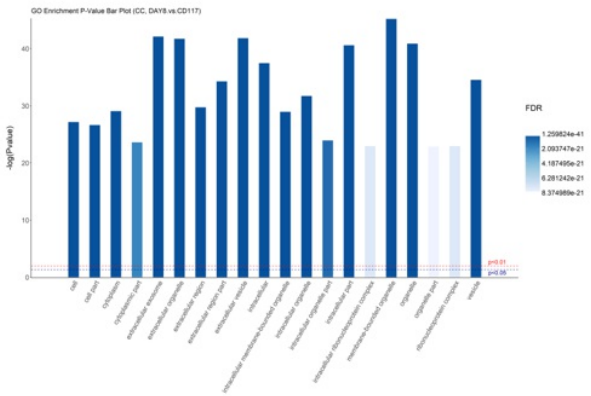


Fig.3B

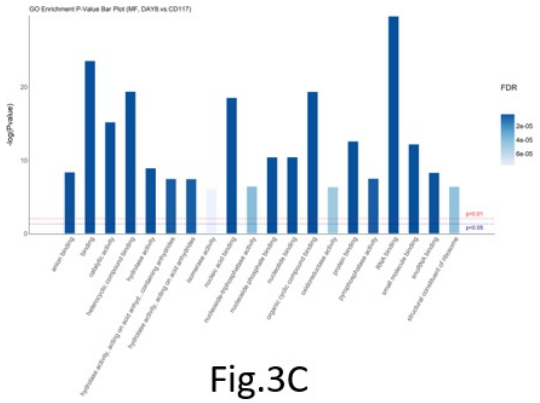


Fig.3C

Figure 3

Go functional annotation of DEPs The x-axis shows GO classification, and the y-axis indicates corresponding p-values (A).BP(biological process); (B).CC (cell -ular component); (C).MF(molecular function).

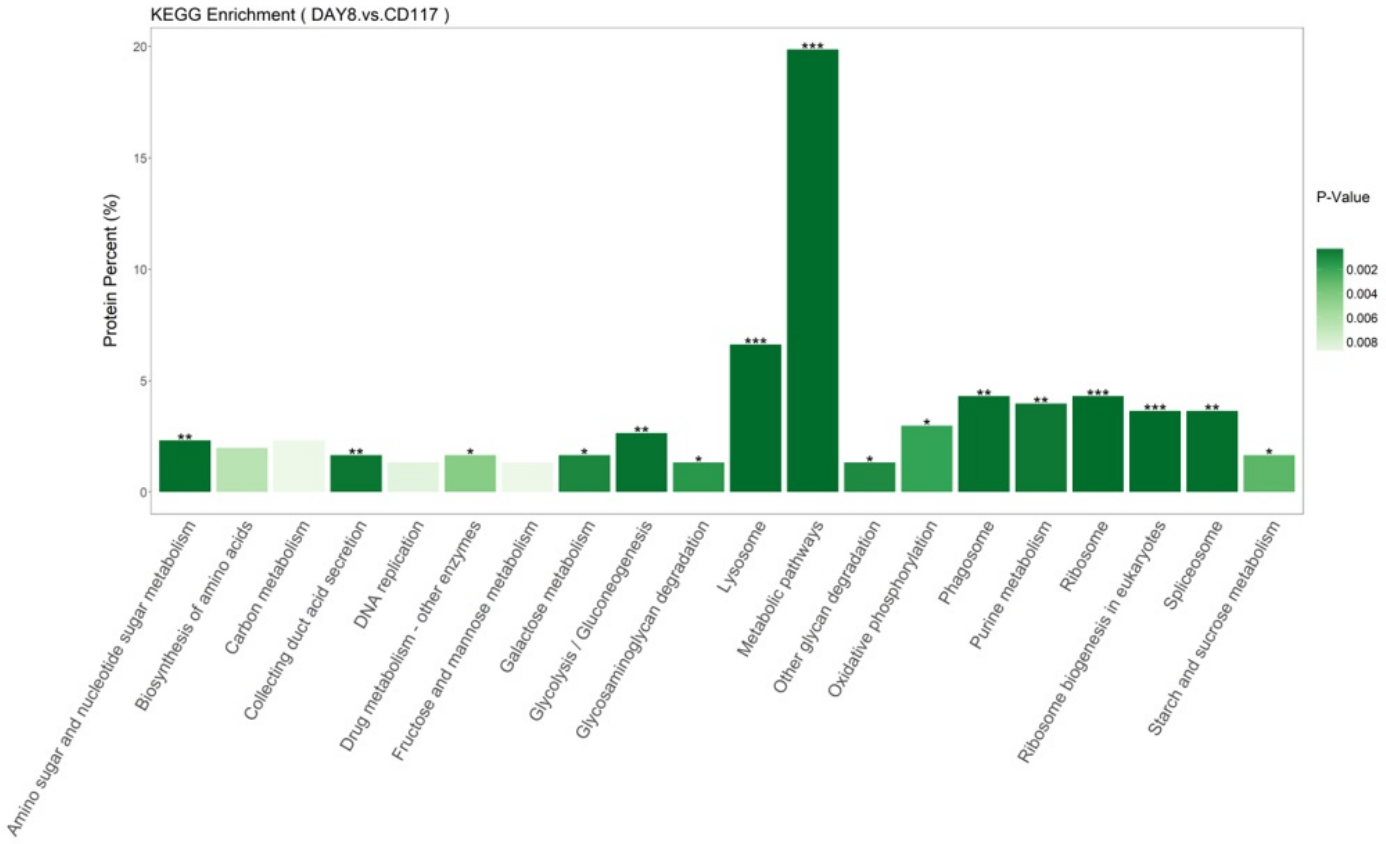


Figure 4

The top20 representative KEGG pathway enrichment analysis of the DEPs The x-axis shows representative enriched KEGG pathways, and the y-axis indicates corresponding protein percent(%).

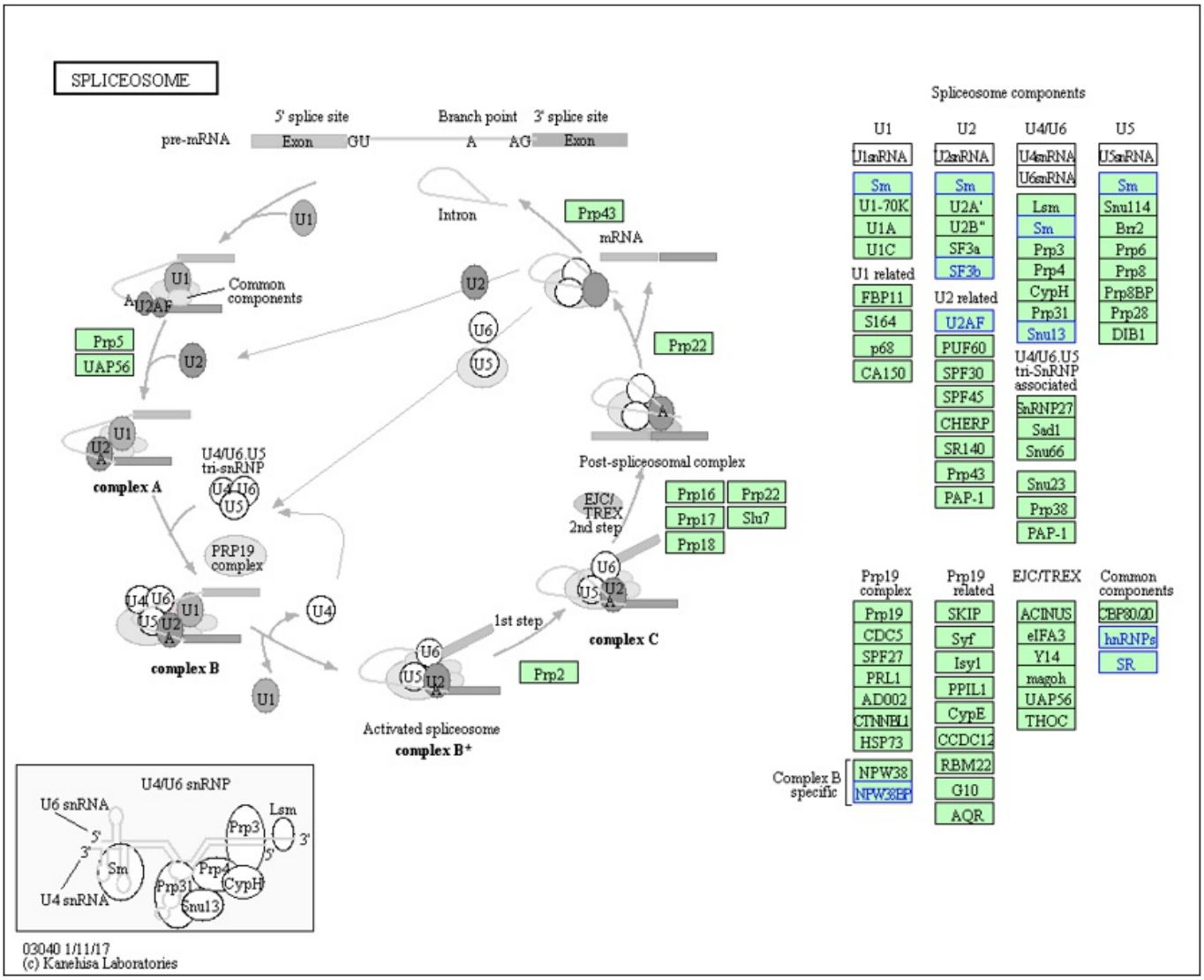


Figure 5

Representative KEGG pathway for mRNA splicing Blue signifies down- regulated proteins related to mRNA splicing in aging group compared with young group.

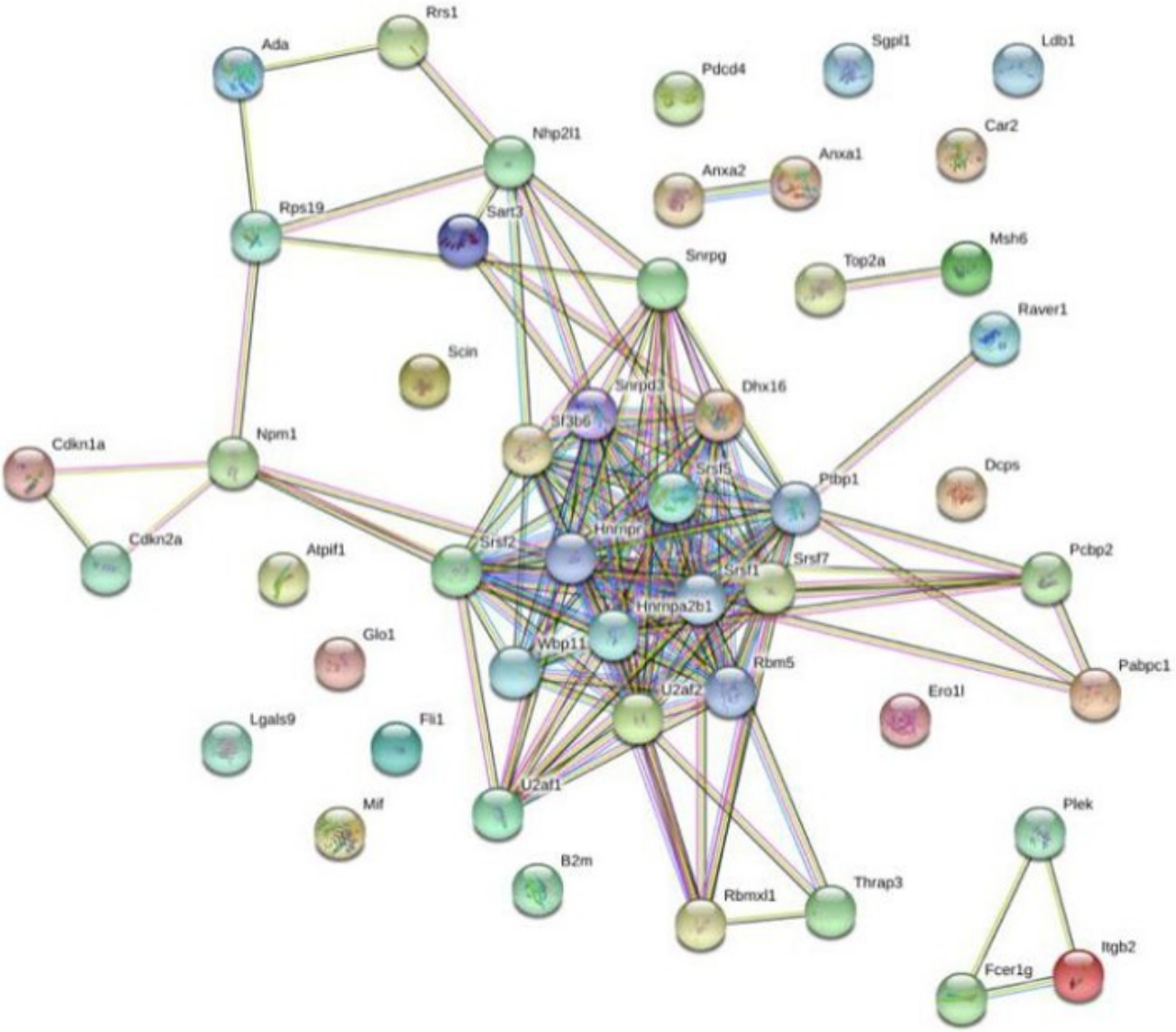


Figure 6

Interaction network of the DEPs involved in mRNA splicing, aging and hemopoiesis

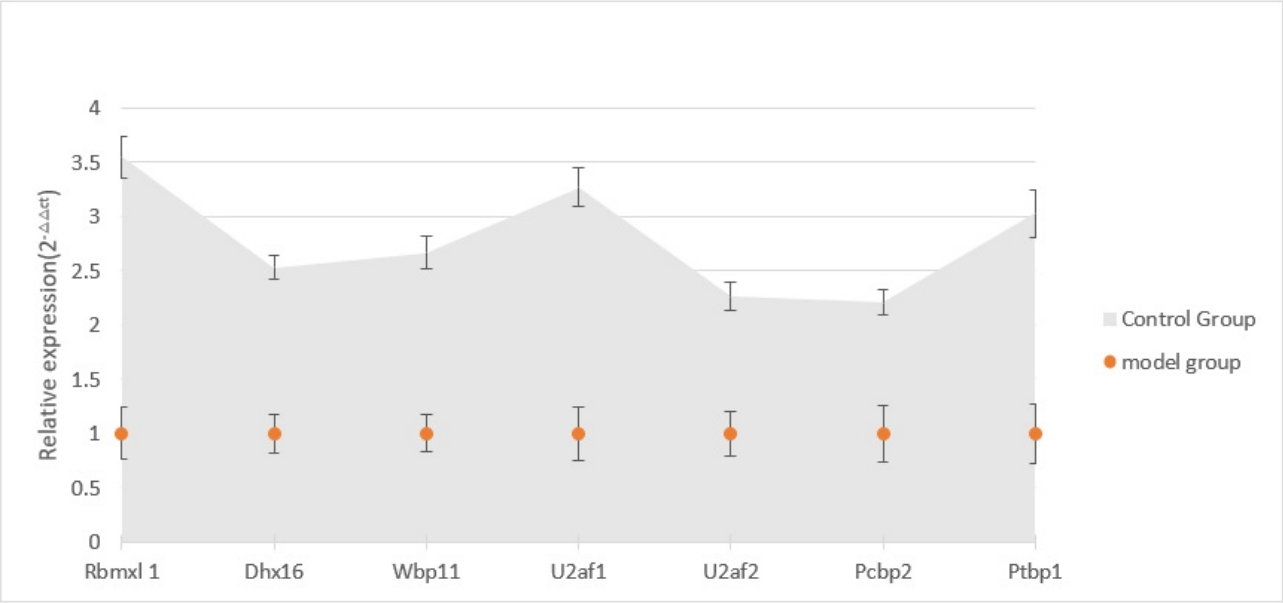


Figure 7

qRT-PCR analysis of representative proteins related to mRNA splicing

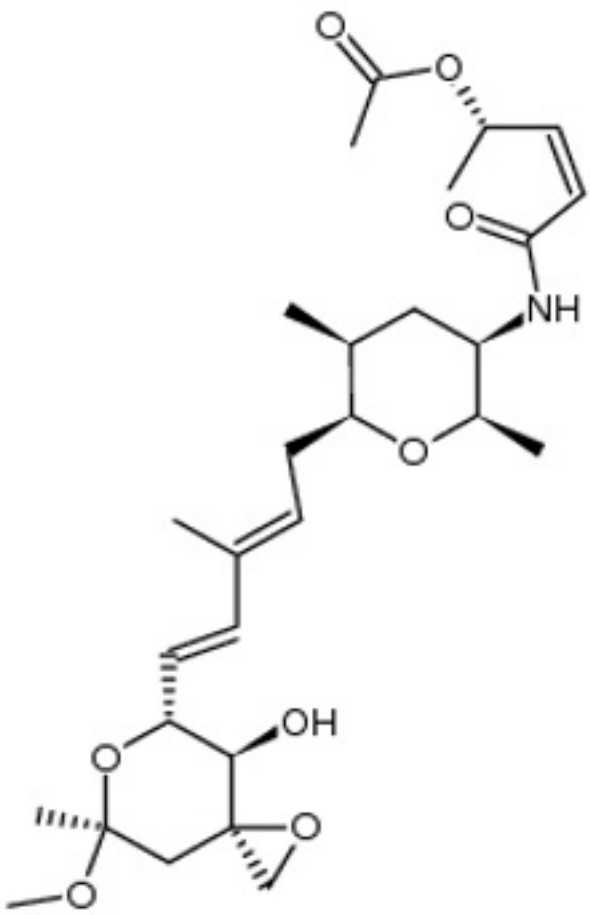


Figure 8

SSA

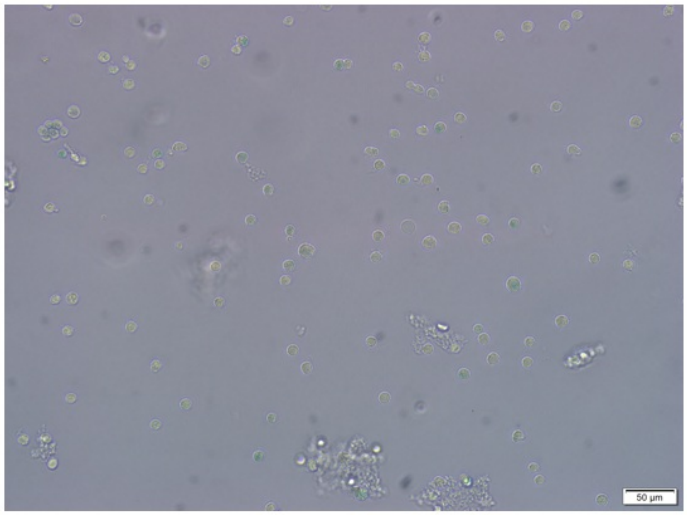


Fig.9A

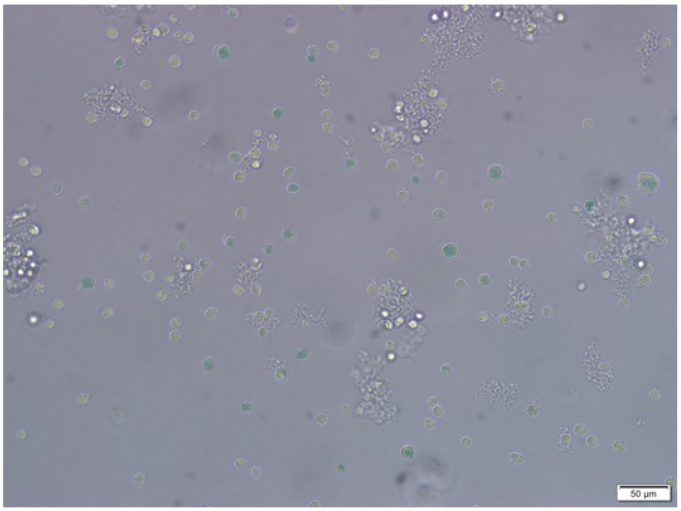


Fig.9B

Figure 9

Photomicrographs of SA-β-gal staining(×200) The percentage of SA-β-gal stain-positive cells was significantly increased in SSA group compared with the control group, n=10.

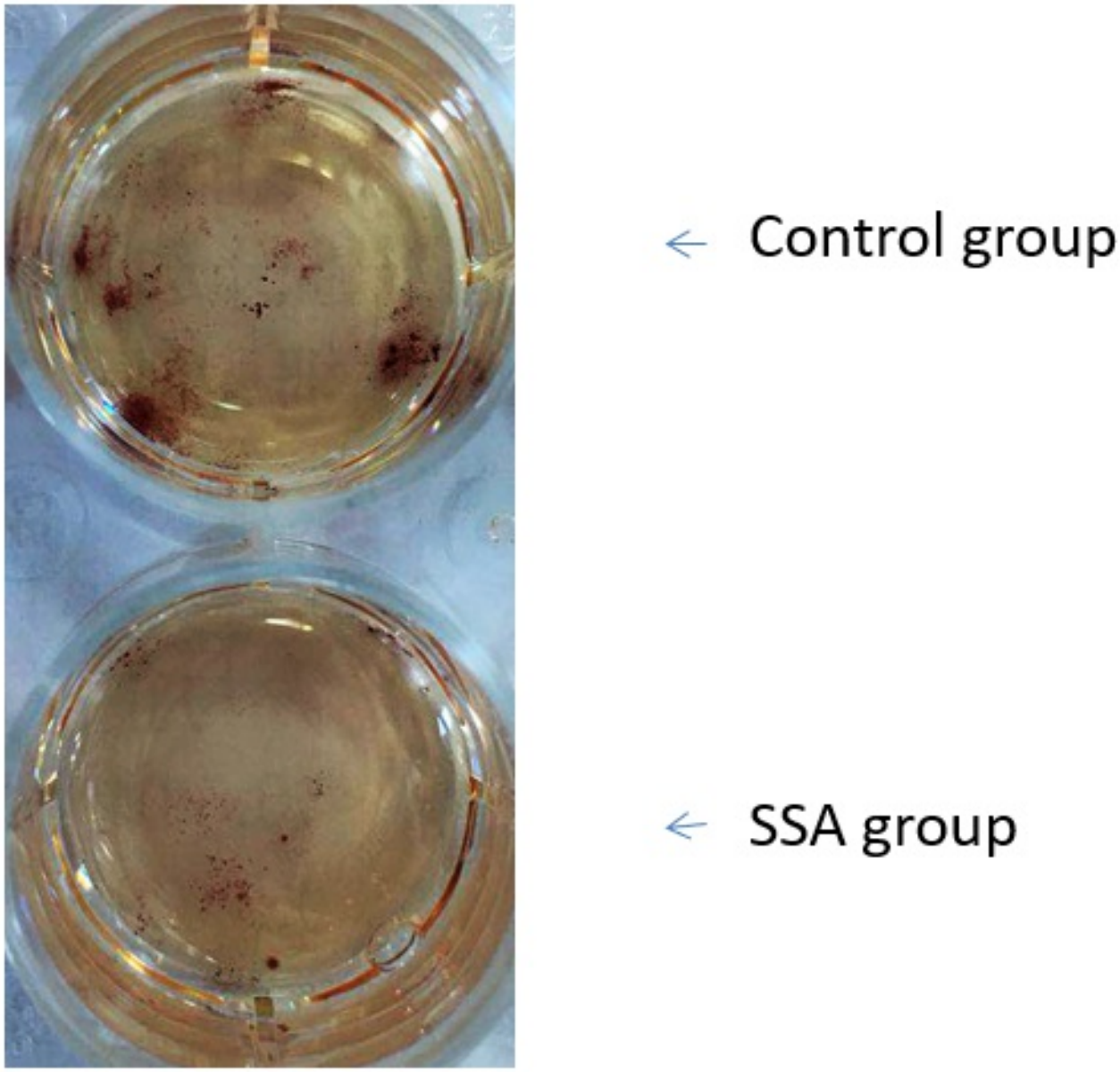


Figure 10

Photomicrographs of CFU-Mix The size and number of CFU-Mix significantly decreased in SSA group compared with young group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

NC3RsARRIVEGuideline191211.pdf
supplementTable.S1.xlsx
supplementTable.S2.xlsx
supplementFig.docx