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Identification and analysis of senescence-related genes in caudal fin cells of triploid crucian carp

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ABSTRACT

This research aims to identify the hub genes associated with the senescence of triploid caudal fin cells. Transcriptomic data are obtained from the high and low generation (P6, P60) of triploid crucian carp caudal fin cells by high-throughput sequencing technology. Initially, all differential genes between the high and low generations are screened, yielding 4140 significantly upregulated genes and 3724 significantly downregulated genes. Subsequently, an aging gene set containing 950 genes is downloaded from the CellAge database to extract the differentially expressed genes associated with caudal fin cell aging, totaling 29 genes. GO and KEGG enrichment analyses are performed on these 29 aging differential genes. The GO analysis shows enrichment mainly in cellular processes related to aging, such as regulation of cell division, chromatin organization, cell cycle regulation. KEGG analysis reveals that the 29 aging-related genes are primarily involved in cell cycle and cellular senescence pathways. A PPI network of aging-related genes is constructed using the STRING database and Cytoscape software. Top-ranked genes were identified by using Degree, MCC, MNC, and Closeness algorithms in the Cytohubba plugin in Cytoscape, resulting in hub genes *EZH2, JUN, MYD88, RBL2, BMP4, CCND1, NFKB2, MMP9*. Lastly, qRT-PCR validation of these eight hub genes further confirmed the involvement of four genes: *EZH2, RBL2, BMP4,* and *CCND1*. The hub gene screened in this study may become a potential biomarker of fish caudal fin cell senescence, which provides a valuable experimental basis for the senescence of fish caudal fin cells, especially the senescence of caudal fin cells in polyploid fish, and the reproduction and breeding improvement of polyploid fish. It also provides meaningful data for elucidating the molecular mechanism of polyploid formation in animals, as well as the formation of aging and tumour in human beings.

1. Introduction

From a biological perspective, triploidy refers to organisms where each nucleus contains three complete sets of chromosomes [1]. At the molecular level, polyploidy is defined as an increase in the content of genomic DNA. Polyploidy represents the duplication of an organism's or cell's entire genome, which profoundly impacts genes and genomes, cells and tissues, organisms, and even entire ecosystems. Hence, biological polyploidy is a significant driver of biodiversity and a source of innovation and species diversification. Polyploidy is widespread in plants and less common in animals [2]. However, fish chromosomes exhibit high plasticity compared to other vertebrates, making it easier to obtain duplications. This forms a strong theoretical basis for establishing polyploid fish systems. Prof. Liu Yun from the College of Life Sciences at Hunan Normal University successfully bred the world's first genetically stable and sexually reproductive allotetraploid fish species using integrated cellular engineering and sexual hybridization techniques. By crossing this tetraploid fish with diploid fish, sterile triploid crucian carp (Xiangyun Crucian Carp) can be produced [3]. The triploid crucian carp used in this study is an excellent breed obtained by crossing diploid red carp with tetraploid carp [4] and provides excellent experimental material.

Cell culture of fish started in the 1960s, with the successful establishment of the rainbow trout gamete cell line in 1962 marking the

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maturity of fish cell culture and cell line establishment. Since the first fish cell line was cultured from rainbow trout gonadal juvenile tissue, over 283 cell lines from various fish species have been established [5]. However, fish cell culture in China started a little later than in foreign countries, and our research team has also conducted extensive work in fish cell culture [6–14]. Primary cells cultured in vitro often lose their proliferative ability after a limited number of divisions, a phenomenon known as cellular senescence. Cellular aging is a hot topic in biological research, but it has been less reported in fish, especially in polyploid fish. Therefore, studying senescence and its mechanism in polyploid fish cells has significant theoretical implications.

Bioinformatics has been widely used to screen genomic-level genetic changes [15–17]. Therefore, this study analyzes the transcriptome information of high and low generations of triploid crucian carp caudal fin cells obtained by our team's prior high-throughput sequencing. In combination with the aging genes provided by the CellAge database, we screen for differentially expressed aging genes in caudal fin cells and perform enrichment analysis on these genes. Subsequently, we construct a protein-protein interaction (PPI) network of high and low generation caudal fin aging differential genes using the STRING database and Cytoscape software. We screen for hub genes in the PPI network using the Cytohubba plugin to predict and screen for hub genes related to cellular aging, providing a theoretical basis for further studying the aging mechanism of fish cells, especially polyploid fish cells.

2. Materials and methods

2.1. Ethics statement

All experimental procedures were conducted in accordance with the standards and ethical guidelines established by the Animal Ethical Review Committee of Hunan Normal University, Changsha, China.

2.2. Experimental material

The experimental fish was sourced from the State Key Laboratory of Developmental Biology of Freshwater Fish, a joint establishment of the Hunan Normal University and provincial authorities. Healthy triploid crucian carp were selected, and sterile caudal fin tissues were excised for in vitro culture. Fibroblast lines were established and cultured to high and low generations (P6, P60) with three tubes each, quickly frozen in liquid nitrogen and preserved at −80 °C.

2.3. Screening of aging-related differentially expressed genes

The samples were sent to Shanghai OE Biotech Co., Ltd. for transcriptome sequencing. To obtain high-quality reads suitable for subsequent analysis, raw reads were further filtered for quality. Trimmomatic [18] software was first used for quality control and adapter removal, followed by filtering out low-quality bases and N bases to eventually obtain high-quality clean reads. Trinity software (version: 2.4) [19]was used with a paired-end assembly method to assemble the clean reads into Transcript sequences. The longest sequence was selected based on sequence similarity and length to serve as the Unigene. FPKM [20] and count of the Unigene were analyzed using bowtie2 [21] and eXpress [22] software. The number of reads falling into each sample's Unigene was obtained using the eXpress software. The data were normalized using the estimateSizeFactors function of the DESeq (2012) [23] R package, and *p*-values and fold-change values for differential comparisons were calculated using the nbinomTest function. Significant differences were defined as $|\log_2\text{foldchange}| > 1$ and *p*-value < 0.05. A set of 950 characteristic cell aging genes were downloaded from the CellAge database, and these were compared with the significantly different genes obtained above to extract the significantly different cell aging genes for subsequent analysis.

2.4. Functional enrichment analysis

To better understand the crucial signaling pathways in which these aging-related differentially expressed genes participate, the DAVID software (https://david.ncifcrf.gov/) [24] was used for GO and KEGG functional enrichment analysis. Visualization of the data was carried out at http://www.bioinformatics.com.cn/.

2.5. Construction of PPI network and screening of hub genes

To further study the relationships between the proteins encoded by the aging-related differentially expressed genes, these genes were imported into the STRING database to construct a protein-protein interaction (PPI) network. The selected organism was Cypriniformes, and the minimum required interaction score was set at medium confidence (0.400). The interaction data analyzed by the STRING database were visualized and further analyzed using Cytoscape 3.9.1. The Cytohubba plugin in Cytoscape was used to calculate the top 10 genes according to Degree, MCC, MNC, and Closeness algorithms, and the intersection of these results was taken to obtain the final Hub genes.

2.6. Validation of hub genes by qRT-PCR

RNA was extracted using the HiPure Total RNA Mini Kit, followed by gel electrophoresis and concentration and OD value detection using a multi-function enzyme marker. TAKARA reverse transcription kit was employed to transcribe RNA into cDNA. The selected eight hub genes from the triploid crucian carp P6 and P60 caudal fin cells (*EZH2, JUN, MYD88, RBL2, BMP4, CCND1, NFKB2, MMP9*) were examined using Life Technologies' 4472908 real-time fluorescence quantitative reagent kit on an ABI 7500 Real-time PCR instrument. The primers used are shown in Table 1. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with *β-actin* as an internal reference gene. Graphed Prism 9 was employed for graphing and differential analysis, with a $p < 0.05$ indicating significant differences. Specific methods can be found in Refs. [4,7,25].

3. Results

3.1. Selection of aging-related differential genes

Based on a criterion of $|\log_2\text{foldchange}| > 1$ and $p < 0.05$, a total of 4140 significantly upregulated genes and 3724 significantly downregulated genes were identified (Fig. 1A). Comparing these to 950 cellular aging marker genes downloaded from the CellAge database, we found 29 genes with significant differences between high and low-generation triploid crucian carp caudal fin cells, including 15 significantly upregulated genes and 14 significantly downregulated genes (Fig. 1B and C).

3.2. Functional enrichment analysis

To predict and analyze the functions of individual genes, we performed GO functional enrichment analysis on aging-related genes in low (P6) and high (P60) generation triploid crucian carp cells. The results of GO functional enrichment analysis showed that the 29 aging-related differential genes are enriched in two main categories: biological process (BP) and cellular component (CC), mainly involved in regulating cell division, chromatin organization, cell cycle regulation, transcription control by RNA polymerase II, transcription factor complex, and cellular nucleus, which are associated with cellular aging (Fig. 2A). To further explore cellular aging-related signaling pathways, we carried out KEGG enrichment analysis, which revealed that the 29 aging-related genes are enriched in Cell cycle, Cellular senescence, NOD-like receptor signaling pathway and Apoptosis pathways, etc. (Fig. 2B), further establishing the connection between these differential genes and cellular aging.

Table 1
qRT-PCR primers.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)	Primer product(bp)	Tm(°C)
EZH2	CACTCCTTCCATACGCTCT	ATTCTCCATGTTCTTACGCTTG	105	53.1
JUN	TACCGAAACCAGGCCACGAAC	GCATGTCTCCACCCGGCATC	166	60.7
MYD88	AGCTTTAAACTGCAATTTCCGA	TTTCCCACTCCGTTAAGACC	170	54.3
RBL2	GTATCCGTCCCTCCGCACT	GTTTATCTCTCGCAGCCGGTT	160	58.4
BMP4	CGCATCAGTCGCTCCTTG	CGTCGCTGAAGTCCACATA	190	56.1
CCND1	AGAACAGAAATGCGAAGA	AACATACAAGTTGCTCCTAA	117	47.6
NFKB2	ACTTCAATATATGCACGAACCT	TCATAGCGAAATCTGAATCCTC	123	52.3
MMP9	ACAAAAGAGAACGCAAAGGTC	TCGATTTTACGTGGTCCGAAC	169	55.5
β-actin	ATACTCCTGCTTGCTAATCCAC	ATGTACCCTGGCATTGCT	174	57.0

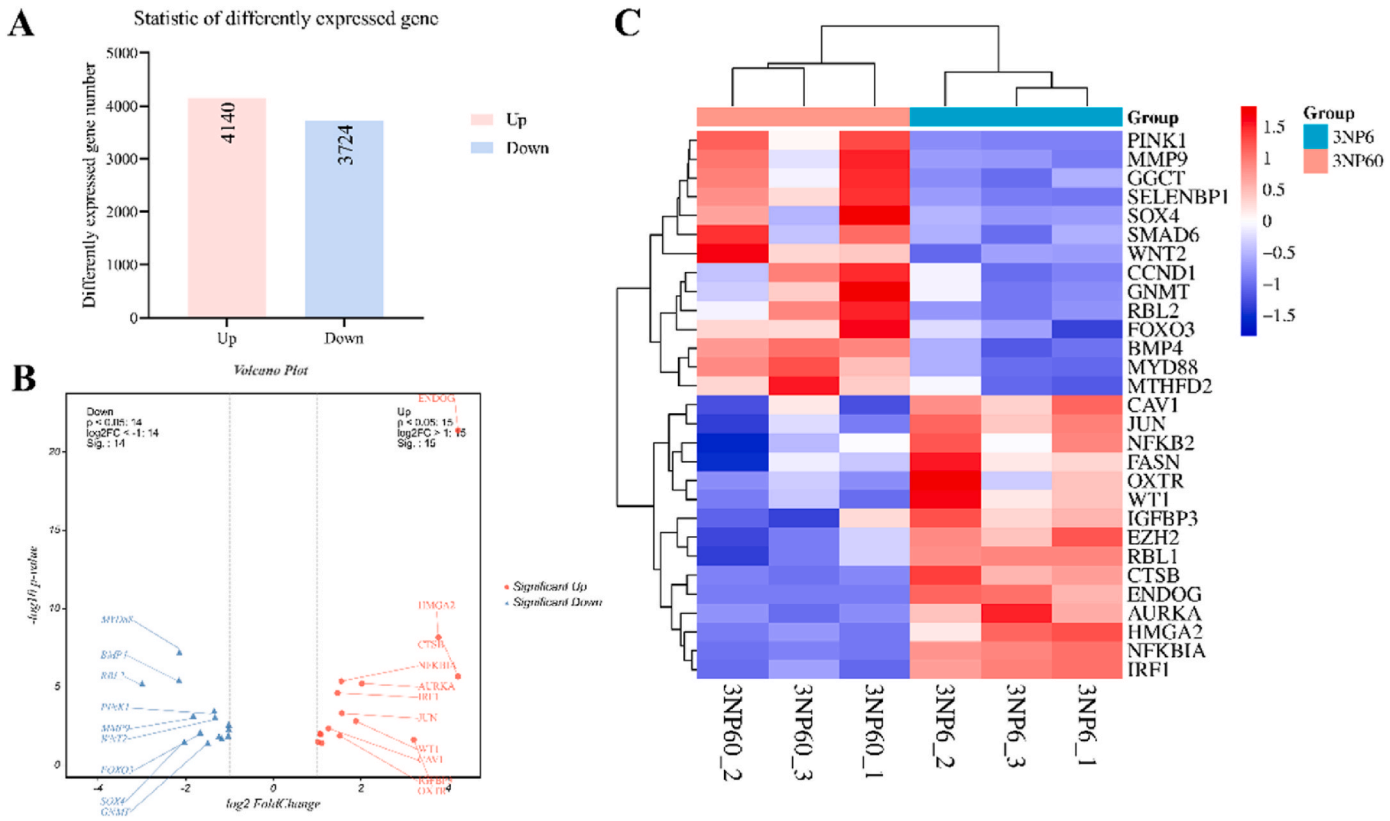

Fig. 1. Selection of aging-related differential genes. (A) Differential analysis of all genes. (B) Volcano plot of aging-related differential genes. (C) Heatmap of aging-related differential genes.

3.3. Construction of PPI network and screening of hub genes

To gain insights into gene functions and the regulatory role and mechanism of their encoded proteins in cellular aging, we used the STRING database and Cytoscape software to construct a protein-protein interaction network with the 29 aging differential genes, hiding nodes with no interactions (Fig. 3). Node sizes highlight that proteins such as *CCND1, MYD88, MMP9, EZH2*, and *JUN* have more interacting protein nodes. To further identify aging-related marker genes, we used four different algorithms (Degree, MCC, MNC, Closeness) in Cytohubba to score the top 10 genes, then took their intersection, finally identifying 8 hub genes: *EZH2, JUN, MYD88, RBL2, BMP4, CCND1, NFKB2, MMP9* (Fig. 4).

3.4. qRT-PCR verification

To further validate the reliability of the above four algorithms, we conducted qRT-PCR verification of the 8 hub genes selected in section 3.3 (Fig. 5). The results, apart from *MMP9*, aligned with the transcriptome data, and we discovered significant differences in the high and low generations for four genes (*EZH2, RBL2, BMP4, and CCND1*). This further confirms that the selected hub genes may be markers for cellular aging, thus validating the feasibility of the earlier algorithms.

4. Discussion

Since triploids are sterile, their chromosomes may be unstable during cell division, and there is a link between chromosome stability and cell senescence, which makes triploid fish a good experimental material to study cell senescence. The caudal fin cells are more convenient for experimental manipulation than those of adult fish, so we chose the caudal fin cells of triploid crucian carp as the experimental material for this study. In this study, a cell line was established from the caudal fin tissue of triploid crucian carp. The development and characterization of this cell line have provided valuable indicators for the focus and pace of research activities involving fish cells and tissues [26]. Throughout the sub-culturing process, we observed that the cells gradually aged as the number of generations increased. Aging is a complex multi-factorial

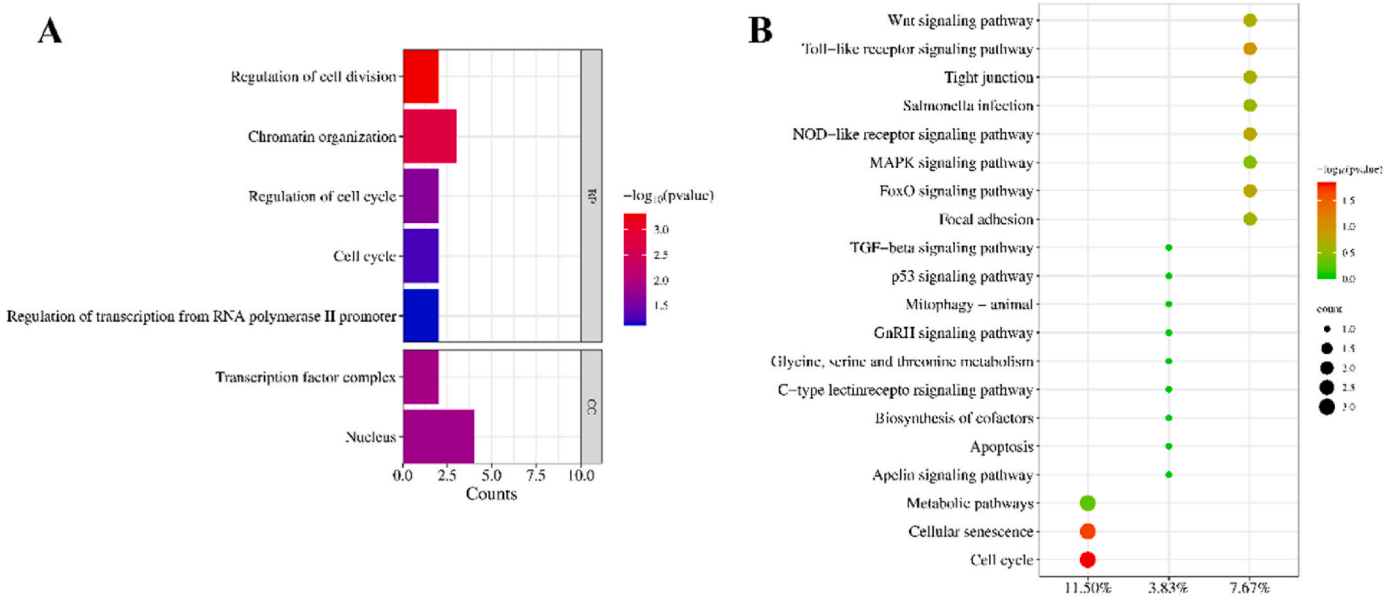

Fig. 2. Functional enrichment analysis. (A) Go enrichment analysis. (B) KEGG enrichment analysis.

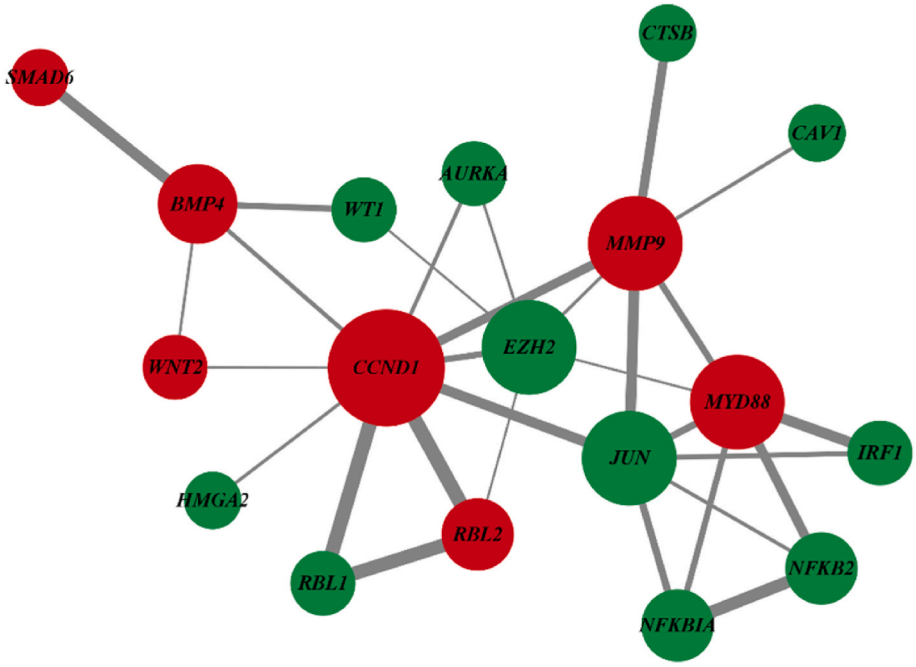

Fig. 3. PPI network of aging differential genes in 3 NP60 *vs* 3 NP6. The red represents upregulation, the green downregulation, the line thickness represents combined score, and node size corresponds to the number of interacting protein nodes.

biological process that encompasses growth and development, gene regulation, gene expression, and more. This process applies to all living organisms [27] and is a hot topic in biological research, with aging-related studies spanning the entire life course of an organism [28]. Various aging theories have been proposed by scholars around the world, such as the Telomere Theory [29], Free Radical Damage Theory, and Aging Gene Theory [30]. However, the research on aging in aquatic animals is still insufficient. To further investigate the related mechanisms of aging in triploid crucian carp caudal fin cells, our study utilized a combination of bioinformatics and molecular validation methods. We analyzed the transcriptome data of high and low generation (P6, P60) caudal fin cells, identifying characteristic genes for tail fin cell aging, with the aim to provide substantial evidence for the molecular mechanism of fish tail fin cell aging.

Through differential analysis of all genes annotated in the transcriptome and in conjunction with cell aging-related genes obtained from the CellAge database, a total of 29 differential genes related to tail fin cell aging were extracted. Following this, we performed enrichment analysis on these differential genes. GO enrichment analysis revealed their primary involvement in regulating cell division, chromatin organization, cell cycle control, cell cycle, and transcription initiation by RNA polymerase II, among other biological processes. The cell cycle is orderly and follows the sequence of G1-S-G2-M, where the G1 phase is hub for initiating the cell cycle. One of the most prominent characteristics of cell aging is the sustained metabolic activity over an extended period while being arrested at the G1 phase, losing the ability to respond

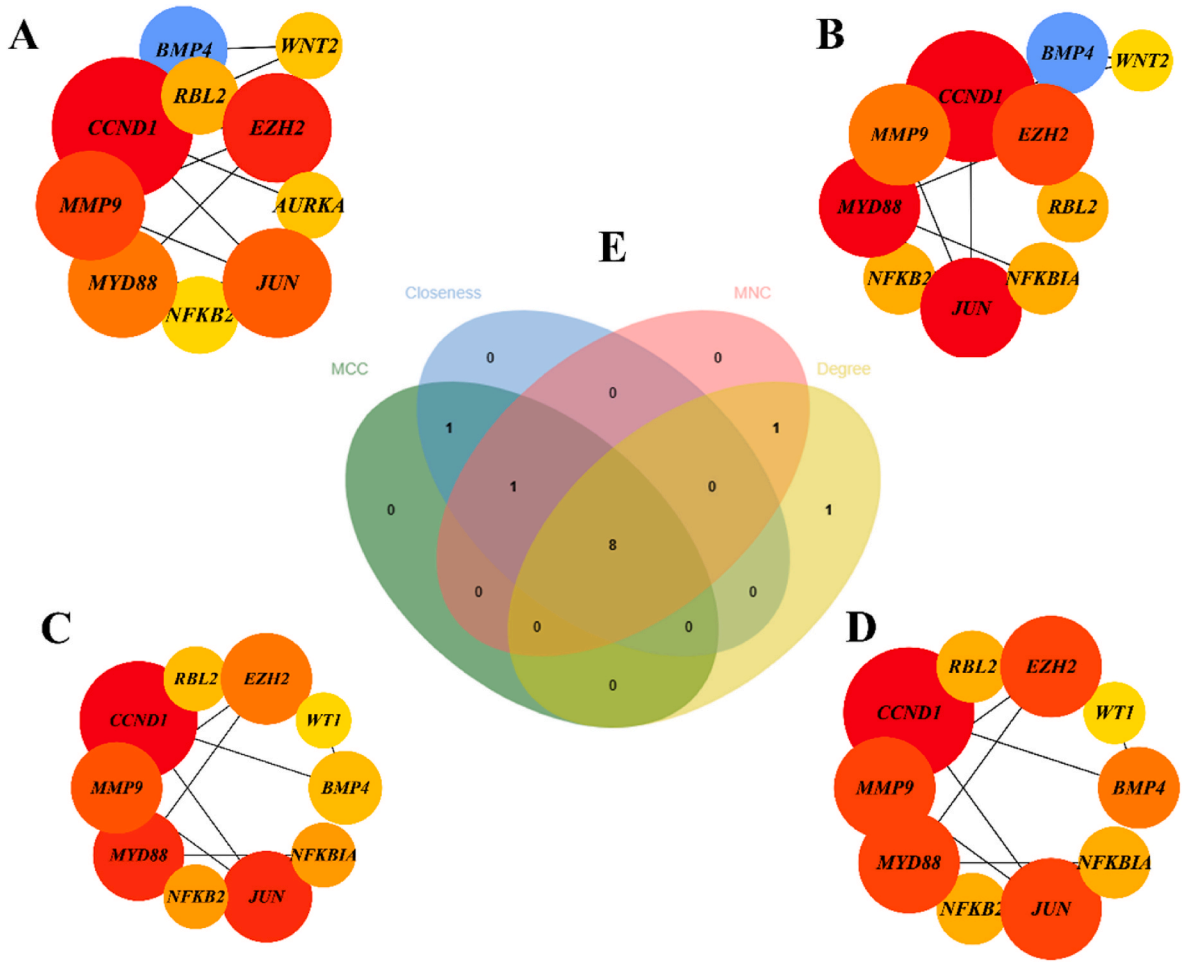

Fig. 4. Hub gene selection. (A) Top 10 hub genes selected by Closeness algorithms. (B) Top 10 hub genes selected by MNC algorithms. (C) Top 10 hub genes selected by MCC algorithms. (D) Top 10 hub genes selected by Degree algorithms. (E) Venn diagram.

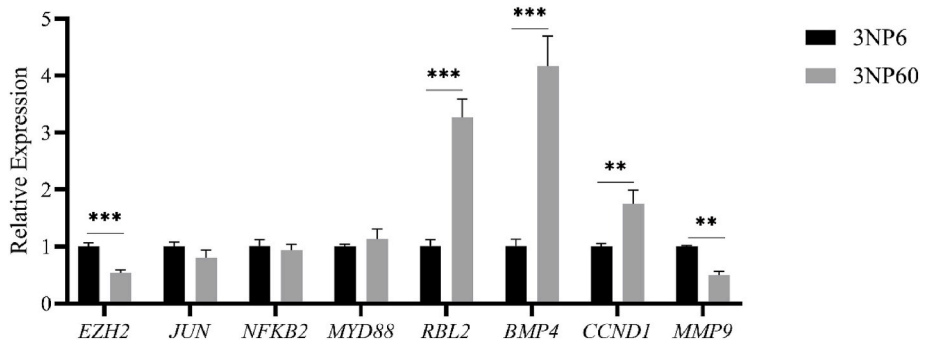

Fig. 5. qRT-PCR to verify the expression trend of hub gene. ** represents $P < 0.01$, *** represents $P < 0.001$.

to mitotic stimuli and synthesize DNA, and unable to enter the S phase [31]. This hints that the aging of caudal fin cells might be accompanied by a stagnation in the cell cycle. Research shows that the velocity of RNA polymerase II (Pol II) increases with age, which might affect gene expression and cell function. Additionally, aging cells may exhibit changes in chromatin structure [32], indicating a potential role for RNA polymerase II in the regulation of cell aging. GO analysis of cellular components shows that aging-related genes are mainly located in transcription factor complexes and the cell nucleus. Studies have demonstrated that the PBRM1-SWI/SNF complex participates in cell cycle control through E2F1 in renal cell carcinoma cells [33], suggesting a potential connection between transcription factor complexes and cell aging through cell cycle regulation. GO analysis highlights the possibility that these differentially expressed aging genes may influence caudal fin cell aging through the regulation of cell cycle, RNA polymerase II synthesis rate, chromatin structure changes, and more.

KEGG enrichment analysis found that aging-related differential genes are mainly activated in two signaling pathways: cell cycle and cellular aging. The latter includes pathways like FOXO, mTOR, P53, etc. P53 is a classical pathway related to cell aging where p53, a transcription factor, stabilizes protein levels and induces elevated cellular p53 levels through post-translational modifications like phosphorylation and acetylation in response to various stress signals such as DNA damage and oncogene activation [34]. The most noticeable results of p53 activation

are cell cycle arrest and aging apoptosis, regulated by phosphorylation and dephosphorylation [35]. FOXO1 has been involved in the aging of osteoblasts [36], mouse hematopoietic stem cells [37], ovarian granules [38], etc., but has not been reported in fish. These studies further illustrate that aging differential genes might affect fish tail fin cell growth through cell cycle changes and relevant signaling pathways for cell aging.

The construction of the PPI network of aging-related differential genes and the selection of hub genes revealed more accurate aging-related differential genes: *EZH2, JUN, MYD88, RBL2, BMP4, CCND1, NFKB2, MMP9*. Subsequent qRT-PCR further identified *EZH2, RBL2, BMP4*, and *CCND1* as potential markers for tail fin cell aging. The histone-lysine N-methyltransferase EZH2 (enhancer of zeste homolog 2) is the core component of the PCR2 complex. It exhibits methyltransferase activity and catalyzes the trimethylation of lysine 27 on histone H3 (H3K27me3). Such modifications on various target gene promoters lead to gene expression silencing [39]. Elevated expression of the *EZH2* gene has been noted in prostate cancer, breast cancer, and lymphoma [40–43]. Some studies have indicated that reducing *EZH2* expression, which in turn decreases H3K27me3 levels, can promote ovarian cancer cell aging [44]. This underscores the correlation between the *EZH2* gene with tumor development and cellular aging. However, no reports are available on the role of the *EZH2* gene in aging of fish caudal fin cells. *RBL2* (Retinoblastoma-like protein 2) has been shown in zebrafish to be associated with eye cell apoptosis. An upregulation of *RBL2* expression can influence the cell cycle and initiate the apoptosis pathway [45]. Our research suggests that the *RBL2* gene might also be related to the aging of caudal fin cells, and, based on enrichment analysis results, might also be linked to the cell cycle. Overexpression of the *BMP4* gene during bone formation can stimulate cell apoptosis, affecting the formation of fingers or toes. Moreover, studies have indicated that the chemotherapy drug doxorubicin can inhibit tumor growth by inducing premature aging in lung cancer cells, with *BMP4* playing a pivotal role in this aging process induced by doxorubicin [46,47]. This suggests that *BMP4* is associated with both cell apoptosis and cellular aging, indicating its potential as a biomarker for caudal fin cell aging. *CCND1* (Cyclin D1) is widely recognized for its crucial role in cell cycle regulation. Most of the current research on *CCND1* focuses on tumors and cancers. Overexpression of *CCND1* can lead to abnormal cell proliferation, causing cancer and contributing to cancer invasion and metastasis. Moreover, *CCND1* has shown significant clinical value in the early classification, treatment, and prognosis prediction of various tumors [48,49].

In conclusion, this study combined bioinformatics and molecular experimental verification, identified four potential markers of tail fin cell aging, *EZH2, RBL2, BMP4*, and *CCND1*. These findings lay a solid foundation for further exploration of the molecular mechanisms of cellular aging in polyploid fish species. Additionally, it offers guidance for the selection and cultivation of fish strains with enhanced resistance.

Authorship contribution statement

Yamei Xiao, Canhui Jiang, Zhen Huang , ; Lingwei Tang, Fangyuan Peng: Design the experiments and organize and write the manuscript. Canhui Jiang, Zhen Huang , ; Lingwei Tang, Fangyuan Peng: Carry out the experiments. Canhui Jiang, Zhen Huang; prepared the Figs. 1–5. Yamei Xiao, Canhui Jiang, Zhen Huang: conduct the statistical analysis and wrote the discussion. All authors read the manuscript and agreed to list their names as coauthors.

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Declaration of competing interest

The authors confirm that this article content has no conflicts of interest.

A list of full gene names

Gene name	*Description*
EZH2	[histone H3]-lysine27 N-trimethyltransferase EZH2 [EC:2.1.1.356]
JUN	transcription factor AP-1
MYD88	myeloid differentiation primary response protein MyD88
RBL2	retinoblastoma-like protein 2
BMP4	bone morphogenetic protein 4
CCND1	G1/S-specific cyclin-D1
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
MMP9	matrix metalloproteinase-9 (gelatinase B) [EC:3.4.24.35]

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