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Parishin treatment alleviates cardiac aging in naturally aged mice

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ABSTRACT

Background: Cardiac aging progressively decreases physiological function and drives chronic/degenerative aging-related heart diseases. Therefore, it is crucial to postpone the aging process of heart and create products that combat aging.

Aims & methods: The objective of this study is to examine the effects of parishin, a phenolic glucoside isolated from traditional Chinese medicine *Gastrodia elata*, on anti-aging and its underlying mechanism. To assess the senescent biomarkers, cardiac function, cardiac weight/body weight ratio, cardiac transcriptomic changes, and cardiac histopathological features, heart tissue samples were obtained from young mice (12 weeks), aged mice (19 months) treated with parishin, and aged mice that were not treated.

Results: Parishin treatment improved cardiac function, ameliorated aging-induced cardiac injury, hypertrophy, and fibrosis, decreased cardiac senescence biomarkers $p16^{Ink4a}$, $p21^{Cip1}$, and IL-6, and increased the "longevity factor" SIRT1 expression in heart tissue. Furthermore, the transcriptomic analysis demonstrated that parishin treatment alleviated the cardiac aging-related *Gja1* downregulation and *Cyp2e1, Ccna2, Cdca3,* and *Fgf12* upregulation in the heart tissues. The correlation analysis suggested a strong connection between the anti-aging effect of parishin and its regulation of gut microbiota and metabolism in the aged intestine.

Conclusion: The present study demonstrates the protective role and underlying mechanism of parishin against cardiac aging in naturally aged mice.

1. Introduction

Aging is a common, inevitable, and ongoing physiological process that involves the deterioration of cells, tissues, and organs in terms of their material, morphological, and functional aspects. Aging is also the major contributor to the development of cardiovascular disease and death. The aging heart shows unique structural and physiological changes, including left ventricular hypertrophy

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and decline in diastolic function [1]. Cardiac aging usually accompanies by various functional declines, including increased DNA damage response [2], oxidative stress [3], mitochondrial dysfunction [4], apoptosis [5], senescence-associated secretory phenotype (SASP) and systemic inflammation [6,7]. The progression of these abnormal physiological processes ultimately results in heart conditions, including myocardial ischemia, atherosclerosis, hypertension, and cardiac fibrosis, ending with heart failure [8,9]. Hence, it is imperative to create innovative techniques to mitigate heart injuries caused by aging and enhance the well-being of older individuals.

Most cardiomyocytes are considered postmitotic and have limited proliferative capacity. It was reported that the length-independent telomere damage in cardiomyocytes activates the classical senescence-inducing pathways, $p21^{Cip}$ and $p16^{Ink4a}$, and results in a noncanonical SASP, and pharmacological clearance of these senescent cells can alleviate detrimental features of cardiac aging, including myocardial hypertrophy and fibrosis [10]. Parishin is a phenolic glucoside isolated from traditional Chinese medicine *Gastrodia elata*, with the molecular formula of $C_{45}H_{56}O_{25}$. The formation of this gastrodin citrate ester occurs through the esterification of three gastrodin molecules with three terminal carboxyl groups of citric acid.

Parishin has various biological and pharmacological activities, such as ameliorating neurological injury by reducing oxidative stress and inflammatory responses [11], increasing hypoxia tolerance, resisting cardiomyocyte apoptotic [12], repairing intestinal mucosal barrier and delaying intestinal aging [13]. Parishin significantly prolongs yeast lifespan by regulating oxidative stress against the Sir2/Uth1/TOR signaling pathway [14], indicating its anti-aging potential. Our previous study demonstrates that parishin administration reduces the systemic inflammatory response induced by gut microbes-produced toxic lipopolysaccharide (LPS) in the D-galactosyogenic aging mice model [13]. Currently, few studies address whether parishin delays cardiac cellular senescence. We found that parishin ameliorated systemic aging phenotype, including cardiopulmonary injury, in naturally aged mice, and this effect is closely related to the altered gut microbiota in mice under parishin treatment [15]. These results imply a possible causal association between the anti-aging effect of parishin on cardiomyocytes and its health-promoting effect on the aging-type gut.

This study investigates the cardiac histopathology, cardiac function, senescence-associated biomarker, and cardiac transcriptomic changes in naturally aged mice to explore further the role of parishin against cardiac aging and its underlying molecular mechanism. This study's results can provide a scientific basis for the clinical application of the promised natural anti-aging drug parishin.

2. Materials and methods

2.1. Drug preparation

The parishin solution is prepared as described previously [15]. Qi Jianhua's research team at Zhejiang University provided parishin. It was dissolved in normal saline at a 2 mg/mL concentration.

2.2. Animal Experiment design

Male specific pathogen free adult (C57BL/6, 12 weeks of age) and naturally aged (19 months of age) mice were provided by Zhejiang Experimental Animal Center. All the mice were provided with a regular diet and kept at room temperature (RT) with a 12/12 h day/night cycle. Following a two-week period of acclimation, the mice were divided into three groups (N = 10 each group, 5 per cage) and treated with different solutions once daily as follows: the young group (adult mice given normal saline via gavage), the aged group (aged mice given normal saline via gavage), and the parishin group (aged mice given20 mg kg^{-1} d^{-1} parishin in normal saline via gavage). Body weight was recorded weekly. After eight weeks of treatment, the mice were administered pentobarbital sodium for anesthesia prior to being sacrificed. The heart was quickly removed, and the residual blood was washed with phosphate buffer (PBS). After drying the heart with absorbent paper, it was weighed. A portion of the heart tissues was removed for histopathological evaluation, immunohistochemical staining, and transcriptomic analysis. The remaining heart tissues were stored at −80 °C until use.

2.3. Echocardiography

The mice were anesthetized with 1.5%–2.0 % isoflurane. Echocardiography was performed on anesthetized mice using a Vevo2100 (Fuji, Tokyo, Japan) with a 30-MHz transducer at the end of the experiment. The following parameters were measured: left ventricular (LV) ejection fraction(EF), LV fractional shortening(FS) LV diameter at end diastole (LVEDD), LV diameter at end systole (LVESD), left ventricular posterior wall end-diastolic thickness (LVPWd), and other indicators.

2.4. Hematoxylin-Eosin (H&E) and Masson's trichrome staining (MS)

The heart tissue samples were fixed with 10 % formalin (Solarbio Technology Co., Ltd., Beijing, China) at RT for 24 h. Subsequently, the samples were embedded in paraffin wax and sliced into 3-μm-thick sections. To assess the cardiac architecture and fibrosis, the H&E or Masson's trichrome stained slices were examined under an inverted light microscope (Leica, Berlin, Germany), respectively. The degree of cardiac fibrosis was quantified using ImageJ software and expressed as a percentage of the fibrotic area of the whole region. The cross-sectional area of cardiac cells was quantified using HALO software v3.5.3577.

2.5. Immunohistochemistry

The paraffin sections (4 μm) were deparaffinized with xylene and then gradually rehydrated using a series of ethanol. After that,

they underwent a 20-min alkaline repair with 100 °C EDTA (pH = 9). Following three rounds of rinsing with 1 × PBS for 15 min (three times, 5 min each), the sections were blocked with 3 % hydrogen peroxide for 10 min and then rinsed again with 1 × PBS for 15 min. The sections were incubated with primary antibodies against $p16^{Ink4a}$, $p21^{Cip1}$, interleukin-6 (IL-6), γH2AX, poly ADP-ribose polymerase 1 (PARP1), silent mating type information regulation 1 homolog, (SIRT1), Caspase-3 and Caspase-9 respectively overnight at 4 °C (Table 1). Then, the sections were rinsed with PBS for 15 min and incubated with the appropriate secondary antibodies (Leica, Berlin, Germany) for 15 min at RT. After rinsing with 1 × PBS for 15 min, immunoreactivity was detected with 3,3′-diaminobenzidine substrates (Leica) for 10 min, and the samples were washed with 1 × PBS for 15 min. The slides were stained with hematoxylin dye solution at RT for 3 min and mounted with neutral gum. All the above steps are performed on Leica Bond RX and quantitatively analyzed with HALO software v3.5.3577.

2.6. Transcriptome analysis

The total RNA was isolated from each heart sample and purified with TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The amount and purity of the total RNA were qualitatively controlled using NanoDrop ND-1000 (ThermoFisher Scientific). The integrity of the RNA was checked using Bioanalyzer 2100 (Agilent, CA, USA). The polyadenylate (PolyA) mRNA was specifically isolated through two rounds of purification utilizing oligo (dT) magnetic beads (ThermoFisher Scientific) through two rounds of purification. The captured mRNA was fragmented and reverse-transcribed to construct the cDNA library following the protocol from the mRNA-Seq sample preparation kit (Illumina, San Diego, CA, USA). Finally, Illumina NovaseqTM 6000 (Illumina, San Diego, CA, USA) was utilized to sequence it at the double end following standard operations in the sequencing mode of PE150. The raw sequencing data were filtered to acquire high-quality clean data. Then, the trimmed reads were aligned to mouse genome version mm10 using RNA STAR 2.6.0b with default parameters. DESeq2 was used to analyze differential expression by quantifying and normalizing the number of clean reads mapped to each gene to the number of fragments per kilobase of exon model per million mapped fragments (FPKM) using FeatureCounts. Benjamini and Hochberg's approach was utilized to adjust the resulting *P*-values in order to control the false discovery rate. Genes that were assigned as differentially expressed had a P_{adj} value less than 0.05 according to DESeq2.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The transcription of differences associated with cardiac aging was verified using RT-qPCR. The total RNA was converted into cDNA using HiScript Reverse Transcriptase (Vazyme, Jiangsu, China) according to the manufacturer's manual and then measured in triplicate with 2 × T5 Fast qPCR Mix (Takara Biomedicals, Kusatsu, Japan) using a ViiA7 real-time PCR system (Applied Biosystems, Waltham, MA, USA). GAPDH was defined as an internal control. Gene transcription was converted into relative expression based on the internal control using the $2^{-\Delta\Delta CT}$ method. The primer sequences for the indicated genes are provided in Table S1.

2.8. Statistical analysis

The Shapiro–Wilk method was employed to assess the distribution of the data from each group while comparing echocardiography data, heart weight, body weight, heart weight/body weight ratio, quantitative cardiac histopathology results, and cardiac $p16^{Ink4a}$, $p21^{Cip1}$, IL-6, γH2AX, PARP1, Caspase-3, Caspase-9 and SIRT1 expression. To compare data sets that were not normally distributed, the Mann–Whitney *U* test was employed. In cases where there were more than two groups, the Kruskal–Wallis test was used. Otherwise, the one-way ANOVA followed by the Student–Newman–Keuls method was applied. Spearman's rank test was utilized to examine the correlations between every two significant factors. Benjamini-Hochberg method was used to adjust *P*-values for the false discovery rate when necessary (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

3. Results

3.1. Parishin treatment alleviates myocardial hypertrophy, cardiac dysfunction, and histopathological changes in naturally aged mice

The heart weight (HW), heart weight/body weight ratio (HW/BW) and the cross-sectional area of cardiac cells were calculated in

Table 1
The details of primary antibodies used in this study.

Antibody	Clone	Company	Catalog No.	Dilution
$p16^{Ink4a}$	monoclone	Abcam	Ab241543	1:640
$p21^{Cip1}$	polyclone	ABclone	A11454	1:100
IL-6	monoclone	Abcam	Ab290735	1:100
γH2AX	polyclone	Abcam	Ab11174	1:2000
PARP1	monoclone	ABclone	A19596	1:100
SIRT1	monoclone	Abcam	Ab110304	1:100
Caspase-3	polyclone	ABclone	A16793	1:100
Caspase-9	monoclone	ABclone	A11910	1:100

each group to evaluate the protective effect of parishin on cardiac hypertrophy in aged mice. Before parishin treatment, aged mice had significantly higher BW, HW, and HW/BW ratios ($P < 0.01$) than young mice. Although there was no difference in BW after eight weeks of parishin treatment between the aged and parishin groups, it significantly reduced the increase in HW and HW/BW ratio caused by aging (Fig. 1A). Additionally, parishin treatment significantly reduced the elevation of cardiac cell cross-sectional area in aged mice, making it no different from the young group (Fig. 1D). This observation indicated that parishin might prevent myocardial hypertrophy, which is prevailing in the aged population.

The cardiac function of aged mice was monitored using echocardiography after parishin treatment. Compared to young mice, the aged mice had significant cardiac dysfunction, including decreased EF, and FS, and increased LVESD and LVEDD. After treatment with parishin, EF and FS were significantly improved in the aged mice (Fig. 1B and C) and became insignificant compared to young mice. Simultaneously, parishin reduced LVESD to improve cardiac function compared to the aged group.

Cardiac fibrosis is crucial in heart failure, particularly in the aged population. We conducted a heart biopsy using H&E and MS staining to explore the cardioprotective effect of parishin further. Photomicrographs showed that the heart tissue in aged group exhibited hypertrophy of cardiac cells, proliferation of sarcomere, proportional increase in surface area of transverse tubules, and increased connective tissue between myocardial fibers (Fig. 1D). and became significantly neat after parishin treatment. MS staining

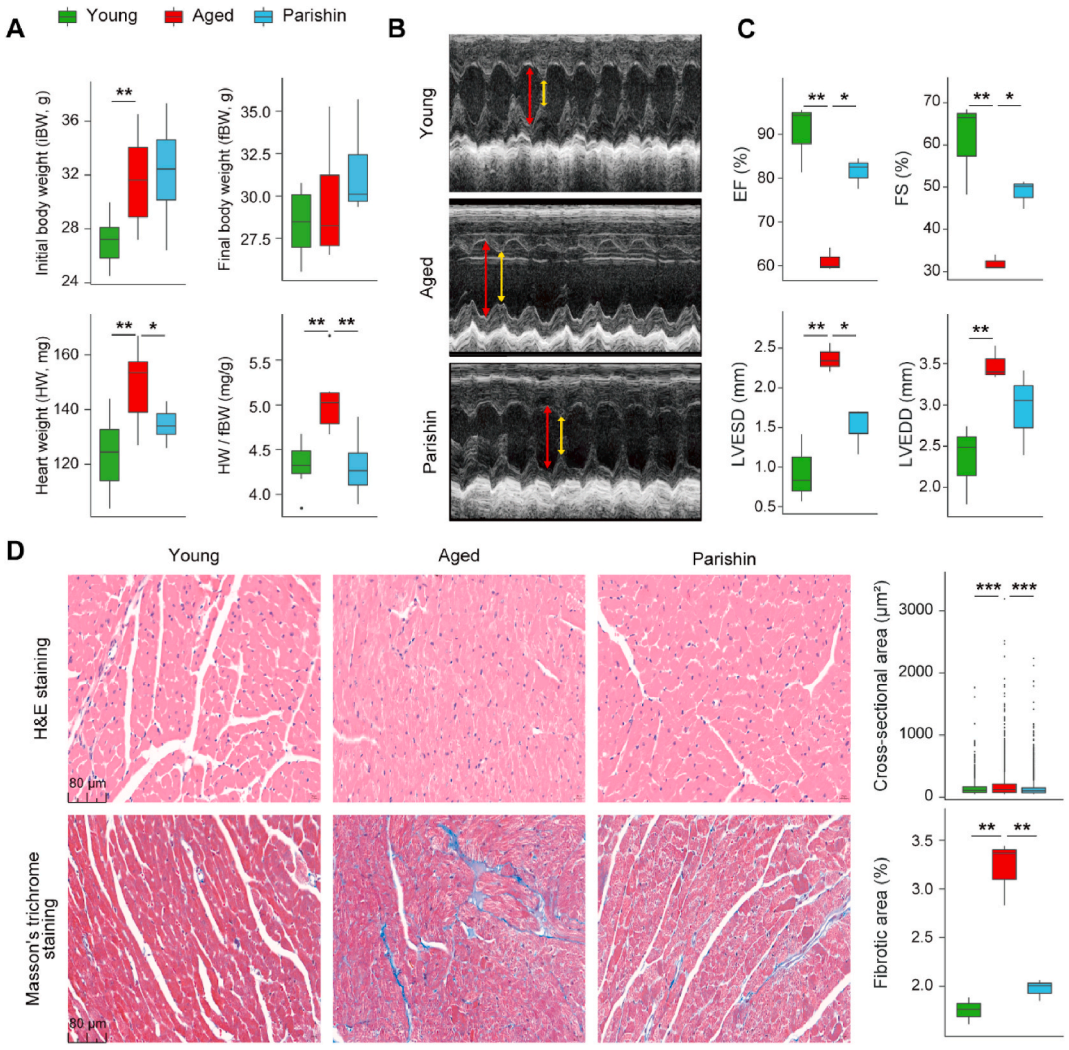

Fig. 1. Echocardiography findings, cardiac histopathological changes, and cardiac fibrosis in natural aging mice. A. Parishin treatment reduced the elevation of heart wight and heart weight/body weight ratio caused by aging. B. Representative echocardiographic M-mode images from mice with young, aged, and parishin groups. The long red arrow indicates cardiac diastole, and the yellow arrow displays cardiac systole. C. Parishin prevented cardiac dysfunction in naturally aged mice. Ejection fraction (EF), fractional shortening (FS), LV diameter at end diastole (LVEDD) and LV diameter at end systole (LVESD) were measured using echocardiography. D. Morphological changes were assessed using H&E staining, while the cardiac fibrosis was investigated using Masson's trichrome staining. The cross-sectional area of cardiac cells was quantified by HALO software, the cardiac fibrotic area was semi-quantified by Image J software. *$P < 0.05$; **$P < 0.01$, n = 10 each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

displayed a large area of collagen accumulation in the aged group, implicating a high level of fibrosis, while parishin treatment alleviated collagen accumulation (Fig. 1D). Furthermore, the semi-quantitative analysis of the MS assay confirmed that parishin significantly attenuated fibrotic area in the parishin group (Fig. 1D).

3.2. Parishin treatment alleviates cardiac senescence biomarker overexpression and reduces DNA damage in naturally aged mice

Cellular senescence has been documented during aging. The cyclin-dependent kinase inhibitors $p16^{Ink4a}$ and $p21^{Cip1}$ and cytokine IL-6 are important biomarkers. We conducted immunohistochemical staining combined with quantitative analysis to determine the cardiac $p16^{Ink4a}$, $p21^{Cip1}$, and IL-6 expressions and monitor cellular senescence. We discovered that these protein expressions were upregulated in an aged group compared to the young group (Fig. 2A and B) and downregulated after parishin treatment.

DNA damage is the primary cause of aging, and may eventually leading to apoptotic cell death. The γH2AX and PARP1 were the first responder and repair factors to monitor genomic integrity [16,17]. The aged group had higher levels of γH2AX and PARP1 than the young group (Fig. 2A and B), indicating that aged mouse cardiac cells accumulate significantly more DNA damage. After parishin treatment, γH2AX and PARP1 expressions were downregulated and maintained a similar level with the young group. In line with these findings, the expression of apoptosis marker proteins Caspase-3 and Caspase-9 in the heart tissue of aged mice was also significantly reduced after parishin treatment. Thus, we speculated that parishin reduced DNA damage to maintain genomic stability and alleviate

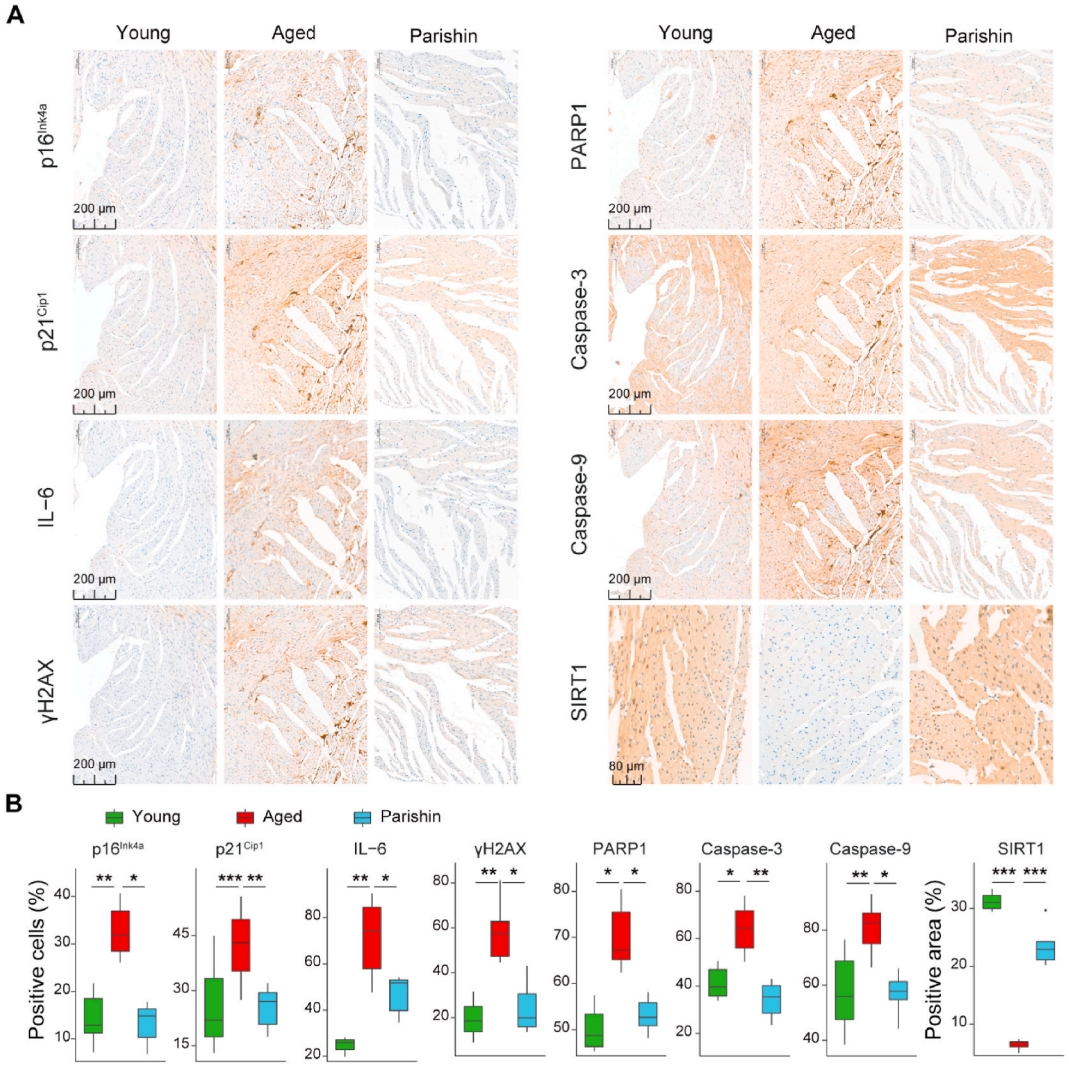

Fig. 2. Parishin decreased the senescence marker, inflammatory factors, DNA damage/repair factor, and apoptosis marker expression, and increased SIRT1 expression in the cardiac tissues of aged mice. A. The expression of $p16^{Ink4a}$, $p21^{Cip1}$, IL-6, γH2AX, PARP1, Caspase-3, Caspase-9 and SIRT1 in cardiac tissue was examined using immunohistochemical staining. B. The percentage of positive area of $p16^{Ink4a}$, $p21^{Cip1}$, IL-6, γH2AX, PARP1, Caspase-3, Caspase-9 in the slice was calculated using HALO software, and the integrated optical density value of the SIRT1-positive cells was calculated using Image-Pro Plus software. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n = 10 each group.

cell apoptosis in aged cardiac cells.

Parishin has been reported to improve the oxidative stress capacity and prolong the lifespan of yeast by upregulating the sirtuin 2 [13]. Moderate overexpression of SIRT1, a mammalian homolog of sirtuin 2, was reported to attenuate age-dependent increases in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of cellular senescence markers in mouse model [18]. Therefore, expression of SIRT1 in the cardial tissue of mice was examined using immunohistochemical staining. The results exhibited that the aged group had significantly lower SIRT1 expression than the young group and parishin treatment increased it (Fig. 2A and B). These findings suggest that senescent cardiac cells may obtain benefits from parishin treatment due to the alleviated increase in aging

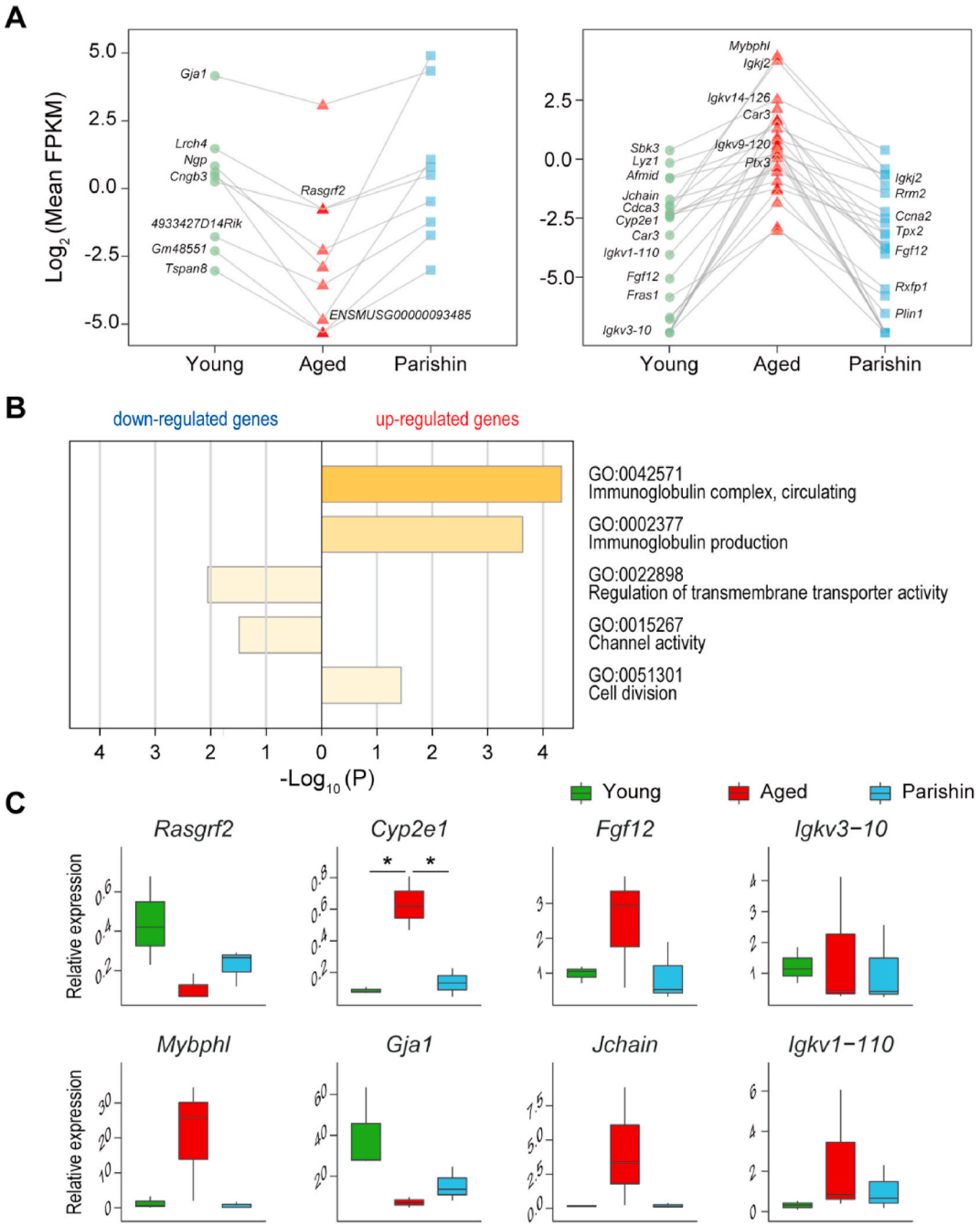

Fig. 3. Parishin partially reverses transcriptional regulatory changes. A. Thirty-three cardiac genes were differentially transcribed between young, aged, and parishin groups ($P_{adj} < 0.05$). Among them, parishin reversed the upregulation of 24 genes and the downregulation of nine genes induced by cardiac aging. B. KEGG pathway enrichment analysis of these differentially transcribed genes. C. Relative expression of representative genes using RT-qPCR. $^*P < 0.05$, n = 3 in each group.

biomarkers, DNA damage and cell apoptosis.

3.3. Parishin partially reversed the aging-induced transcriptional changes in cardiac cells

RNA-sequencing of heart tissue samples was performed to investigate the global regulatory role of parishin treatment in anti-cardiac aging at the transcriptional level. Among the transcripts of 32,477 mapped genes, 33 genes with significant differences between young and aged groups returned to normal levels after treatment with parishin. Parishin treatment reversed the upregulation of 24 cardiac genes, including *Igkj2*, *Ccna2*, *Fgf12*, *Cyp2e1,* and *Rxfp1*, and the downregulation of nine genes, including *Gja1*, *Lrch4*, *Ngp*, *Cngb3*, *Rasgrf2*, *Tspan8*, *Gm48551*, *Gm20708,* and *Gm43951* (Fig. 3A). KEGG pathway enrichment analysis indicates that these genes involved the immunoglobulin complex, circulating, immunoglobulin production, regulation of transmembrane transporter activity, channel activity, and cell division (Fig. 3B). The transcription of eight representative genes, including *Rasgrf2*, *Cyp2e1*, *Fgf12*, *Gja1*, *Mybphl*, *Jchain*, *Igkv1-110* and *Igkv3-10*, were quantified using RT-qPCR to validate the transcriptomic results. We discovered that their expression was similar to those in the transcriptome analysis (Fig. 3C).

3.4. Altered cardiac senescence biomarkers, cardiac function, cardiac fibrosis, and cardiac genes were closely associated with parishin-regulated gut microbes and metabolites

The anti-aging effect of parishin was closely related to its regulation of microbiota in the aged intestine [15]. We analyzed the correlations between parishin treatment-altered gut microbes and metabolites, cardiac function indicators, fibrosis markers, aging markers, DNA damage indicators, and transcripts in naturally aged mice using Spearman's rank test to clarify their relationship further. Significant results were identified by using both a correlation coefficient (r) with an absolute value greater than 0.4 and a *P*-value (*P*) less than 0.05 as the screening threshold.

First, we discovered that the gut bacterial taxa, including *Desulfovibrio*, *Tannerellaceae*, *Lachnospiraceae*, *Beijerinckiaceae*, *Saccharimonas*, *Turicibacter*, *Parabacteroides*, *Oscillospiraceae* sp., *Oscillibacter*, uncultured *Clostridium,* and *Erysipelatoclostridium*, positively correlated with gene transcripts *Afmid*, *Fgf12*, *Cdca3*, *Cyp2e1*, *Tpx2*, *Car3*, *Ccna2*, *Ptx3*, *Rxfp1*, *Fras1*, *Jchain*, *Mybphl*, *Lyz1*, *Igkj2*, *Sbk3*, *Igkv1-110*, *Igkv14-126*, *Ighv1-55* and *Trpc2*, and/or negatively correlated with *Tspan8*, *Gja1*, *Cngb3*, *Lrch4*, *Gm20708*, *Gm43951,* and

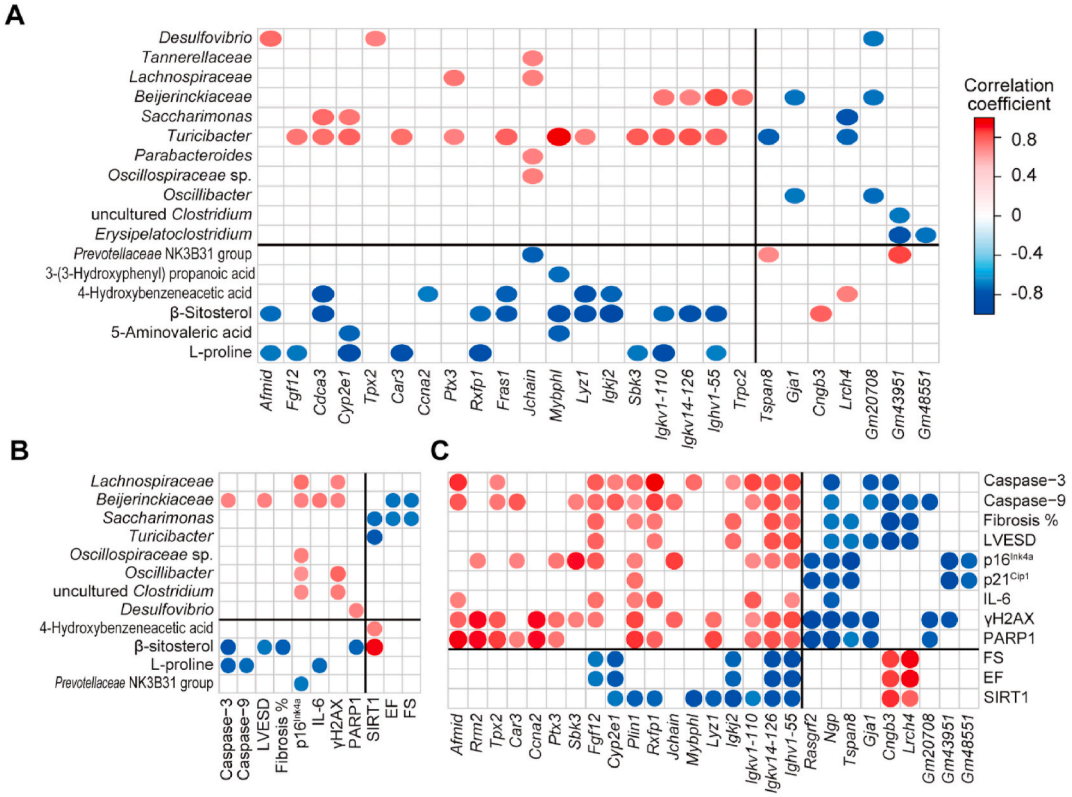

Fig. 4. Associations among gut microbes and metabolites, cardiac genes, aging biomarkers, and function indicators influenced by parishin treatment ($|r| > 0.4$, $P < 0.05$). A. Correlations of parishin-altered gut microbes and metabolites with cardiac genes; B. Correlations of parishin-altered gut microbes and metabolites with cardiac aging biomarkers and function indicators. C. Correlations of parishin-altered cardiac genes with cardiac aging biomarkers and function indicators.

Gm48551. The correlations between *Prevotellaceae* NK3B31 group, gut metabolites 3-(3-Hydroxypheny) propanoic acid, 4-Hydroxybenzeneacetic acid, β-Sitosterol, 5-Aminovaleric acid, L-proline, and these gene transcripts are opposite to the above bacterial taxa (Fig. 4A). Second, the gut bacterial taxa, including *Lachnospiraceae*, *Beijerinckiaceae*, *Saccharimonas*, *Turicibacter*, *Oscillospiraceae* sp., *Oscillibacter*, uncultured *Clostridium,* and *Desulfovibrio*, were positively correlated with cardiac LVESD and fibrosis percentage and Caspase-3, Caspase-9, $p16^{Ink4a}$, IL-6, γH2AX and PARP1, and negatively correlated with cardiac SIRT1, EF, and FS. However, the correlations between *Prevotellaceae* NK3B31 group, gut metabolites 4-Hydroxybenzeneacetic acid, β-Sitosterol, L-proline, and those cardiac indicators are opposite (Fig. 4B). Third, the cardiac fibrosis percentage, LVESD, Caspase-3, Caspase-9, $p16^{Ink4a}$, $p21^{Cip1}$, IL-6, γH2AX, and PARP1 were positively correlated with gene transcripts *Afmid*, *Rrm2*, *Tpx2*, *Car3*, *Ccna2*, *Ptx3*, *Sbk3*, *Fgf12*, *Cyp2e1*, *Plin1*, *Rxfp1*, *Jchain*, *Mybphl*, *Lyz1*, *Igkj2*, *Igkv1-110*, *Igkv14-126,* and *Ighv1-55*, and/or negatively correlated with *Rasgrf2*, *Ngp*, *Tspan8*, *Gja1*, *Cngb3*, *Lrch4*, *Gm20708*, *Gm43951,* and *Gm48551*. In contrast, the correlations between FS, EF, SIRT1, and these gene transcripts are opposite (Fig. 4C).

4. Discussion

Cardiac dysfunction is one of the greatest health risks for the elderly. The pathobiology of cardiac dysfunction includes cellular senescence, chronic inflammation, DNA damage, apoptosis, oxidative stress, and mitochondrial dysfunction [2,19]. Parishin is extracted from *Gastrodia elata*, a traditional Chinese medicine with life prolonging effect in *vitro* [14] and anti-inflammatory and antioxidant activities in *vivo* [11]. In this study, we investigated its health-promoting effect on cardiac aging in naturally aged mice.

We discovered that the cardiac function, cardiac hypertrophy, and cardiac fibrotic degree of aged mice were improved after parishin treatment. Moreover, parishin treatment alleviated the increased cardial $p16^{Ink4a}$, $p21^{Cip1}$, IL-6, γH2AX, PARP1, Caspase-3 and Caspase-9 in the aged mice. The age-dependent increase in expression of senescent biomarkers $p16^{Ink4a}$ and $p21^{Cip1}$ and the SASP IL-6 in aged heart tissue are reported [10]. The γH2AX and PARP1 are well-known DNA damage response factors that function as key regulators during DNA damage. They cooperate with most aging phenotypes as major causes of senescence, providing a potential target for the intervention against aging-related diseases [20]. Our findings suggest that parishin treatment may reduce the genomic DNA damage of senescent cardiac cells and maintain their chromosome integrity, and may eventually alleviate cell apoptosis.

On the other hand, age-independent cellular senescence and associated pathological expression of those senescent biomarkers and SASP can also induce cardiac dysfunction and degenerative defect. Studies have shown that senescent cell removal by senolytics, such as dasatinib and quercetin, can be an effective treatment for cellular senescence-related heart injury caused by diabetes or myocardial infarction [21,22]. Although there is no evidence that parishin contributes to the clearance of senescent cells, its regulatory effects on SASP and geroprotective proteins have been reported: parishin can promote macrophage switch from pro-inflammatory M1 state to anti-inflammatory M2 state through JAK/STAT1 pathway and attenuate pro-inflammatory cytokine, such as IL-6 and TNF-α, secretion in macrophages [23]; parishin administration can increase the expression of α-Klotho, an anti-aging protein protects cells from SASP, thereby delaying vascular dysfunction [24]. In this study, we found that parishin treatment eliminated the aging-induced down-regulation of SIRT1. The "longevity factor" SIRT1 plays a pivotal role in mediating the cell death/survival process and has been implicated in the pathogenesis of cardiovascular disease [25–27]. It is worth noting that SIRT1-dependent deacetylation blocks PARP1 activity under stress conditions, it protects cells from PARP1-mediated cell death; and SIRT1 negatively regulates the activity of the PARP1 gene promoter [28]. That is in line with our findings that the decrease in PARP1 level in the aged group after parishin treatment is accompanied by an increase in SIRT1, which may help promote stress-resistance, DNA repair, and cardiac function.

The involvement of gut bacteria has been proposed in almost all major diseases including cardiovascular disease [29,30]. The previous study revealed that parishin attenuated the depletion of some potentially beneficial gut microbes, such as *Prevotellaceae* NK3B31, and the enrichment of some opportunistic pathogens, such as *Clostridium*, *Oscillibacter*, *Lachnospiraceae*, *Desulfovibrio*, *Oscillospiraceae* sp., and *Beijerinckiaceae* [15]. In this study, correlation analysis revealed that potentially beneficial bacteria, such as the *Prevotellaceae* NK3B31 group, were negatively correlated with $p16^{Ink4a}$ expression, while potentially harmful bacteria, such as uncultured *Clostridium*, *Oscillibacter*, *Lachnospiraceae,* and *Beijerinckiaceae*, were positively associated with $p16^{Ink4a}$ and γH2AX, and *Beijerinckiaceae* was positively associated with Caspase-3 and IL-6. The cardio-protective/pathogenic effects of those beneficial bacterial taxa such as *Prevotellaceae* and opportunistic pathogens such as *Oscillibacter* and *Desulfovibrio* have been reported to closely related to their metabolites short-chain fatty acids (sCFAs) and trimethylamine-N-oxide (TMAO) [9,31]. The elevated level of circulating TMAO can accelerate cellular senescence by inhibiting SIRT1 expression and activating the p53/p21/Rb pathway [32]. TMAO is a gut microbes-dependent metabolite, indicating that parishin's protective effect on cardiac aging may be related to its inhibition of TMAO circulation by regulating gut microbiota, which necessitates further investigation. Fecal metabolites bridge the interaction between the host and the gut microbes. Our previous study discovered that gut metabolites L-proline and β-sitosterol of aged mice returned to normal level after parishin treatment [15]. In this study, β-sitosterol correlated negatively with cardiac LVESD, fibrosis percentage, Caspase-3 and PARP1 and correlated positively with SIRT1, and L-proline negatively correlated with Caspase-3, Caspase-9 and IL-6. β-sitosterol can prevent diabetes-induced cardiac injury [33] and be reported to reverse the elevation of TNF-α and IL-6 and the reduction of eNOS in heart tissue by reducing oxidative stress response, thus playing a cardiac protective role [34]. These results indicate that parishin treatment-altered gut microbes and metabolites also played important roles in promoting aging heart health.

Moreover, we revealed that parishin treatment ameliorated the transcriptional upregulation of cardiac genes *Cyp2e1*, *Ccna2*, *Cdca3,* and *Fgf12* and the downregulation of *Gja1* in naturally aged mice. First, the cytochrome *Cyp2e1* is upregulated in various heart diseases and causes damage mainly via reactive oxygen species (ROS) production [35,36]. In mice, the increased *Cyp2e1* expression induced cardiomyocyte apoptosis, and endogenous *Cyp2e1* knockdown attenuated the pathological development of dilated cardiomyopathy [37]. Our correlation analysis result indicated that *Cyp2e1* transcription correlated positively with conditionally harmful gut microbes

Saccharimonas and *Turicibacter*, apoptosis marker Caspase-3 and Caspase-9, and negatively with gut metabolites L-proline and 5-aminovaleric acid, cardiac function indicators EF and FS, and the "longevity factor" SIRT1. *Turicibacter* has promoted heart disease and correlated positively with obesity and host inflammation [38,39]. The 5-aminovaleric acid, a γ-aminobutyric acid analog, serves as a significant compound in the pathways of L-lysine degradation and L-proline degradation [40,41]. It is commonly found in elevated levels in the heart, muscle, and brown adipose tissue with health-promoting effects. Second, cyclin A2 (CCNA2), a key regulator of cell proliferation, plays a crucial role in cardiomyocyte growth in fetal and neonatal hearts and is sustained and expressed in the adult heart to induce cardiomyocyte proliferation [42]. Cell division cycle-associated protein 3 (CDCA3), also known as trigger of mitosis entry 1 (Tome-1), has been reported to mediate cell cycle progression [43]. Although the majority of the adult cardiomyocyte s are considered postmitotic, the adult heart retains limited potential for cardiomyocyte proliferation [10]. We indicated that *Cdca3* transcription correlated positively with *Saccharimonas* and *Turicibacter* and negatively with 4-hydroxybenzeneacetic acid and β-sitosterol. *Ccna2* correlated positively with DNA damage markers γH2AX and PARP1 and negatively with 4-hydroxybenzeneacetic acid. The 4-hydroxyphenylacetate, produced from gut microbial polyphenols and amino acid metabolism, has been shown to inhibit the growth of pathogenic bacteria [44], and decreases in age-related intestinal metabolic dysfunction [45]. These findings imply the health promoting effect of the parishin-modulated gut microbiota on cardiac aging. Third, the fibroblast growth factor (FGF) family has numerous biological functions, including cell growth, tissue regeneration, embryonic development, metabolism, and angiogenesis. Fgf12 can interact with ion channels in the nervous and cardiac systems and play a role in various arrhythmias, such as ventricular tachycardia [46]. *Fgf12* is also an important regulator of vascular smooth muscle cell homeostasis and a promoter of organ fibrosis [47,48]. We revealed that the transcription *Fgf12* correlated positively with *Turicibacter*, cardiac fibrosis percentage, LVESD, Caspase-3, Caspase-9, $p16^{Ink4a}$, γH2AX, and IL-6 and negatively with L-proline, FS, and EF. These findings indicate that *Fgf12* may be a potential cardiac fibrosis, injury, and dysfunction marker. Fourth, the gap junction protein alpha 1 (GJA1), also known as connexin 43, is involved in cellular communication and tissue homeostasis maintenance. It can be expressed by atrial and ventricular cardiomyocytes [49] and has been reported to decreased in cardiomyocytes of aged rat. Moreover, inducible *Gja1* deletion was related to cardiac arrhythmia development [50]. Consistent with this, we discovered that *Gja1* transcription correlated negatively with LVESD, γH2AX, PARP1, Caspase-3, Caspase-9 and the conditionally harmful bacteria *Beijerinckiaceae* and *Oscillibacter*.

In conclusion, this work demonstrates the protective role and underlying mechanism of parishin against cardiac aging in naturally aged mice. Parishin treatment significantly alleviated aging-induced cardiac dysfunction, histopathological abnormalities, DNA damage response, transcriptional changes, and aging biomarker overexpression. Based on our previous research, we confirmed that these health-promoting effects of parishin are closely related to its regulation of microbes and metabolites in the aged-type gut. These findings indicated the potential anti-aging applications value of parishin.

Ethical statement

All experimental procedures were conducted following the Instructive Notions with Rspect to Caring for Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China and approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (approval number: 2020 Experimental Kuaishen No. 1446).

Data availability statement

All data included in article are referenced in the article. The raw data of mRNA sequencing can be found in the publicly available repository of NCBI Sequence Read Archive (SRA) with BioProject ID PRJNA916094.

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Gene	Forward (5'→3')	Reverse (5'→3')
GAPDH	ACTCTTCCACCTTCGATGCC	TGGGATAGGGCCTCTCTTGC
Rasgrf2	CTGAGTAAAGTCCGCCTGGG	CCATCGGGCTCCTCAATCAA
Gja1	GTCCTTGGTGTCTCTCGCTC	GGTGAGGAGCAGCCATTGAA
Jchain	CGACCATTCTTGCTGACAACAAA	TCCACAGGATCGCATTTCTTACA
Igkv3-10	ACCAGCAGAAACCAGGACAG	GCCTCCACAGGATCAATGGT
Igkv1-110	GTTGCCTGTTAGGCTGTTGG	TCTGCAAGAGATGGAGGCTTG
Cyp2e1	TGGTCCTGCATGGCTACAAG	CGGGCCTCATTACCCTGTTT
Fgf12	ACCCCAGCTGAAAGGGATTGT	GTAGTCGCTGTTTTCGTCCTTG
Mybphl	CTCCCAAGTTTACCCAGCCG	AGGCTTGCGGATTTCTAGGG

CRediT authorship contribution statement

Shixian Zhou: Writing – review & editing, Writing – original draft, Data curation. **Xinxiu Zhao:** Writing – original draft, Formal analysis. **Li Wu:** Writing – original draft, Data curation. **Ren Yan:** Visualization, Validation, Methodology, Investigation. **Linlin Sun:** Resources, Data curation. **Qin Zhang:** Resources, Data curation. **Caixia Gong:** Investigation, Data curation. **Yang Liu:** Investigation, Data curation. **Lan Xiang:** Resources. **Shumin Li:** Validation, Data curation. **Peixia Wang:** Formal analysis, Data curation. **Yichen Yang:** Formal analysis, Data curation. **Wen Ren:** Data curation. **JingJin Jiang:** Resources. **Yunmei Yang:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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