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Original Article

Identification of differential gene expression in endothelial cells from young and aged mice using RNA-Seq technique

Liang Zhang[1], Manli Liu[1], Qing Li[1,2], Bing Shen[3], Chaojie Hu[1,2], Rui Fu[1], Mengdie Liu[1], Jie Deng[1], Qi Cao[4], Yiping Wang[4], Yuanmin Wang[5]

[1]The Central Laboratory of Medical Research Center, Anhui Provincial Hospital, Anhui Medical University, Hefei 230001, Anhui, PR China; [2]The Central Laboratory of Medical Research Center, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui, PR China; [3]School of Basic Medical Sciences, Anhui Medical University, Hefei 230032, Anhui, PR China; [4]The Centre for Transplantation and Renal Research, Westmead Institute for Medical Research, University of Sydney, Sydney, NSW, Australia; [5]Centre for Kidney Research, Children's Hospital at Westmead, Sydney, NSW, Australia

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Abstract: Aging is a complex phenomenon. Endothelial cell senescence is regarded as a vital characteristic of cardiovascular diseases. This study aims to identify differentially expressed genes in vascular endothelial cells (ECs) of different age groups by RNA sequencing (RNA-Seq) technique, and to explore which molecular pathways differentially expressed genes (DEGs) may enrich in. In this study, we used RNA-Seq to analyze DEGs in primary endothelial cells of young and old mice, and further analyzed them by gene ontology (GO) enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Our results showed that in total identified 229 of the DEGs, 104 were upregulated and 125 were downregulated in endothelial cells of aged mice compared with young mice. Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the involvement of these DEGs in the regulation of morphogenesis of a branching structure, angiogenesis, upregulation of cell proliferation, and extracellular matrix (ECM)-receptor interaction. These results provided a novel insight to understand the molecular mechanisms underlying aortic endothelial cell senescence, and some of the novel candidate genes identified in this study may be valuable in elucidating the molecular mechanisms underlying endothelial cell senescence.

Keywords: Endothelial cells, RNA-seq, gene expression, senescence

Introduction

The average life expectancy of human beings is high. Also, the proportion of people aged more than 65 years has increased worldwide [1]. Cardiovascular diseases are the leading cause of death in the aged population. Aging is an inevitable part of life and, unfortunately, an important risk factor for cardiovascular diseases. Despite a large number of studies in the cardiovascular field, the molecular mechanism underlying vascular endothelial cells (ECs) senescence has not been fully investigated.

The high-throughput RNA sequencing (RNA-Seq) technique has emerged as an useful tool for transcriptome analysis and exploring unknown genes [2]. RNA-Seq has been used to study the proliferation or senescence of several cells such as hematopoietic stem cells [3], neurons [4], and fibroblasts [5]. The RNA-Seq technique could find many differentially expressed genes (DEGs) in clinical cases. For example, 95 differential genes were found in patients with psoriasis [6]. These studies provided a broad understanding of the role of DEGs in vascular endothelial cell senescence. However, the molecular mechanism underlying vascular endothelial cell senescence needs to be further explored.

The RNA expression profiles of endothelial cells in young and aged mice were compared using the RNA-Seq technology to investigate the mo-

lecular mechanisms and regulatory genes in endothelial cell senescence. A total of 229 DEGs were identified. The Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed the involvement of these DEGs in the regulation of morphogenesis of a branching structure, angiogenesis, upregulation of cell proliferation, and extracellular matrix (ECM)-receptor interaction. These results provided a novel insight to understand the molecular mechanisms underlying aortic endothelial cell senescence.

Materials and methods

Ethics statement and experimental animals

Male mice (n=21; C57BL/6 background) were purchased from the Nanjing University-Nanjing Biomedical Institute (Nanjing, China). Before the study, all animals were fed the same diet for 1 week. They were then divided into two groups based on their age: young group (8 weeks, n=9) and aged group (18 months, n=12). The mice were housed in groups of five per cage at a temperature of 25°C in a controlled-atmosphere room and a 12-h light-dark cycle. During the experiment, the mice had free access to food and tap water, and their body weights were recorded weekly. The study protocol was approved by the Biomedical Ethics Committee of Anhui Provincial Hospital (Hefei, Anhui, China). All handling and management procedures were in accordance with the guidelines of experimental animal administration.

Endothelial cell acquisition and culture

The aorta was isolated from mice, washed six times with sterile phosphate-buffered saline (PBS), cut and then digested with type II collagenase for 30 min. The enzyme solution was aspirated, and the cells were cultured at 37°C in an RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified 5% CO_2 incubator. The medium was changed after 1 h and then every 3 days. The cells were subcultured or harvested when they occupied 70%-80% of the flask.

Flow cytometry

Harvested mouse endothelial cells (1×10^6 cells/well) were labeled with APC-conjugated anti-mouse CD105 (MJ7/18 clone, eBioscience, USA), phycoerythrin-conjugated anti-mouse CD31 (390 clone, Miltenyi Biotec, Germany), and appropriate isotype controls. The cells were incubated with antibodies for 30 min at room temperature in the dark. Subsequently, they were washed two to three times (5 min each time) with PBS and analyzed by flow cytometry using FACS Canto II flow cytometer with BD FACSDiva Software (Becton Dickinson, CA, USA). The frequency of endothelial cells was expressed as a percentage of $CD105^+CD31^+$ cells. Five replicates were performed.

RNA isolation, library construction, and sequencing

Total RNA was isolated from the endothelial cells of young and aged using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The quality and concentration of RNA were determined using 1.2% agarose gels and an Agilent 2100 Bioanalyzer system (Agilent Technologies, CA, USA). Degradation of RNA was determined using 1.2% agarose gels. The concentration and purity of RNA were detected using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). Its integrity was confirmed using an Agilent Bioanalyzer 2100 system (Agilent Technologies). Sequencing libraries were generated using a NEBNext1 Ultra_RNA Library Prep Kit for Illumina (NEB, MA, USA). Subsequently, 3 μg RNA per sample was used to purify mRNA using the oligo (dT) magnetic beads, and then the purified mRNA was randomly sheared into pieces of approximately 200 base pairs using the fragmentation buffer. The fragmented mRNAs were then used for the first-strand cDNA synthesis using reverse transcriptase and random hexamer primers. The second-strand cDNA was synthesized using DNA polymerase I and RNase H. After the fragments were ligated to adaptors, they were isolated as polymerase chain reaction (PCR) templates. The quality of the libraries was evaluated using an Agilent 2100 Bioanalyzer and the real-time PCR system. The libraries were sequenced using an Illumina HiSeq 2500 platform (Illumina, CA, USA).

Analysis of RNA-Seq data

The sequences were removed according to the following criteria: low-quality sequence (more than 30% of <Q20 bases) and more than 10% unknown nucleotide (N) reads and adapter.

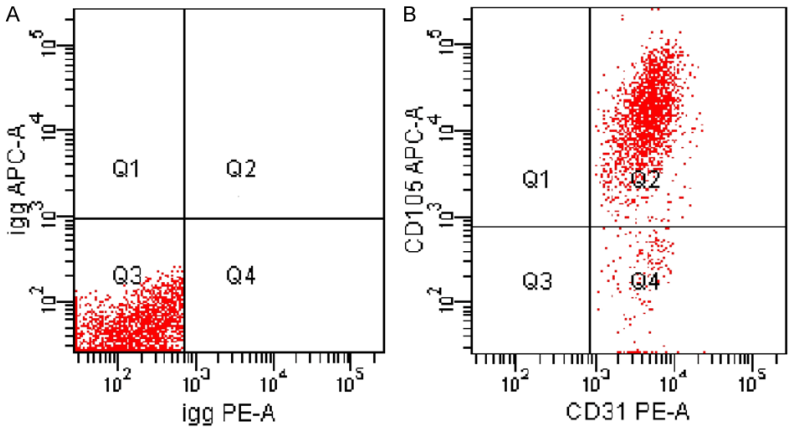

Figure 1. Flow cytometry plot of isotype control (A) and $CD31^+CD105^+$ expression rate (B) in primary endothelial cells. Expression rate of $CD105^+CD31^+$ cells (Q2) in primary endothelial cells was (97.3±2.1)%.

Then, the clean reads were acquired. The mouse genome sequence (GRCm38.p6) was downloaded from the ENSEMBL database (http://www.ensembl.org/index.html). All clean reads were mapped to the mouse genome using the HISAT software. The assembly of transcripts was performed using the Cufflinks software. The gene expression level was calculated using the normalized number of fragments per kilobase of transcript per million fragments (FPKM) method. The Cuffdiff 2 software was used to identify the DEGs between the estrus and proestrus groups using the following filter criteria: *P* value <0.05 and absolute value of $\log_2$ (FPKM_AGED/FPKM_YOUNG) >1.

GO and pathway enrichment analysis

DEGs were annotated using the GO database (http://www.geneontology. org/) by the hypergeometric test to examine the biological functions and pathways of these genes. The *P* value was calculated, and the GO terms were considered as significantly enriched when the *P* value was less than 0.05. The background genes were genes involved in the whole genome. Pathway analyses were performed using the KEGG database (http://www.genome.jp/kegg/), and those with a *P* value less than 0.05 were considered the significant pathways.

Statistical analysis

The RNA-Seq data were statistically analyzed using the R Programming language. The data were normalized using the software. The DEGs were screened using fold change (expression difference multiple) and *P* values.

Results

Endothelial cell identification

The cultured cells were identified by flow cytometry. The results showed that the frequency of $CD105^+CD31^+$ cells was 97.3%±2.1%, indicating that more than 97% of the cultured cells were endothelial cells (**Figure 1**).

Sequencing data of endothelial cells

In this study, 7 cDNA libraries from two groups (Sample A1, A2, A3, A4 from aged and Sample B2, B3, B4 from young mice arterial endothelial cells) were constructed and sequenced. The major characteristics of the sequencing and annotation data are described in **Table 1**. More than 52 million clean reads were obtained for 7 libraries after filtering out low-quality and adaptor sequences were filtered out. Among these clean reads, more than 88.54% had quality scores at a ratio of Q30 (a base quality >30 and error rate <0.001) level. Furthermore, 55.51%-90.36% of the clean reads were mapped onto the mouse reference genome (GRCm38.p6) using the TopHat program.

Identification of DEGs

A total of 21,991 genes were detected in the 7 cDNA libraries. The FPKM method was used to evaluate the gene expression level. The transcriptome differences between the young and aged groups were analyzed. A total of 229 significant DEGs were identified, of which 104 were downregulated and 125 were upregulated (*P* value <0.05 and $|\log_2 FC| > 1$) (**Figure 2**).

GO enrichment analysis

The DEGs were annotated using the GO terms in the GO database to further discover their molecular characterization. The DEGs were assigned to three categories: biological processes, molecular functions, and cellular components (**Figure 3**).

Table 1. RNA-Seq data statistics and annotation information results

Sample	Sample_A1	Sample_A2	Sample_A3	Sample_A4	Sample_B2	Sample_B3	Sample_B4
Raw reads	90377160	58310646	57300710	64604804	74225868	59098442	139108196
Raw bases	13556574000	8746596900	8595106500	9690720600	11133880200	8864766300	20866229400
Clean reads	86290958	52729344	54558042	58193490	67315864	53967034	126238758
Clean bases	12927284757	7899764429	8175306064	8718825989	10085407744	8086174256	18914740741
Valid bases	0.9535	0.9031	0.9511	0.8997	0.9058	0.9121	0.9064
Q30	0.9039	0.8863	0.9093	0.8854	0.8878	0.8943	0.8917
GC	0.595	0.53	0.56	0.49	0.54	0.49	0.5
Total reads	12927284757	7899764429	8175306064	8718825989	10085407744	8086174256	18914740741
Total mapped	55.51%	80.55%	74.44%	90.36%	80.08%	90.03%	89.06%

Note: RNA Sample A1, A2, A3, A4 were from arterial endothelial cells of aged mice and Sample B1, B2, B3 were from those of young mice.

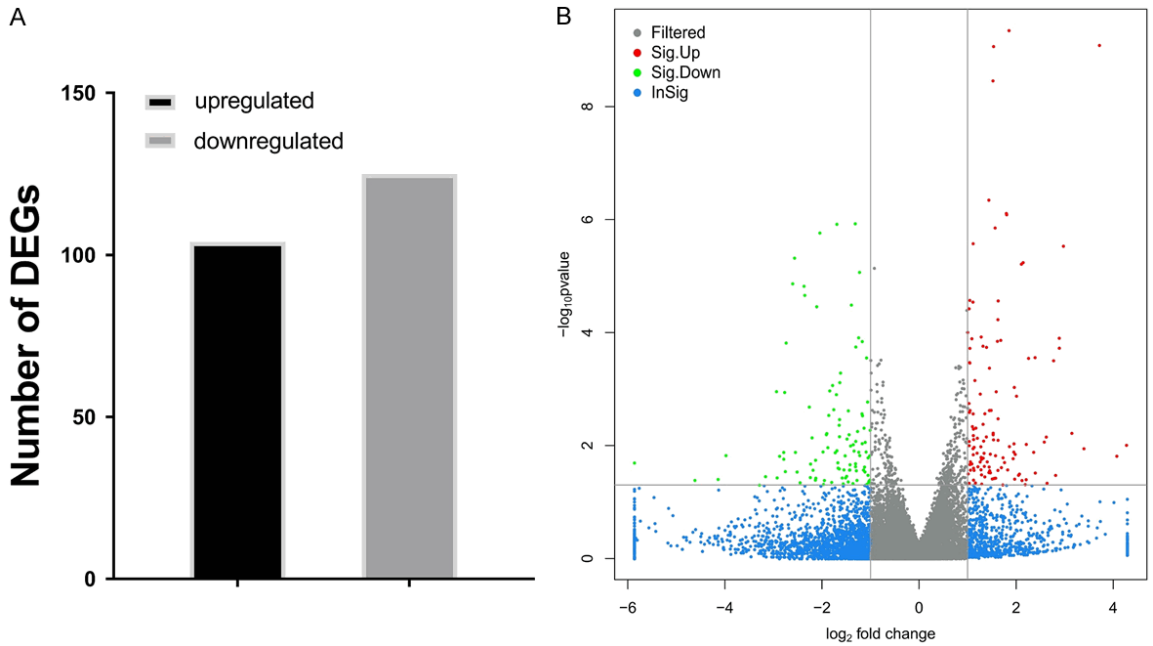

Figure 2. Distribution of DEGs. A. The number of downregulated and upregulated DEGs in the arterial endothelial cells of young mice compared with the aged mice arterial endothelial cells. B. The volcano plot displaying DEGs. The *y*-axis displays the value of -$\log_{10}$ (*P* value), and the *x*-axis shows the $\log_2$ fold change value. The significantly upregulated and downregulated genes are displayed by the red and green dots respectively, while the black dots and blue dots represent genes with no significant changes.

In the GO category biological process, DEGs were involved in the regulation of morphogenesis of a branching structure, angiogenesis, positive regulation of cell proliferation, cell adhesion, regulation of branching involved in prostate gland morphogenesis, extracellular matrix organization, membranous septum morphogenesis, positive regulation of smooth muscle cell proliferation, and negative regulation of mitotic nuclear division. Among the DEGs related to the biological process, bone mineralization had the highest -$\log_{10}$ *P* value, with the maximum difference. These were all inextricably linked with the aging process.

For the cellular component annotation, the most significant term was located in the proteinaceous extracellular matrix. The major molecular function category was heparin binding.

Pathway analysis

A KEGG pathway analysis was performed to identify the pathways of DEGs involved in the

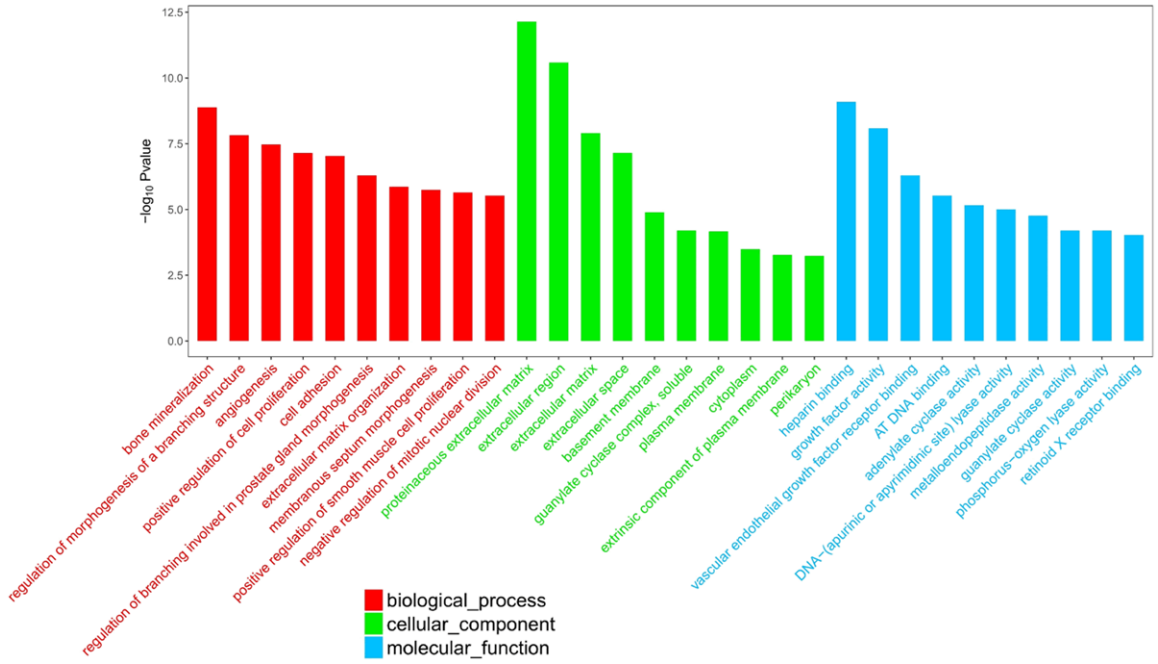

Figure 3. GO analysis of DEGs. Genes were classified into biological process, cellular component, and molecular function. The y-axis shows the value of -$\log_{10}$ (*P* value). The x-axis indicates the GO terms. The red columns represent the biological process, the green columns represent the cellular component, and the blue columns represent the molecular function, respectively.

aging process. A total of 229 DEGs were mapped to 178 KEGG pathways, and 94 pathways were significantly enriched ($P \leq 0.05$) (**Figure 4**). In the significantly enriched pathways, several main pathways were represented, including dilated cardiomyopathy, pathways in cancer, PI3K/Akt signaling pathway, and ECM-receptor interaction.

Discussion

Aging is an important cause of cardiovascular diseases [1]. Decreased cell proliferation and increased apoptosis are important features of cellular senescence. The aging of endothelial cells is involved in the aging of blood vessels, which in turn promotes the occurrence of various cardiovascular diseases. In the present study, the natural aging mouse models were used to explore differential gene expression in endothelial cells in the aging process using the RNA-Seq technique. The aorta was isolated from young (8 weeks old) and aged mice (18 months old), and primary endothelial cells were obtained by type II collagenase digestion and identified using flow cytometry. The purity of endothelial cells was found to be about 97%, indicating that harvesting and culture of endothelial cells were successful in this study.

The RNA-Seq technology is a powerful tool for analyzing and exploring unknown genes. At present, the RNA-Seq technology has been applied to study the proliferation and senescence of various cells, including hematopoietic stem cells, neurons, and fibroblasts. In this study, the RNA-Seq technology was used to identify DEGs in aortic endothelial cells from young and aged mice. A total of 229 genes were significantly differentially expressed in the aged mice compared with the young mice. Of these, 125 genes were significantly upregulated and 104 genes were downregulated in aged mice compared with young mice based on the criteria of $|\log_2 FC| > 1$ with P value <0.05. The GO and KEGG pathway analyses showed that these DEGs were involved in bone mineralization, regulation of morphogenesis of a branching structure, angiogenesis, upregulation of cell proliferation, heparin binding, growth factor activity, vascular endothelial growth factor receptor binding, gap junction, ECM-receptor interaction, focal adhesion, and PI3K/Akt signaling pathway.

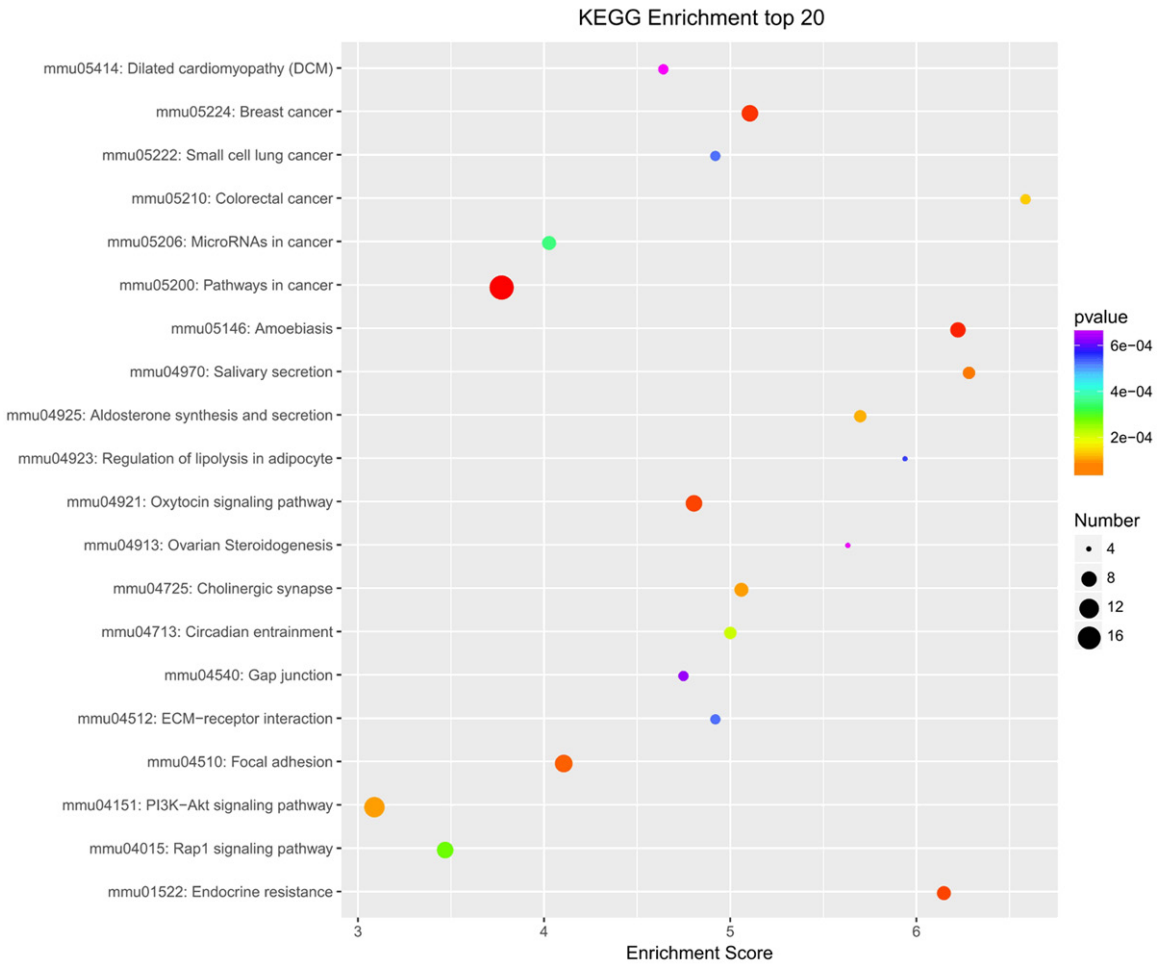

Figure 4. Top 20 significantly enriched KEGG pathways. The x-axis shows the concentration score, and The y-axis displays the enrichment to KEGG pathways. The circle size marks the number of genes, and the color indicates *P* value.

Angiogenesis is the basic process of neovascularization, involving the proliferation, migration, and remodeling of endothelial cells, and the dynamic adhesion of endothelial cells to the extracellular matrix is important in all of these events. The key regulators of endothelial cell adhesion and migration are the αvβ3 and uPA-uPAR complexes, and the αvβ3 integrin heterodimer is a receptor for extracellular matrix components such as statin. Galvagni et al. Marthandan et al reported that the FOSL1 transcription factor of the AP-1 family was pivotal in the regulation of the level of the αvβ3 and uPA-uPAR complexes on the surface of endothelial cells [7]. Also, FOSL1 controls the assembly of endothelial cells into capillary tubes by direct repression of αv and β3 integrin transcription [8]. In this study, the RNA-Seq results showed that the expression of FOSL significantly increased in the aged group compared with the young group. The FOS protein may affect angiogenesis by regulating endothelial cell proliferation and differentiation, thus affecting vascular senescence. In addition, FOSL1 can also regulate the inflammatory response [9].

Among DEGs highly expressed in the aged group, many are associated with apoptosis. Hmga 2 is an oncogene overexpressed in many tumor tissues. It has an important role in stem cell self-renewal, and proliferation, and proliferation [10]. The Hmga 2 protein specifically accumulates in the chromatin of senescent cells. The ectopic expression of Hmga 2 can induce the growth of primary cells, followed by the accumulation of aging phenotypes and DNA damage [11]. Also, Hmga 2 can induce apoptosis in human primary cells and DNA damage [12]. In addition, the results of the present study showed that Nptx1 was highly expressed

in the aged group. This is a novel gene that may be associated with increased endothelial cell apoptosis [13]. Another study found cleavage of caspase-3 and matrix NPTX1 immunoreactivity, accompanied by Akt signaling, conduction inhibition, and mitochondrial dysfunction, in the endometrium using long-acting progestogen contraceptives. *In vitro* culture experiments also found that NPTX1 increased HEEC apoptosis and cytochrome c levels [14]. Netrin is a secretory factor that acts as an anti-angiogenic agent by inhibiting endothelial cell function, and Dcc is one of the netrin homologous receptors. These receptors are involved in endothelial cell recruitment [15]. Plod 2 is associated with increased vascular density in young animals. It is required for angiogenesis and basal layer reconstruction. It is also involved in germination and remodeling [16]. In this study, Plod 2 was highly expressed in the young group compared with the aged group. However, in view of the small sample size, the current results need to be verified using the RT-qPCR technique and the sample size needs to be expanded. Some DEGs are not related to aging, which requires further exploration of the mechanisms.

Few studies have evaluated the role of DEGs in senescence of vascular endothelial cells and pathogenesis of cardiovascular diseases. The present study provided new insights for future studies on the molecular mechanisms of cardiovascular diseases.

In summary, RNA-Seq revealed 229 DEGs, in which 104 were down-regulated and 125 were up-regulated. KEGG pathway analysis revealed 94 pathways were significantly enriched, including PI3K/Akt signaling pathway and ECM-receptor interaction, etc. This study put forward useful data for the potential mechanism of vascular endothelial cell senescence.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qing Li, The Central Laboratory of Medical Research Center, Anhui Provincial Hospital, Anhui Medical University, 17 Lujiang Road, Hefei 230001, Anhui Province, PR China. E-mail: liqing-2001@163.com

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