

# Endogenous hsa-circ\_0007113 binds hsa-miR-515-5p to regulate senescence in human embryonic lung fibroblasts

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D – writing the article; E – critical revision of the article; F – final approval of the article

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## Conflict of interest

None declared

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## Abstract

**Background.** Cellular senescence can lead to many diseases. However, the roles and regulation of circular RNAs (circRNAs) in senescence are poorly understood.

**Objectives.** To investigate the altered expression pattern and mechanism of circRNA during cellular senescence and find potential targets to prevent senescence.

**Materials and methods.** The Arraystar Human circRNA Array and bioinformatics were used to profile the differentially expressed circRNAs in human embryonic lung fibroblasts (IMR-90) between young cells and senescent cells and quantification in the clinical materials. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed. The miRNA targets were predicted using TargetScan and miRanda.

**Results.** A total of 113 differentially expressed circRNAs were identified, including 109 upregulated and 4 downregulated circRNAs (fold change >2 and p-value <0.05). Real-time qualitative polymerase chain reaction (qPCR) showed that the expression levels of 4 circRNA were significantly increased in senescent cells, and that of hsa\_circ\_0007113 was significantly decreased, consistent with the microarray. siRNA against hsa\_circ\_0007113 increased p21 and p53 expression levels and β-gal staining. The hsa\_circ\_0007113 has a binding site for miR-515-5p, which is involved in regulating the p53/p21 signaling pathway. The expression level of hsa\_circ\_0007113 was also decreased in aged people.

**Conclusions.** The study showed an altered circRNA expression pattern in cellular senescence, which might play important roles in senescence-related physiological processes. These findings provide a new direction for studying the molecular mechanism underlying senescence and a new possibility for the treatment of senescence by modulating circRNAs.

**Key words:** senescence, human circRNA array, hsa\_circ\_0007113, hsa-miR-515-5p, P53/P21 pathway

## Background

The essence of aging is cellular senescence, in which cellular functions gradually decrease or are lost, leading to a loss of tissue function. Cell senescence can be accelerated or enhanced by external environmental factors such as radiation, oxidizing agents and therapeutic agents.<sup>1,2</sup> Numerous mechanisms participate in cellular senescence, including DNA damage, telomeres, oncogenes, activated MAPK cascade, and p53 and p16<sup>Ink4a</sup> pathways.<sup>3–7</sup>

Non-coding RNAs play a vital role in the regulation of all cellular pathways.<sup>8</sup> Recently, several studies revealed that non-coding RNAs are linked to the control of cellular senescence.<sup>9–11</sup> Circular RNA (circRNA) is a special class of non-coding RNA, which has a closed circular structure protecting them from exonuclease R. Circular RNAs are conserved in evolution, stable, abundant, and show specific tissues and developmental stage expression.<sup>14,15</sup> Moreover, they have been shown to regulate microRNA (miRNA) expression at the transcriptional or post-transcriptional levels.<sup>12,13</sup> Specific circRNAs play important roles in human diseases such as cancer, stroke, ischemia, neurodegenerative disorders, and heart disease.<sup>16–22</sup> However, so far, few studies have evaluated circRNA changes in cell senescence.<sup>21,23,24</sup>

Circular RNA can bind miRNA-induced silencing complexes via the miRNA response element and affect miRNA concentration to regulate the activity of the downstream gene.<sup>25,26</sup> However, the differential expression of circRNAs during senescence has only rarely been reported. Therefore, this study aimed to use the Arraystar Human circRNA Array to detect the changes in circRNA expression profiles during cellular senescence. Next, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analyses were carried out to anticipate the potential functions of circRNAs during senescence. These findings could provide a better understanding of cellular senescence and eventually slow down senescence associated with pathological conditions.

## Objectives

We aimed to find the relationship between senescence and circRNAs with high-throughput quantitative analysis of circRNA. Furthermore, we quantified circRNAs in clinical samples to clarify the potential biomarkers of human aging, which can provide a certain clinical reference value for clinicians.

## Materials and methods

### Cell culture

The human embryonic lung fibroblast cell line IMR-90 was seeded in a CO<sub>2</sub>-incubator containing 5% CO<sub>2</sub> at 37°C

in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% of penicillin and streptomycin (Gibco, Invitrogen, Waltham, USA). IMR-90 cells were used as young cells in population doublings (PDL) between 15 and 25, and senescent fibroblasts were utilized in PDL 55–65 following additional culture time. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Waltham, USA).

### Labeling and hybridization

Array hybridization and specimen labeling were carried out in accordance with the manufacturer's instructions (Arraystar, Rockville, USA). Total RNA was digested with RNase R (Epicentre, Madison, USA), and the enriched circRNA was amplified and converted into fluorescent cRNA utilizing the random method (Arraystar Super RNA Labeling Kit; Arraystar). The RNeasy Mini Kit was then used to purify the labeled cRNAs (Qiagen, Hilden, Germany). Using a NanoDrop ND-1000, the labeled cRNAs (pmol Cy3/μg) were measured (Thermo Fisher Scientific, Waltham, USA), and 1 μg of cRNA was cleaved by adding 5 μL of a ×10 blocking agent and 1 μL of a ×25 fragmentation buffer. To dilute the labeled cRNA, 25 μL of ×2 hybridization buffer was added to the mixture, and it was incubated for 30 min at 60°C. Finally, space slides containing 50 μL of the hybridization solution were distributed and assembled on the microarray slide for the circRNA expression (Agilent Technologies, Santa Clara, USA). The slides were incubated for 17 h at 65°C in a mixed oven, and then the hybridized arrays were cleaned, fixed, and scanned using the Agilent Scanner G2505C (Agilent Technologies).

### Arraystar human circRNA array analysis

The gathered array images were examined using Agilent Feature Extraction software (v. 11.0.1.1; Agilent Technologies). Then, utilizing the R software package (R Foundation for Statistical Computing, Vienna, Austria), quantile normalization and data processing were carried out (Bioconductor, Github, CRAN; <https://www.bioconductor.org/>). Through volcano map screening, the statistically significant differential expression of circRNAs between the 2 groups was determined, which was displayed as hierarchical clustering. Fold changes ≥2 and p-values <0.05 indicated significant differences in the circRNA expression.

### Comprehensive analysis of the circRNAs-miRNAs-mRNAs networks

The software StarBase (v. 2.0; <http://starbase.sysu.edu.cn>) was used to anticipate the preferred miRNAs of selected circRNAs. The target miRNAs of the identified circRNAs were anticipated using Mireap, Miranda (v. 3.3a; [https://cloud.oebiotech.com/task/detail/array\\_miranda\\_plot](https://cloud.oebiotech.com/task/detail/array_miranda_plot)) and TargetScan (v. 7.0; <http://www.targetscan.org>).

The circRNA-miRNA-mRNA regulation networks were analyzed using miRTarBase (v. 6.1; <https://miRTarBase.cuhk.edu.cn>), and the culminating correlations were clarified with Cytoscape (<https://cytoscape.org/>).

## Gene Ontology and KEGG pathway analyses

We used the gene function classification system, GO, to determine the characteristics and functions of our genes of interest.<sup>27</sup> In the GO database (<http://www.geneontology.org>), all source genes are mapped to GO terms, and the determination of whether a gene fits a term is calculated using an false discovery rate (FDR) threshold of 0.05. Kyoto Encyclopedia of Genes and Genomes pathway analysis identified the significantly enriched pathways in the source genes, as compared to the whole genome background.<sup>28</sup> The calculation equation is identical to that used in GO analysis, and the cascades with  $FDR \leq 0.05$  were deemed as significant enrichment.

## siRNA transfection and SA- $\beta$ -galactosidase activity

The small interfering RNAs (siRNAs) employed for cell transfection were obtained from RiboBio (Guangzhou, China) with the following sequences: circRNA\_0007113 siRNA (5'-CAA GUG UUG CCA ACC CAU CUG AUG GA-3') and Ctrl siRNA (5'-AAU UCU CCG AAC GUG UCA CGU-3'). The siRNA was transfected with Lipofectamine™ 2000 (Invitrogen) at a final concentration of 100 nM. Aging-related senescence-associated (SA)  $\beta$ -galactosidase activity was validated using a kit purchased from Cell Signaling Technology (CST; Danvers, USA).

## Human blood sample collection and real-time quantitative polymerase chain reaction analysis

Total blood specimens were obtained from 40 healthy individuals, aged 30–39 or 60–69 years (male, body mass index (BMI) = 20–26 kg/m<sup>2</sup>) who visited the hospital for routine health examinations. All participants gave their informed consent for inclusion in the study prior to participation. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Hospital and Medical College of Yangzhou University (approval No. YXYLL-2020-02).

TRIzol reagent was used to extract the total cellular RNA, which was then transcribed into cDNA with a Reverse Transcription Kit (Takara, Shiga, Japan). Real-time qualitative polymerase chain reaction (qPCR) was conducted using a kit following the manufacturer's instructions (Takara Bio SYBR Green; Takara). The reaction parameters were as follows: 95°C for 30 s, then 35 amplification cycles

(5 s at 95°C, 30 s at 60°C). All specimens were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the experiment was repeated 3 times. Finally, SDS v. 1.4 software (Applied Biosystems, Foster City, USA) was used to analyze the data based on the  $2^{-\Delta\Delta C_t}$  method. Origin v. 9.0 software (OriginLab, Northampton, USA) was utilized to analyze the histogram.

## Protein extraction and western blotting

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) was used to lyse IMR90 cells, and protein was measured using a Bicinchoninic (BCA) protein assay kit (Bio-Rad, Hercules, USA). Total protein (35  $\mu$ g) was isolated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, St. Louis, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween (TBST) buffer, and then the primary anti-p53 and anti-p21 antibodies were introduced and incubated overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, USA). Membranes were washed thrice in TBST, and incubated with the secondary horseradish peroxidase (HRP)-conjugated antibodies (1:5,000 in TBST; Beijing Zhong-Shan Biotechnology, Beijing, China) for 1 h at room temperature. The protein was exposed using an improved chemiluminescence reagent (Millipore Sigma), and the reactive bands were analyzed for relative intensity using ImageJ software v. 1.46 (National Institutes of Health, Bethesda, USA).

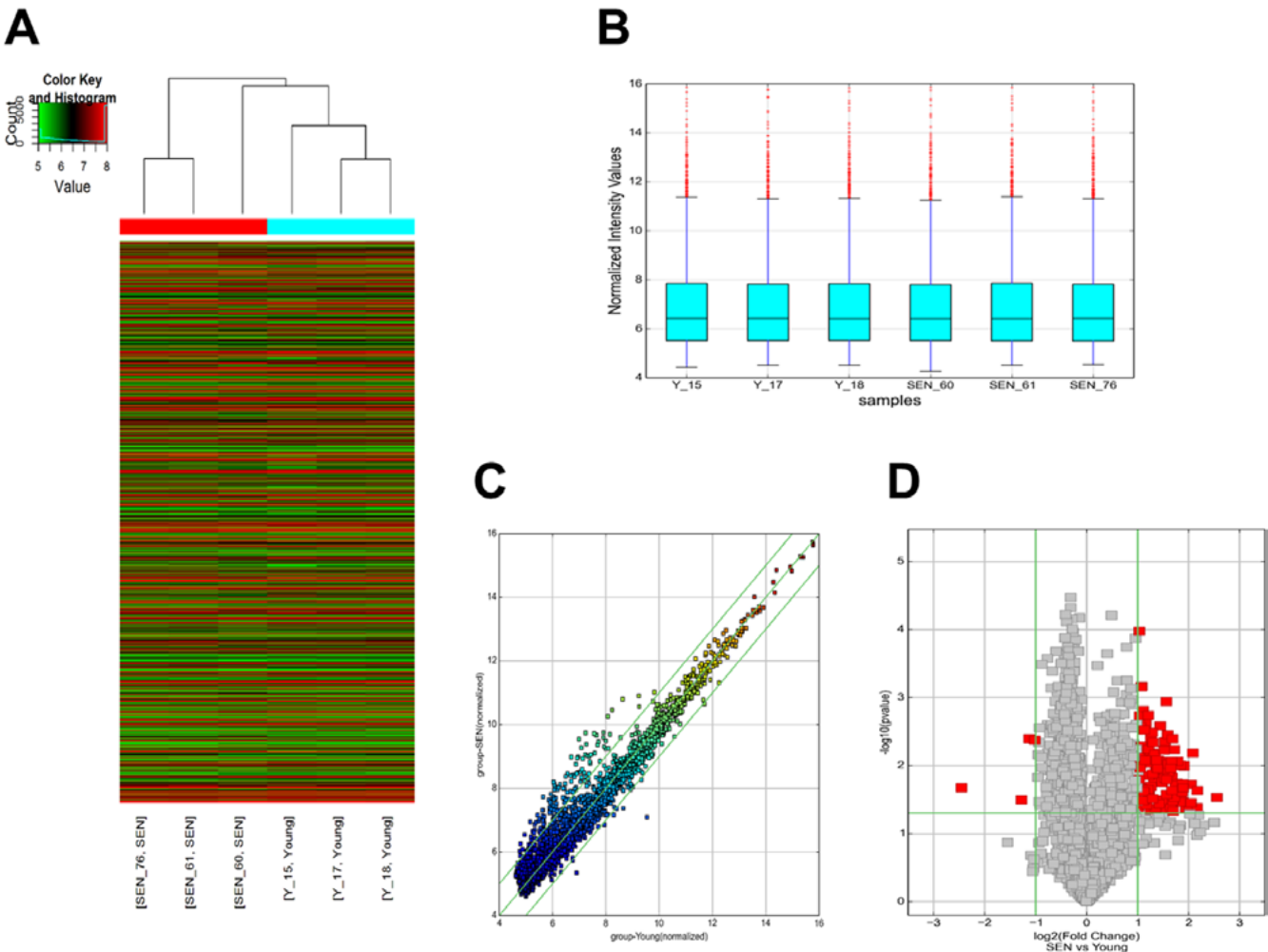
## Statistical analyses

All observations in this study were made in triplicate, and the results were analyzed using GraphPad Prism v. 8 software (GraphPad Software Inc., San Diego, USA). The one-sample nonparametric test was used to compare the difference between candidate circRNA and its corresponding control. The Shapiro–Wilk test and Bartlett test were used to determine the variables' normality, whereas homogeneity and a t-test (if not specifically indicated) or Wilcoxon Mann–Whitney test were used to compare the differences to control samples. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Differential expression profile analysis of circRNA between young and senescent human embryonic lung fibroblasts

A total of 3,936 circRNAs were identified using the circRNA microarray and displayed as hierarchical clustering (Fig. 1A) and box plot analysis (Fig. 1B). Scatter (Fig. 1C) and volcano plots (Fig. 1D) were used to identify differences



**Fig. 1.** Chip analysis of the circular RNAs in proliferating (Y\_15, Y\_17, Y\_18) and senescent (SEN\_60, SEN\_61, SEN\_76) IMR-90 cells. A. Hierarchical clustering result analysis; B. Box plot result analysis. The boxplot was generated using R. The outliers are represented as red dots beyond the upper and lower whisker boundaries. They are defined as values  $> (Q3 + 1.5 \times IQR)$  or  $< (Q1 - 1.5 \times IQR)$  ( $IQR = \text{interquartile range}$ ); C. Scatter plot result analysis; D. Volcano plot analysis. Total 113 differentially expressed circRNAs (difference  $> 2.0$  times,  $*p < 0.05$ ), including 109 upregulated and 4 downregulated. More than 80% were of the exon type

**Table 1.** The upregulated circRNAs in this study

circRNA	Alias	circRNA type	Gene symbol	Fold change	p-value
hsa_circRNA_102602	hsa_circ_0052318	exonic	ZNF418	5.87	0.029
hsa_circRNA_100748	hsa_circ_0020926	exonic	STIM1	4.49	0.023
hsa_circRNA_001405	hsa_circ_0001167	intronic	PREX1	4.48	0.042
hsa_circRNA_103517	hsa_circ_0067997	exonic	FNDC3B	4.23	0.007
hsa_circRNA_001350	hsa_circ_0000253	intronic	BLNK	4.13	0.019
hsa_circRNA_100358	hsa_circ_0000139	exonic	GON4L	4.12	0.035
hsa_circRNA_000942	hsa_circ_0001303	antisense	UBA7	4.03	0.041
hsa_circRNA_000454	hsa_circ_0001703	intronic	SEPT7P2	3.85	0.043
hsa_circRNA_000618	hsa_circ_0000708	intronic	FAM65A	3.74	0.010
hsa_circRNA_104600	hsa_circ_0005927	exonic	VDAC3	3.70	0.021
hsa_circRNA_100057	hsa_circ_0008275	exonic	VPS13D	3.69	0.034
hsa_circRNA_103542	hsa_circ_0068464	exonic	EIF4A2	3.68	0.019
hsa_circRNA_103178	hsa_circ_0062577	exonic	CABIN1	3.67	0.025
hsa_circRNA_100018	hsa_circ_0009361	exonic	GNB1	3.60	0.031
hsa_circRNA_100063	hsa_circ_0010039	exonic	CASP9	3.58	0.010

**Table 1.** The upregulated circRNAs in this study – cont.

circRNA	Alias	circRNA type	Gene symbol	Fold change	p-value
hsa_circRNA_101295	hsa_circ_0030777	exonic	PCCA	3.44	0.009
hsa_circRNA_001747	hsa_circ_0000246	exonic	MCU	3.41	0.033
hsa_circRNA_100147	hsa_circ_0004240	exonic	EIF3I	3.41	0.018
hsa_circRNA_101643	hsa_circ_0036750	exonic	C15orf38-AP3S2	3.34	0.019
hsa_circRNA_102978	hsa_circ_0004525	exonic	RBCK1	3.29	0.029
hsa_circRNA_103665	hsa_circ_0070033	exonic	NUP54	3.29	0.005
hsa_circRNA_001547	hsa_circ_0001874	intronic	BICD2	3.28	0.030
hsa_circRNA_103410	hsa_circ_0003266	exonic	LRIG1	3.23	0.046
hsa_circRNA_102333	hsa_circ_0047303	exonic	ZNF521	3.22	0.032
hsa_circRNA_104693	hsa_circ_0003691	exonic	ASAP1	3.21	0.004
hsa_circRNA_104126	hsa_circ_0076798	exonic	GCLC	3.19	0.023
hsa_circRNA_101491	hsa_circ_0034762	exonic	MAPKBP1	3.19	0.028
hsa_circRNA_000593	hsa_circ_0000550	antisense	SLC10A1	3.18	0.014
hsa_circRNA_100395	hsa_circ_0015278	exonic	KLHL20	3.17	0.037
hsa_circRNA_104323	hsa_circ_0079534	exonic	MACC1	3.17	0.009
hsa_circRNA_104551	hsa_circ_0083294	exonic	TNKS	3.14	0.010
hsa_circRNA_101524	hsa_circ_0035360	exonic	UNC13C	3.13	0.040
hsa_circRNA_100802	hsa_circ_0009018	exonic	EXT2	3.13	0.030
hsa_circRNA_001026	hsa_circ_0000141	intronic	SMG5	3.09	0.009
hsa_circRNA_104044	hsa_circ_0075447	exonic	GMDS	3.04	0.010
hsa_circRNA_101037	hsa_circ_0025767	exonic	TMTC1	3.04	0.013
hsa_circRNA_104553	hsa_circ_0083335	exonic	MTMR9	3.00	0.004
hsa_circRNA_001255	hsa_circ_0000630	intronic	BBS4	2.98	0.010
hsa_circRNA_102979	hsa_circ_0059151	exonic	RBCK1	2.97	0.027
hsa_circRNA_103863	hsa_circ_0001495	exonic	CCNB1	2.96	0.016
hsa_circRNA_102728	hsa_circ_0006110	exonic	USP34	2.96	0.001
hsa_circRNA_100244	hsa_circ_0000075	exonic	FGGY	2.95	0.045
hsa_circRNA_101591	hsa_circ_0036282	exonic	ARID3B	2.95	0.009
hsa_circRNA_000578	hsa_circ_0000487	intronic	DLEU2	2.95	0.009
hsa_circRNA_100749	hsa_circ_0020927	exonic	STIM1	2.92	0.010
hsa_circRNA_000250	hsa_circ_0000848	intronic	SMAD7	2.89	0.004
hsa_circRNA_100921	hsa_circ_0023920	exonic	PICALM	2.89	0.011
hsa_circRNA_100850	hsa_circ_0006857	exonic	PACS1	2.88	0.015
hsa_circRNA_104803	hsa_circ_0087354	exonic	UBQLN1	2.87	0.006
hsa_circRNA_101956	hsa_circ_0041551	exonic	ANKFY1	2.85	0.032
hsa_circRNA_101742	hsa_circ_0004683	exonic	C16orf62	2.85	0.011
hsa_circRNA_001653	hsa_circ_0001568	intronic	DUSP22	2.79	0.034
hsa_circRNA_001503	hsa_circ_0001191	intronic	DYRK1A	2.79	0.018
hsa_circRNA_100384	hsa_circ_0002093	exonic	SFT2D2	2.78	0.011
hsa_circRNA_000921	hsa_circ_0001120	intronic	SNED1	2.77	0.045
hsa_circRNA_100752	hsa_circ_0020976	exonic	OR51B5	2.74	0.009
hsa_circRNA_101958	hsa_circ_0041555	exonic	UBE2G1	2.72	0.003
hsa_circRNA_104426	hsa_circ_0081188	exonic	SLC25A13	2.68	0.037
hsa_circRNA_103278	hsa_circ_0001265	exonic	MTMR14	2.65	0.008
hsa_circRNA_102247	hsa_circ_0046462	exonic	TBCD	2.62	0.011
hsa_circRNA_104780	hsa_circ_0001861	exonic	GRHPR	2.58	0.008
hsa_circRNA_101401	hsa_circ_0032641	exonic	MLH3	2.56	0.010

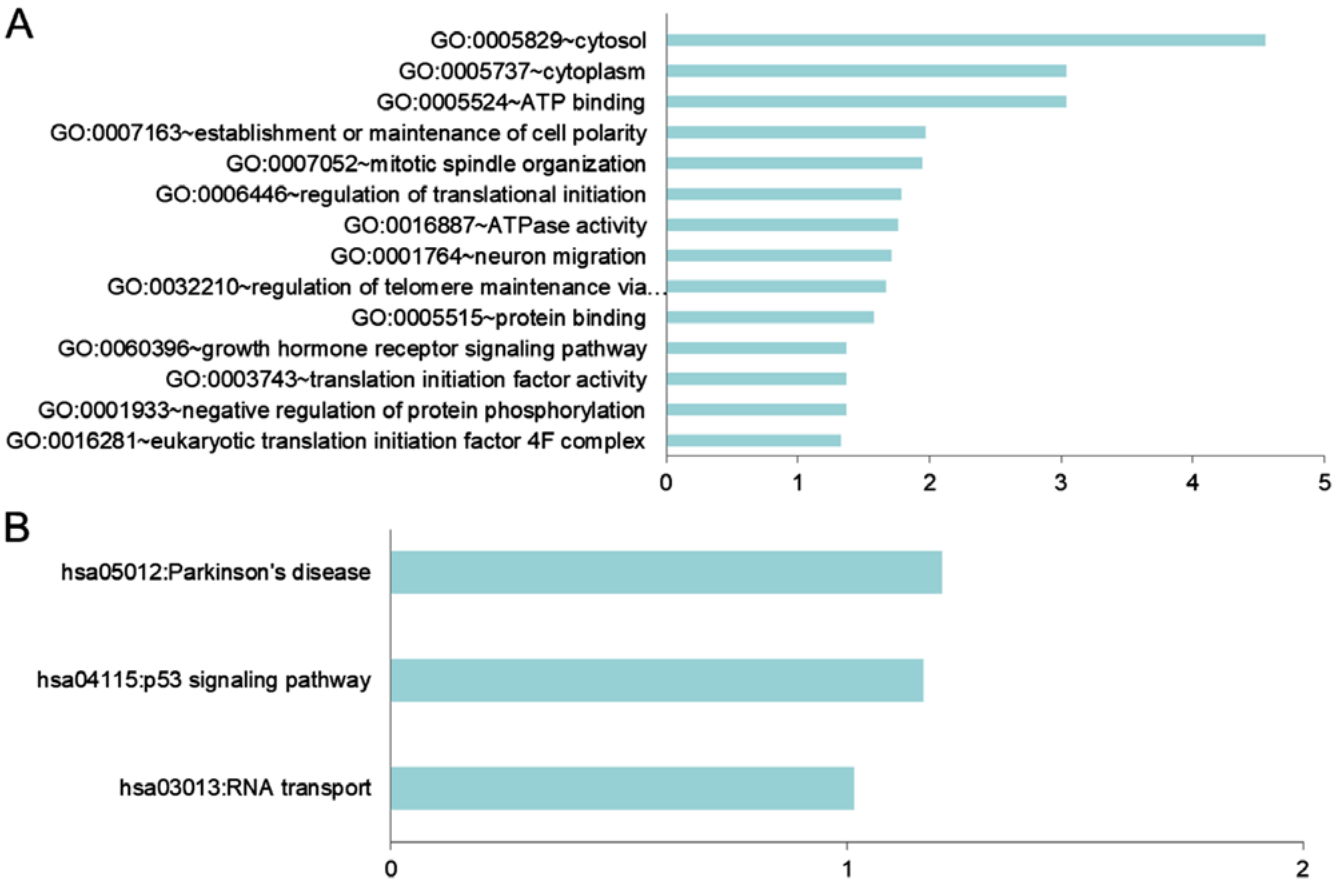
**Table 1.** The upregulated circRNAs in this study – cont.

circRNA	Alias	circRNA type	Gene symbol	Fold change	p-value
hsa_circRNA_102246	hsa_circ_0046449	exonic	TBCD	2.54	0.042
hsa_circRNA_104401	hsa_circ_0005513	exonic	GTF2I	2.53	0.007
hsa_circRNA_000644	hsa_circ_0000861	antisense	XLOC_012735	2.50	0.015
hsa_circRNA_000274	hsa_circ_0000919	intronic	ATP13A1	2.49	0.015
hsa_circRNA_102851	hsa_circ_0008032	exonic	HAT1	2.46	0.005
hsa_circRNA_001587	hsa_circ_0000979	intronic	XLOC_001374	2.45	0.007
hsa_circRNA_101759	hsa_circ_0038608	exonic	EARS2	2.45	0.013
hsa_circRNA_104948	hsa_circ_0001897	exonic	POMT1	2.45	0.031
hsa_circRNA_103140	hsa_circ_0061891	exonic	PDXK	2.43	0.003
hsa_circRNA_101746	hsa_circ_0038349	exonic	C16orf62	2.40	0.009
hsa_circRNA_000042	hsa_circ_0000036	intronic	THEMIS2	2.40	0.004
hsa_circRNA_100442	hsa_circ_0002274	exonic	LPGAT1	2.39	0.004
hsa_circRNA_000422	hsa_circ_0001545	intragenic	TCOF1	2.39	0.020
hsa_circRNA_000679	hsa_circ_0001248	intronic	TTC38	2.38	0.045
hsa_circRNA_100100	hsa_circ_0010931	exonic	TMEM50A	2.34	0.028
hsa_circRNA_100588	hsa_circ_0018293	exonic	ANXA8L2	2.34	0.014
hsa_circRNA_101635	hsa_circ_0036666	exonic	NTRK3	2.32	0.006
hsa_circRNA_104135	hsa_circ_0007874	exonic	MTO1	2.31	0.002
hsa_circRNA_102251	hsa_circ_0002225	exonic	TBCD	2.30	0.022
hsa_circRNA_104367	hsa_circ_0080170	exonic	TNS3	2.25	0.038
hsa_circRNA_001800	hsa_circ_0001033	intronic	TTC31	2.23	0.002
hsa_circRNA_104694	hsa_circ_0007934	exonic	ZFAT	2.22	0.008
hsa_circRNA_104816	hsa_circ_0087493	exonic	IARS	2.19	0.003
hsa_circRNA_102476	hsa_circ_0007396	exonic	MYO9B	2.18	0.024
hsa_circRNA_000926	hsa_circ_0001022	intragenic	ACTR2	2.16	0.006
hsa_circRNA_104744	hsa_circ_0002606	exonic	MLLT3	2.16	0.033
hsa_circRNA_001380	hsa_circ_0000540	intragenic	FBXO34	2.15	0.002
hsa_circRNA_000082	hsa_circ_0000189	intragenic	NVL	2.15	0.020
hsa_circRNA_101070	hsa_circ_0026512	exonic	EIF4B	2.15	0.033
hsa_circRNA_100699	hsa_circ_0020250	exonic	ATE1	2.14	0.006
hsa_circRNA_100604	hsa_circ_0009172	exonic	DNA2	2.14	0.006
hsa_circRNA_101248	hsa_circ_0029976	exonic	NBEA	2.12	0.022
hsa_circRNA_100981	hsa_circ_0024737	exonic	VWA5A	2.11	0.001
hsa_circRNA_100999	hsa_circ_0025006	exonic	ADIPOR2	2.11	0.005
hsa_circRNA_000046	hsa_circ_0000059	intronic	CAP1	2.10	0.002
hsa_circRNA_102575	hsa_circ_0051527	exonic	EML2	2.10	0.001
hsa_circRNA_102509	hsa_circ_0006446	exonic	LSM14A	2.07	0.003
hsa_circRNA_102551	hsa_circ_0003859	exonic	LTBP4	2.07	0.011
hsa_circRNA_103009	hsa_circ_0003853	exonic	NAPB	2.06	0.008
hsa_circRNA_101743	hsa_circ_0006797	exonic	C16orf62	2.05	0.002
hsa_circRNA_103232	hsa_circ_0002877	exonic	MKL1	2.05	0.000
hsa_circRNA_102074	hsa_circ_0043815	exonic	STAT3	2.04	0.016
hsa_circRNA_000526	hsa_circ_0000248	intronic	ADK	2.03	0.022
hsa_circRNA_103593	hsa_circ_0069031	exonic	TMEM128	2.03	0.007
hsa_circRNA_001104	hsa_circ_0001157	antisense	DHX35	2.01	0.041
hsa_circRNA_102813	hsa_circ_0007052	exonic	CLASP1	2.00	0.008



**Table 2.** The downregulated circular RNAs (circRNAs) in this study

circRNA	Alias	circRNA type	Gene symbol	Fold change	p-value
hsa_circRNA_104700	hsa_circ_0005273	exonic	PTK2	5.48	0.021
hsa_circRNA_104147	hsa_circ_0004905	exonic	IBTK	2.43	0.032
hsa_circRNA_100601	hsa_circ_0007113	exonic	HERC4	2.18	0.004
hsa_circRNA_400011	hsa_circ_0092374	intronic	GADD45A	2.03	0.004



**Fig. 2.** Bioinformatics analyses of 113 differentially expressed circular RNAs (circRNAs). A. Gene Ontology (GO); B. Kyoto Encyclopedia of Genes and Genomes (KEGG)

in circRNAs between young and senescent cells. Among them, as shown in Table 1,2, 113 differentially expressed circular RNAs (circRNAs) were analyzed; 109 circRNAs were upregulated, and 4 circRNAs were downregulated, with  $p < 0.05$  and  $|\log_2(\text{fold change})| > 1$ . More than 80% of the differentially expressed circRNAs belong to exonic circRNA, which is exclusively composed of exons.

### Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses

The mRNAs produced from the parent genes of 113 changed circRNAs were examined using GO and KEGG pathway analysis to hypothesize the pathological and physiological significance of circRNAs throughout cellular senescence. The main supplemented and meaningful GO terminologies and biological process (BP) were “establishment

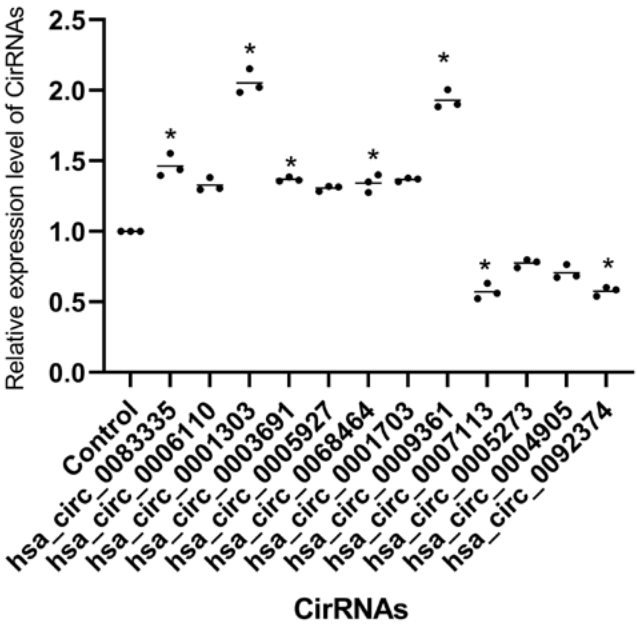
or maintenance of cell polarity”, “regulation of translational initiation” and “ATPase activity”. In terms of molecular function (MF), it was found that most of the circRNA-associated mRNAs were in “ATP binding” and “protein binding” states. As for cellular components (CC), the most enriched CC terms were “cytosol” and “cytoplasm” (Fig. 2A). Gene-enriched KEGG cascade analysis demonstrated that these pathways may be linked with the progression of aging. Most of these circRNAs are target genes linked with Parkinson’s disease, p53 signaling pathway and RNA transport (Fig. 2B).

### Differentially expressed circRNAs’ evaluation with qPCR

Nine significantly upregulated and 4 significantly downregulated circRNAs were chosen for qPCR confirmation. Primers are shown in Table 3. We found that

**Table 3.** Primers used in this study

Name	Forward (5'-3')	Reverse (5'-3')
hsa_circ_0007113	TGGGAAGCATTGTCACTGAG	CAAGCATACACCTGGCCTTT
hsa_circ_0009361	GCCGAGCAACTTAAGAACCA	AGTGCTCTTCAATGCCACCT
hsa_circ_0092374	AGCTCCCACGGACTGAAAG	TTAGCTTCTCCCTGCAA
hsa_circ_0004905	GTTTTGACCTGCTCCGTTTC	AAGAGACGGGTCTCGCTAT
hsa_circ_0006110	ATGGTTCAGTGTACTCTTGAGG	TGCTGCCATTGGAGTCCTTA
hsa_circ_0083335	TTTGTGTGATGGTGGCTTG	GACGGATGAACCTCTGTCCT
hsa_circ_0001303	AAAATAACTGGCAAATATATCATTGAG	AGAAGCCCTGCCCTTCTC
hsa_circ_0003691	GCTGCTTAGACGCTGGATTT	AGAAGCCCTGCCCTTCTC
hsa_circ_0005927	TCCTCTCCAAAATGCCAGAG	ACTCTGCTGCTCGTGCTAC
hsa_circ_0068464	TCATGTTATGCGCTGATTT	ACCAGAGTCTCCCGAATG
hsa_circ_0001703	GCTGGGGTCTTGCTATCTGA	TGCCACTGTGTTACCTTGG
hsa_circ_0005273	TGAGAGAACTTACCATAGAAATTAGCA	AGTCGCTGTGCCATTGTTT
hsa_circ_0004905	CACAACCTCAAACCCGTCT	TCAAGAGGTTGTTGCACAGG
P53	CCCCAGCCAAAGAAGAAAC	AACATCTCGAAGCGCTCAC
P21	GGGATGTCCGTCAGAACCCA	AAGTTCATCGCTCACGGG
18SRNA	CGAACGCTGCGCCTATCAACTT	ACCCGTGGTCACCATGGTA
GAPDH	GAGTCCACTGGCGTCTTCAC	ATCTTGAGGCTGTTGTCATACTTCT



**Fig. 3.** Validation of 9 upregulated and 4 downregulated circular RNAs (circRNAs) using real-time qualitative polymerase chain reaction (qPCR). The Wilcoxon Mann–Whitney test was employed to analyze the differences between each circRNA group and the control group. The hsa\_circ\_0083335, hsa\_circ\_0006110, hsa\_circ\_0001303, hsa\_circ\_0003691, hsa\_circ\_0005927, hsa\_circ\_0068464, hsa\_circ\_0001703, hsa\_circ\_0009361, hsa\_circ\_0001703, hsa\_circ\_0009361, hsa\_circ\_0007113, hsa\_circ\_0005273, hsa\_circ\_0004905, and hsa\_circ\_0092374 vs control:  $Z = -2.087$ ,  $p = 0.037$ . The test assumptions are as follows:  $H_0$ : The relative expression levels of circRNA X exhibit a similar overall distribution to that of the control group;  $H_1$ : The relative expression levels of circRNA X differ from those of the control group,  $\alpha = 0.05$ .

hsa\_circRNA\_0083335, hsa\_circRNA\_0068464, hsa\_circRNA\_0009361 and hsa\_circ\_0001303 expression levels were significantly elevated in senescent cells, while the hsa\_circ\_0007113 and hsa\_circ\_0092374 expression

levels were significantly decreased, which was in line with the microarray results (Fig. 3).

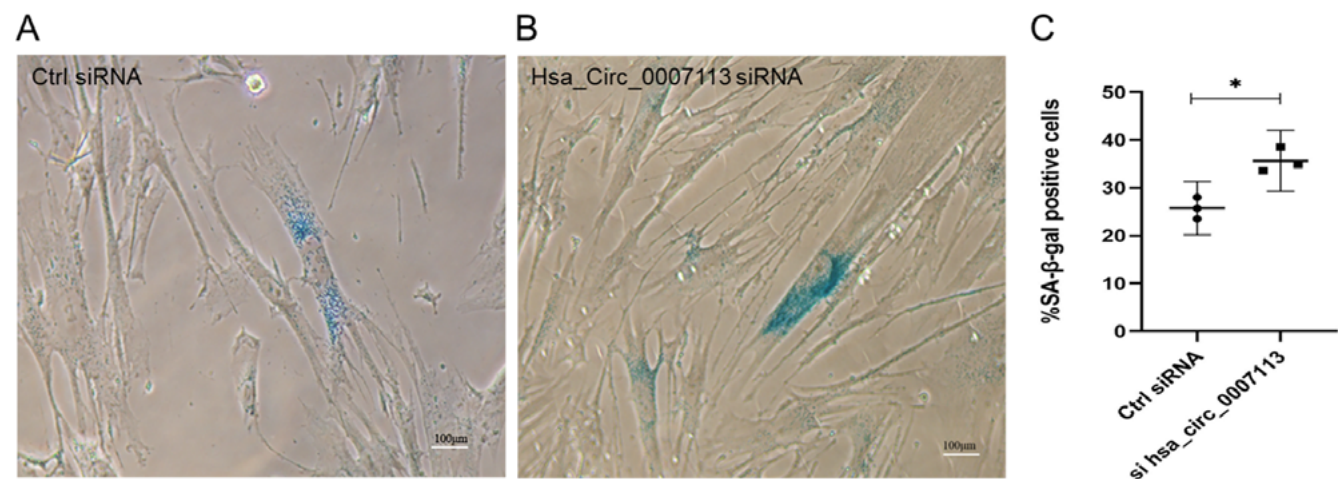
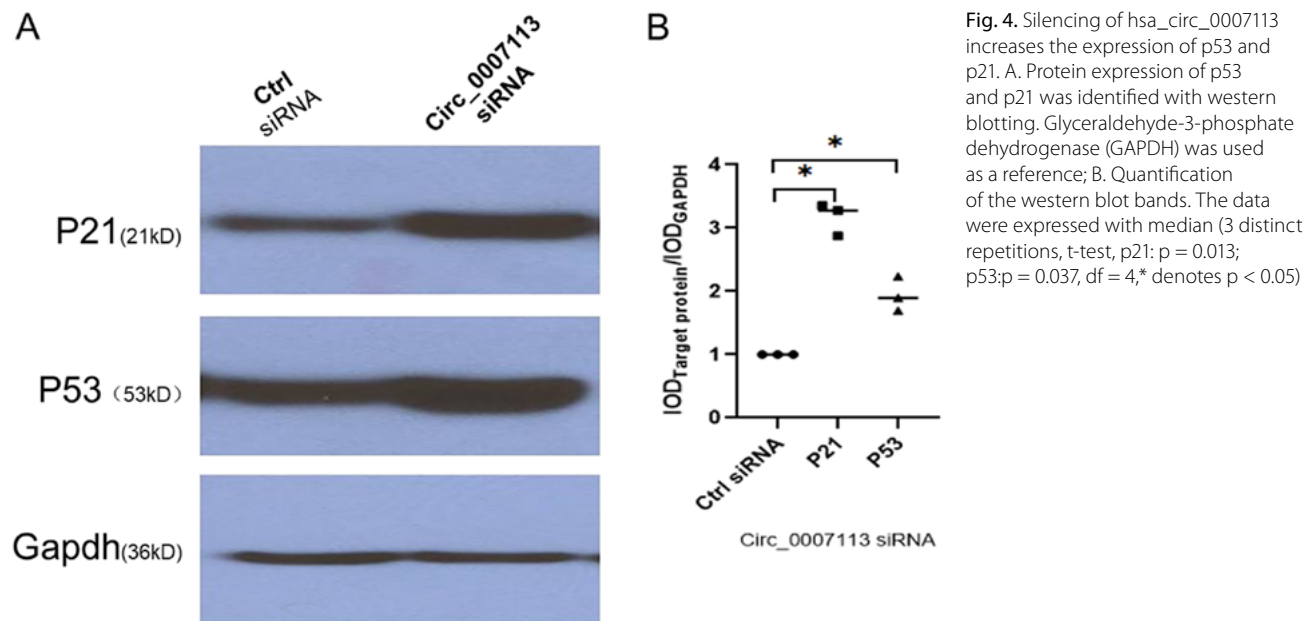
### Cellular senescence was exacerbated after siRNA treatment of hsa\_circ\_0007113

Following the administration of siRNA to silence hsa\_circ\_0007113, the expression levels of p21 and p53 were significantly elevated (Fig. 4AB). Moreover, the signal was significantly increased after  $\beta$ -gal staining, indicating that cell senescence was exacerbated after siRNA treatment against hsa\_circ\_0007113 (Fig. 5A–C).

### hsa\_circ\_0007113 could alleviate cellular senescence via miR-515-5p

CircRNAs can serve as “sponges” for miRNAs to control target gene expression. Therefore, we established a regulation network of circRNAs-microRNAs-mRNAs. The hsa\_circ\_0007113 is anticipated to bind with 275 miRNAs, thereby controlling a wide array of genes. It was found that hsa\_circ\_0007113 has a binding site for miR-515-5p, which is involved in the regulation of p53/p21 signaling pathway (Fig. 6). Five miRNAs, including hsa-miR-515-5p, hsa\_miR-1301-3p, hsa-miR-181c-5p, hsa-miR-22-5p, and hsa-miR-141-5p, were selected and were shown to target cellular processes including inflammation (IL17A, IL11RA, IL17AC), energy metabolism (ATP2B1, ATP7B, ATP12A), cell apoptosis (BCL2, BCL2L13, CASP9, CASP7, CASP10), cell senescence (SIRT2, GPR17, GPR135), cell cycle and division (CDK11A, CDK6), and zinc finger protein (ZNF784, ZNF655, ZNF589).





## The expression of hsa\_circ\_0007113 decreases with aging

The relative expression of hsa\_circ\_0007113 was measured in 40 healthy individuals, 30–39 and 60–69 years old, and was shown to be higher in the 30–39-year-old group compared to the 60–69-year-old group (Fig. 7).

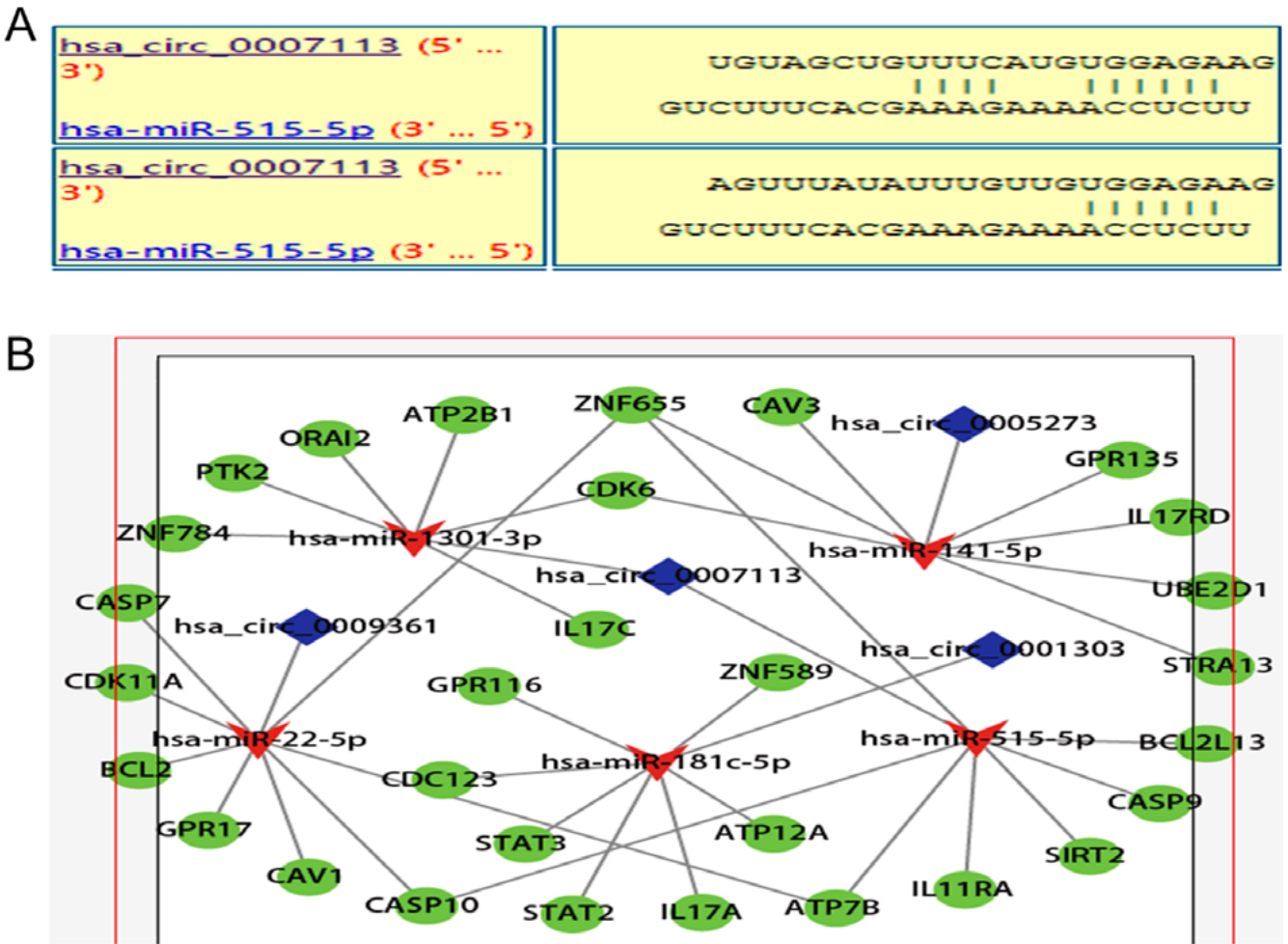
## Discussion

Age-linked diseases like osteoarthritis, atherosclerosis, cancer, Parkinson's disease, Alzheimer's disease, and type 2 diabetes are all impacted by cellular senescence.<sup>16–22,29,30</sup> Therefore, understanding the regulatory mechanism of cellular senescence may enable interventions in these aging-related

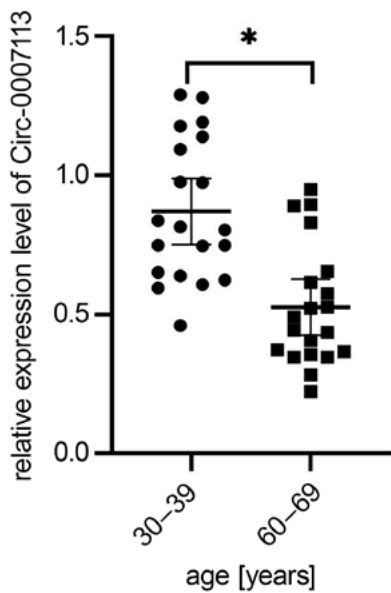
diseases. The main signaling pathways controlling cellular senescence from a mechanical perspective are P53-P21<sup>CIP1</sup> and P16<sup>INK4A</sup>-Rb. Thus, it is thought that P53 and P16<sup>INK4A</sup> are crucial elements in the induction of cellular senescence.<sup>31</sup>

Circular RNAs are important molecules involved in many biological processes, play important roles in regulating many cellular functions, and are expected to be biomarkers or treatment targets for diseases. However, their role in cellular senescence and the mechanisms of how circRNAs regulate it have not been previously described.

Therefore, the present study focused on the examination of alterations in circRNA expression profiles in senescence using the Arraystar Human circRNA Array, showing they are altered during cellular senescence. There were 109 up-regulated and 4 downregulated circRNAs, possibly playing important roles in senescence-related physiological



**Fig. 6.** hsa\_circ\_0007113 alleviates cellular senescence state via the regulation of hsa-miR-515-5p. **A.** hsa\_circ\_0007113 has a binding site for miR-515-5p; **B.** The network of selected target genes of hsa-miR-515-5p, hsa-miR-1301-3p, hsa-miR-181c-5p, hsa-miR-22-5p, and hsa-miR-141-5p



**Fig. 7.** Comparison of whole blood hsa\_circ\_0007113 levels in healthy individuals of 30–39 compared to 60–69 years. The hsa\_circ\_0007113 levels were determined using real-time qualitative polymerase chain reaction (qPCR). The data were expressed as mean with 95% confidence interval (95% CI) (40 distinct repetitions, t-test,  $p = 0.036$ ,  $df = 38$ , \*denotes  $p < 0.05$ )

processes. Most circRNAs related to aging showed a trend towards increased expression, and we speculate that this may be due to the tendency of circRNA to accumulate in aging tissues, which in turn leads to their upregulation. In the microarray results, the most upregulated and down-regulated circRNA were hsa\_circ\_0052318 (fold = 5.78) and hsa\_circ\_00052730 (fold = -5.48), respectively. However, the differential expression of circRNA detected was not as obvious as it is in cancer. This may be because cancer is a pathological process and is affected by drug stimulation, while aging is an overall slower, chronic and progressive physiological process. As this study revealed differentially expressed circRNA in human embryonic lung fibroblasts, our results may offer a new avenue for studying the molecular mechanism underlying senescence, and novel opportunities for senescence medication through the modulation of circRNAs.

Among the differentially expressed circRNA, circRNA\_0007113, a downregulated circRNA, was selected, and the pathological phenotype associated with cellular senescence was investigated. The parent gene of circRNA\_0007113 is ubiquitin ligase E3 (*HERC4*). hsa\_circ\_0007113 was obtained from exons 19 to 23 of the *HERC4* gene. E3 ubiquitin

ligase, a novel protein only discovered in recent years, plays a vital role in the ubiquitin-protease system due to its substrate recognition specificity and is also inextricably linked to cellular aging.<sup>32</sup> Currently, there are limited data on its related functions, such as participating in lung, cervical, breast and liver cancer, and participating in the incidence and development of other tumors as well,<sup>33</sup> but its role in the aging process has not been reported. To begin preliminary investigations on the potential effect of circRNA\_0007113 in cellular senescence, loss-of-function experiments were conducted using siRNA silencing. The results demonstrated that reducing circRNA\_0007113 expression significantly increased the p53 and p21 protein expression levels, which are well known to trigger cell senescence.<sup>34</sup> These results were also confirmed with  $\beta$ -gal staining.<sup>35</sup> The role of circRNA\_0007113 in cellular senescence has not been reported yet, and no other studies are available for comparison. Still, circRNAs have been shown to be involved in all cellular processes, including senescence.<sup>23–26</sup> The present study also suggests that hsa\_circRNA\_0007113 is involved in total body senescence, as hsa\_circRNA\_0007113 levels were lower in older individuals than in younger participants. Therefore, additional studies are necessary to confirm and refine the potential mechanisms.

Competing endogenous RNAs analyses showed that circRNAs modulate miRNA target gene expression. Therefore, a bioinformatics analysis was performed, which suggested that hsa\_circ\_0007113 has a binding site for miR-515-5p. The miR-515-5p was also reported to be involved in the p53/p21 pathway,<sup>36,37</sup> supporting the association of hsa\_circ\_0007113 with cell senescence. Indeed, the levels of p53 and p21 were increased in senescent cells, and studies showed that p53 expression is necessary for the maintenance of senescence.<sup>38–42</sup> However, it is noteworthy that decreasing p53 and p21 expression in senescent cells leads to the restoration of the cell cycle and immortalization.<sup>38–42</sup> Therefore, silencing hsa\_circ\_0007113 would increase miR-515-5p levels, leading to higher levels of p53 and p21, supporting the hypothesis that a reduction in hsa\_circ\_0007113 is a hallmark of cellular senescence. Notably, KEGG pathway analysis showed p53 signaling to be enriched under these circumstances. Thus, hsa\_circ\_0007113 could bind miR-515-5p, modulating the p53/p21 pathway and regulating cell senescence. However, hsa\_circ\_0007113 is only 1 of many factors that regulate cellular senescence. In this study, the new function of one circRNA derived from the *HERC4* gene was elaborated, the function was verified in human lung fibroblasts, and its relationship with aging was confirmed.












## Limitations

The specific molecular mechanism requires further clarification. For example, over-expression of hsa\_circ\_0007113 both in vitro and in vivo would also show the function.

## Conclusions

This study showed that altered circRNA expression patterns are present in cellular senescence, which may play important roles in senescence-related physiological processes. These findings offer a fresh approach to understanding the molecular mechanism underlying senescence, as well as a new way to potentially cure senescence by altering circRNAs. Additional investigations are necessary to recognize circRNA roles in cellular senescence.

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