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ORIGINAL RESEARCH

EIF4A3 Enhances the Proliferation and Cell Cycle Progression of Keloid Fibroblasts by Inducing the hsa_circ_0002198 Expression

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Background: Recent evidence suggests a crucial biological role for Circular RNAs (circRNAs) in keloid diseases, yet the underlying mechanisms remain unclear. This study explored the biological effects and molecular mechanisms of hsa_circ_0002198 in keloid formation.

Methods: Real-time quantitative PCR (qRT-PCR) was employed to assess the expression of circ_0002198 in keloid tissues, normal skin tissues, keloid fibroblasts (KFs), and normal skin fibroblasts (NFs) from nine patients. To investigate the role of circ_0002198 in keloid pathogenesis, cell transfection technology was utilized to knock down circ_0002198. Various experiments including Cell Counting Kit-8 (CCK-8), 5-Ethynyl-2′-deoxyuridine (EdU), Transwell, wound healing assay, flow cytometry, and others were conducted to explore the potential mechanisms associated with circ_0002198 expression. The RNA-binding protein Eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3) binding to circ_0002198 was identified and confirmed through bioinformatics databases prediction and RNA immunoprecipitation (RIP) assay. Finally, the expression of EIF4A3 was assessed, and both silencing and overexpression were employed to verify its role in circ_0002198 regulation.

Results: The expression levels of circ_0002198 and EIF4A3 were notably elevated in keloid tissues and KFs compared to normal skin tissues and NFs. The reduction of circ_0002198 expression in KFs significantly impeded their proliferation, migration, and invasion. It also hindered the cell cycle process and the expression of associated proteins while concurrently promoting apoptosis in KFs. EIF4A3 was identified to bind to the flanks of circ_0002198, enhancing the occurrence of circ_0002198 and its role in regulating the progression of KFs.

Conclusion: Our study offers insights into how Circular RNA may contribute to the pathogenesis of keloid formation, highlighting Circ_0002198 as a potential novel biomarker for keloids in association with EIF4A3. Further research, involving larger study cohorts, is necessary to broaden our understanding of keloid mechanisms and potential treatment approaches.

Keywords: keloid, circular RNA, fibroblasts, hsa_circ_0002198, EIF4A3, cell cycle

Introduction

Keloids develop as benign fibroproliferative tumors during the process of abnormal wound healing, with their pathogenesis primarily characterized by the excessive deposition of extracellular matrix and the proliferation of keloid fibroblasts (KFs).[1] Keloids not only impact one's appearance but also give rise to specific clinical symptoms, including sensations of itching, pain, and disruptions in joint movement as well as psychiatric problems.[2,3] The development of keloids is an intricate biological process, influenced by various systemic, environmental, and genetic factors with many hypotheses have been suggested to explain it is occurrence.[4] Despite promising advancements in current research on the

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pathogenesis and treatment of keloids, the recurrence rate for these lesions remains high. Therefore, it is crucial to enhance ongoing, in-depth explorations into the mechanisms of keloid formation to develop more effective treatments.[5,6]

In recent years, scholars have made significant discoveries indicating the involvement of non-coding RNA in the establishment of crucial gene regulatory systems within tumor biology, hence influencing tumor development.[5] In recent decades, circular RNA, which falls under the category of non-coding RNA, has been postulated to arise from erroneous exon editing.[6] Nevertheless, the advent of high-throughput sequencing technology has facilitated the identification of aberrantly produced circRNAs in several tumor types, revealing their potent capacity for gene regulation. MicroRNAs (miRNAs) have the ability to function as either miRNA sponges or RNA-binding proteins (RBPs), playing a significant role in the development of many diseases. Certain circular RNAs (circRNAs) have been found to possess translation capabilities, hence exerting an influence on cellular physiological processes through diverse ways.[7–9] In a recent investigation, the utilization of circRNA microarrays, bioinformatics analysis, and high-throughput RNA sequencing techniques led to the identification of three circRNA biomarkers, namely hsa_circ_0008259, hsa_circ_0005480, and hsa_circ_0002198 (also referred to as circ_0002198), that exhibit associations with keloids. Notably, circ_0002198 shows a considerably elevated expression level in this context.[10] Nevertheless, whereas circ_0002198 has been identified as a possible diagnostic marker for ovarian endometriosis, its specific roles and activities in keloids have not yet been fully elucidated.[11]

RBPs are a group of proteins that play a significant role in the regulation of gene expression, including the transcription and translation processes of many genes, including circRNA [12,13]. Multiple research studies have demonstrated that RBPs have the capability to interact with circRNA, hence exerting regulatory control on RNA metabolism. This interaction has been observed to be particularly significant in a diverse range of both benign and malignant tumor formations. As an illustration, it has been observed that circular RNA circRPN2 exhibits the ability to interact with enolase 1, thereby facilitating its degradation. Simultaneously, this interaction promotes glycolytic reprogramming through the AKT/mTOR pathway, resulting in the inhibition of liver cancer spread[14]. In their study, Du et al showed that circ-FOXO3 has the ability to bind with P21 and CDK2, resulting in the formation of a complex that hinders the activity of CDK2. This complex consequently impedes the progression of the cell cycle from the G1 phase to the S phase by means of the P21 molecule[15]. Furthermore, upon additional investigation into the functionality of circRNA, scholars have discovered that RBPs may play a role in the process of circRNA formation. According to reports, the interplay between RNA and protein has been found to exert an influence on protein expression and function, while also playing a role in the regulation of circRNA synthesis and degradation[12,16,17].

In this study, we determined that circ_0002198 was highly expressed in keloid cells and tissues. In addition, we found that Circ_0002198 stimulated cell proliferation in vitro and that eIF4A3, which is RBP (eukaryotic translation initiation factor 4A), may mediate the biogenesis of circ_0002198. Moreover, we established that circ_0002198 could potentially stimulate the formation of keloids via activation of the P21/CDK4/Cyclin D/Cyclin E signaling pathway. Based on our findings, circ_0002198 appears to be a potentially effective therapeutic target for keloid.

Materials and Methods

Human Samples

A total of nine patients diagnosed with keloids and nine healthy volunteers without any known medical conditions were selected as participants from the Second Affiliated Hospital of Harbin Medical University, Harbin, China in 2021. All patients had regular pathological diagnoses and had not undergone any previous injections or physical or surgical interventions were selected. The clinical and demographic information of the individuals has been presented in the Table S1. The experiments undertaken in this study were granted approval by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, in accordance with the principles outlined in the Declaration of Helsinki. Furthermore, informed permission was acquired from all participating patients.

Cell Culture

The keloid and normal skin tissue that had been surgically excised has been collected and subjected to three rinses using sterile phosphate-buffered saline (PBS) buffer. Following the removal of the epidermis and subcutaneous fat, the white fibrous tissue was dissected into small pieces of around 1 mm^3 small pieces, and the tissue pieces were moved to T25 culture flask with a gap of about 1cm. A total volume of 2 milliliters of DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) was introduced into the culture and maintained in a controlled environment at 37°C with a 5% concentration of carbon dioxide (CO2) in an incubator. The cellular specimens utilized in this experimental study were derived from 3 to 5 successive generations.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Assays

The extraction of total RNA was performed on frozen tissues or cultured cells using the TRIzol reagent (Sigma, St. Louis, Missouri, USA), in accordance with the instructions provided by the manufacturer. The cDNA synthesis was synthesized from RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), and then the qPCR experiment was conducted using the FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) on Slan®-96p fluorescent quantitative PCR instrument (Hongshit, Shanghai, China). The PCR primers for target genes were synthesized by General Biology (Anhui, China) and summarized in Table 1. Finally, the relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Transfection

Based on the high expression of circ_0002198 in the KFs, three siRNA and one siRNA-NC (GeneseedBiotech, Guangzhou, China) were synthesized to silence the expression of circ_0002198, and qRT-PCR detected the silencing efficiency of three siRNAs. Moreover, an EIF4A3 overexpression plasmid (pcDNA3.1-EIF4A3) and siRNA (GenePharma, Shanghai, China) were designed, synthesized, and transfected into KFs. In brief, KFs were cultured in 6-cm culture dishes and transfected with the vector, siRNA, and negative control for 48 hours using Lipofectamine 8000 (Beytime, Shanghai, China) following the manufacturer's instructions. Subsequently, RNA/protein extraction and additional functional experiments were conducted on the resulting groups. The siRNA sequences used in this study are provided in Table S2.

Cell Proliferation Assays

We checked cell viability using the Cell Counting Kit-8 (CCK-8; Seven, Beijing, China) and the EdU experiment. Untransfected or transfected cells were seeded into 96-well plates with 2×10^3 cells per well. Next, 10 μL of CCK-8 was introduced into the wells in dark conditions. The absorbance of each well was measured at 450 nm wavelength using a spectrophotometer (Molecular, Shanghai, China), and in this assay, at least three independent experiments were performed. For the EdU experiment, we inoculated the cells into each well of a 96-well plate at a density of 4×10^3 cells per well according to the instructions of the EdU Flow Cytometry Assay Kit (Us Everbright, Shanghai, China) and cultured them with 50 μM EdU for 4–6 hours before fixation. The click-IT working solution was then incubated for 30 minutes in the dark, and the image was obtained and analyzed after DNA restaining with Hoechst33342 (Beytime, Shanghai, China) was completed.

Table 1 Details of PCR Primers for Target Genes

Gene	Forward	Reverse
GAPDH	5-TGTGGGCATCAATGGATTTGG-3	ACACCATGTATTCCGGGTCAAT-3
circ_0002198	5-GCAAACCTATATCAGGAAACAGC-3	5TTGAAGAGGTGGCACAACAGT-3
EIF4A3	5-CGCGGACTCTGACATATGGCGACCACGGCCACGATG-3	5-TCCCGCAGGCCCATGGTGTCG-3

Transwell Assay

The upper chamber of the Transwell was pre-treated with 50 μL Matrigel (Solarbio, Beijing, China) (Matrigel was not involved in the migration experiment). KFs at 1×10^5 cells/mL density were then seeded into the upper compartment that lacked fetal bovine serum. DMEM high-glucose medium (10% FBS) was added to the lower chamber. After 37°C 5%CO_2 incubator for another 24 hours, 4% paraformaldehyde was fixed and stained with 0.1% crystal violet. Migrating or invading cells were counted using an inverted microscope (Leica Microsystems, Mannheim, Germany).

Flow Cytometric Analysis

Cell samples from different groups were collected for cell cycle analysis and exposed to 70% ethanol overnight at 4 °C. On the day of analysis, the cells were washed with cold PBS and centrifuged at 1000 RPM for 5 minutes, and Propidium iodide (PI) stain was added to each test tube and incubated at 37 °C for 30 minutes. For apoptosis analysis, cells were treated according to the instructions of the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). All samples were analyzed by flow cytometry using the Beckman CXP500 system. The ModiFit software was used to analyze the percentage of cells at different cell cycle stages, and FlowJo software was used to analyze the apoptosis rate.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The transfected cells were prepared as a cell suspension, which was inoculated into 96-well plates at a cell density of 5×10^3/ well. After 24 hours of culture, the PBS buffer solution was washed once, and 100 μL of TUNEL detection solution (Beyotime, Shanghai, China) was added to each well after fixation and penetration of the cell membrane. After incubation at 37 °C for one hour, nuclei were stained with Hoechst 33342 (Beyotime, Shanghai, China) solution, and the anti-fluorescence quenching sealing solution was dropped to seal the tablets. Finally, fluorescence microscopy was used to observe and photograph each sample.

RNA Immunoprecipitation (RIP)

The correlation between circ_0002198 and EIF4A3 expression was studied using the RIP analysis, following the instructions of the RIP Kit manufacturer (Geneseed, Guangzhou, China). In brief, magnetic beads were incubated with anti-EIF4A3 antibodies, immunoglobulin G (IgG) was used as a negative control, cell lysates were incubated with corresponding antibody-coated beads, and enrichment multiple of circ_0002198 was detected using qRT-PCR.

Western Blotting Assay

Briefly, the cells were lysed with radioimmunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) for protein extraction and quantified using a BCA kit (Beyotime, Shanghai, China). Proteins were isolated using 12.5% or 10% SDS polyacrylamide gels according to their molecular weight. Next, the isolated protein bands were transferred to the polyvinylidene fluoride(PVDF) membrane (Bio-RAD, California, USA). The membrane was sealed with 4% skim milk powder and combined with anti-EIF4A3 (Affinity,1:1000), CyclinE1 (Immunoway,1:1000), CyclinD1 (Immunoway,1:1000), CDK4 (Immunoway,1:1000), P21 (Wanleibio,1:1000), and β-actin (Abcam,1:7000) were incubated overnight at 4 °C. The following day, the membrane was conjugated with a secondary antibody (Abcam,1:5000) and incubated for one hour at 37°C. Finally, the bands were detected by chemiluminescence, in which β-actin was used as an internal control.

Northern Blotting Assay

A 2% agarose gel was prepared according to the size of the product fragment. The mixture was heated in a microwave until fully dissolved, gently stirred, and then poured into a casting tray with a comb. After cooling, the gel solidified over a period of 15–30 minutes and was placed in an electrophoresis tank containing 1×TAE electrophoresis buffer. Subsequent to sample loading, electrophoresis was conducted at 100V for 40 minutes, and the gels were observed and photographed using a gel imager (Invitrogen; Thermo Fisher Scientific, Inc., USA).

Statistical Analysis

Statistical analyses were conducted with GraphPad Prism 9.0 (GraphPad Prism Software Inc., San Diego, CA) and SPSS 28.0 (IBM, SPSS, Chicago, IL, USA). Results are expressed as the mean ± standard deviation (S.D.) based on a minimum of three independent experiments. Group differences were assessed using Student's *t*-test, one-way analysis of variance (ANOVA), or the Chi-square test as appropriate. Statistical significance was set at $P < 0.05$.

Results

circ_0002198 Up-Regulated in Keloid Tissue and Keloid Fibroblasts

By utilizing the circBase (http://www.circbase.org/) and CSCD2 (http://gb.whu.edu.cn/CSCD/) databases to acquire information on circ_0002198, our investigation revealed that circ_0002198 originates from exon 7 to exon 9 of the PDE7B gene located on chromosome 6. This circular transcript, spanning 329 base pairs, is formed through reverse splicing, connecting the 5 ′ end of exon 7 to the 3 ′ end of exon 9 of the host gene PDE7B. To confirm that circ_0002198 was formed by reverse splicing, Sanger sequencing was performed on fluorescent quantitative PCR products, and the results confirmed that circ_0002198 was formed by reverse splicing (Figure 1A). Moreover, we designed the extender and adductor primers to amplify circ_0002198 and linear PDE7B mRNA, respectively. PCR amplification and agarose gel electrophoresis were performed using KFs cDNA and genomic DNA (gDNA). The findings revealed the detection of PDE7B in both cDNA and gDNA, whereas circ_0002198 was exclusively identified in cDNA, eliminating the possibility of its presence in gDNA (Figure 1B).

Following verification and confirmation of its presence, our inquiry expanded to assess the expression levels of circ_0002198 in normal skin tissue, keloid tissue, normal fibroblasts, and keloid fibroblasts through qRT-PCR. The analysis revealed a notably elevated expression of circ_0002198 in keloid tissue compared to normal skin tissue (n=9) (Figure 1C). Circ_0002198 expression was significantly higher in keloid tissue than in normal skin tissue (n=9) (Figure 1C). Similarly, the level of circ_0002198 in keloid fibroblasts was significantly higher than that in normal fibroblasts (Figure 1D). These results suggest that circ_0002198 may play a role in the development of human keloid fibroblasts. To validate circ_0002198 potential biological role in the pathogenesis of keloids, three siRNAs were designed to target the splicing site of circ_0002198 and were transfected into KFs. Subsequently, circ_0002198 expression was assessed using qRT-PCR across various groups. The findings indicated a significant reduction in the expression level of circ_0002198 compared to the control group, particularly with the siRNAs selected for further investigation (Figure 1E).

circ_0002198 Promote Proliferation, Migration and Invasion of Keloid Fibroblasts

Given the notably heightened expression of circ_0002198 in keloids, we sought to unravel its potential biological significance in keloid development. To scrutinize the biological role of circ_0002198 in keloids, we investigated the impact of targeted circ_0002198 knockdown on cell proliferation, migration, and invasion. CCK-8 and EdU experiments illustrated a decrease in the proliferation of keloid fibroblasts after transfection with si-circ-2, as depicted in Figure 2A-C. Correspondingly, wound healing and transwell experiments demonstrated a significant reduction in the number of migrating and invading KFs following si-circ-2 transfection, as well as impaired wound healing (Figure 2D-F). These results imply that circ_0002198 plays a role in promoting keloid fibroblast proliferation, migration, and invasion in vitro.

Down-Regulation of circ_0002198 Induces Cell Cycle Arrest and Apoptosis

To further elucidate the role of circ_0002198 in the advancement of keloids, we delved into cell cycle and apoptosis following circ_0002198 knockdown in vitro. Flow cytometry revealed a significant increase in the proportion of KFs in the G1 phase after silencing circ_0002198 expression, accompanied by a decrease in the S phase, indicating that circ_0002198 downregulation can arrest the KFs cell cycle in the G1 phase (Figure 3A). Moreover, Western blotting was employed to examine the expression of cyclin-related proteins. The results demonstrated a substantial reduction in the expression of cyclin-related factors, including cyclin E1/D1/CDK4/P21, in KFs following circ_0002198 down-regulation (Figure 3B).

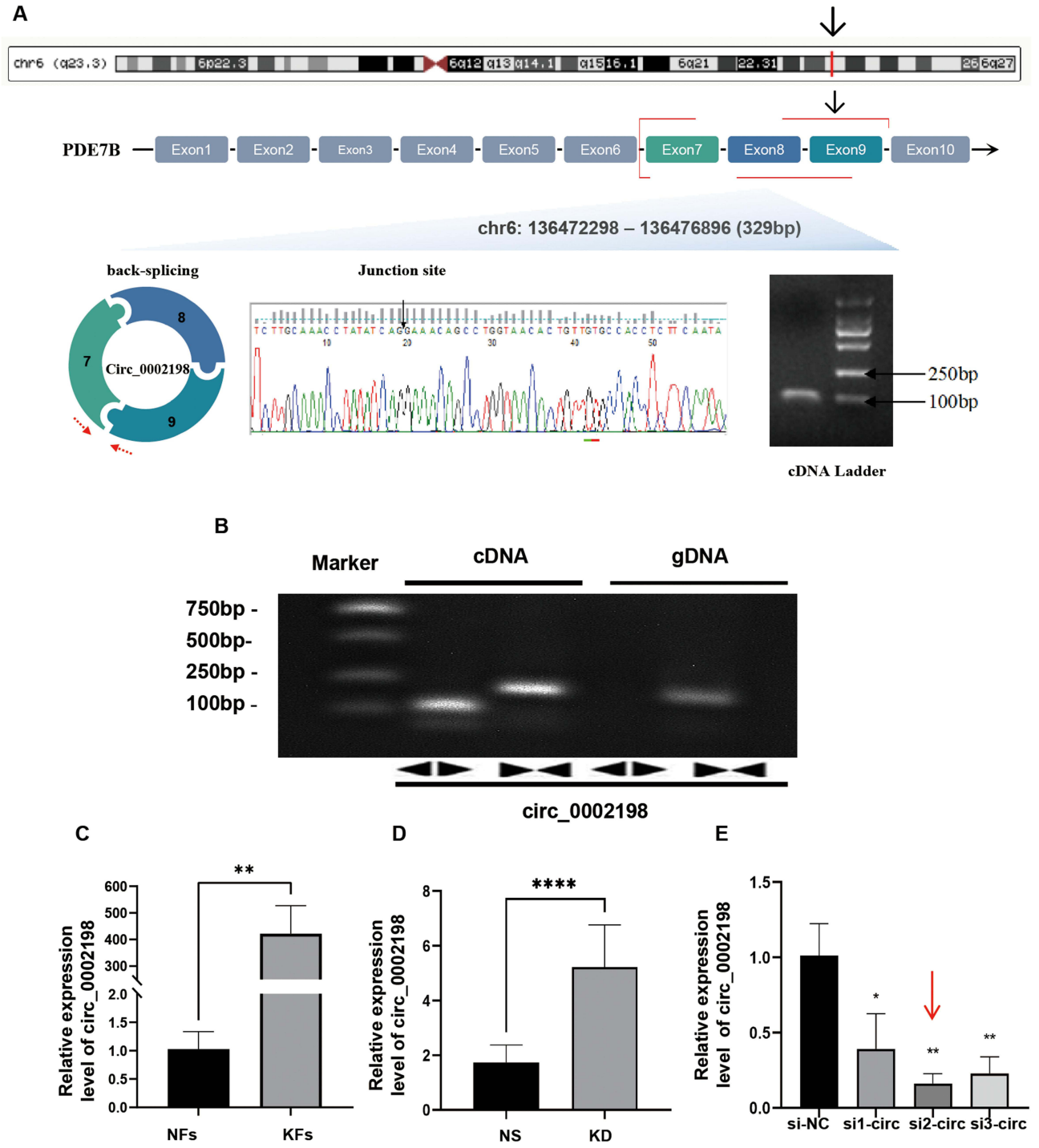

Figure 1 Illustrates the elevated expression of circ_0002198 in keloids. (**A**) presents a schematic diagram depicting the formation of circ_0002198, with Sanger sequencing and agarose gel electrophoresis utilized to confirm the reverse splicing sequence and RT-PCR products of the circRNA. (**B**) shows Northern blot analysis, where circ_0002198 was detected exclusively in the cDNA of keloid fibroblasts, absent in gDNA, confirming its circular nature.(**C** and **D**) demonstrate the differential expression of circ_0002198 in keloid fibroblasts compared to normal skin fibroblasts, as well as in keloid and normal skin tissue samples from nine cases, with expression levels quantified via qRT-PCR. GAPDH served as the internal reference gene. (**E**) further investigates the modulation of circ_0002198 expression in keloid fibroblasts following siRNA-mediated knockdown (si-circ) or negative control (si-NC) treatment. The data, expressed as mean ± SD, represent the results of three independent experiments, with significant differences indicated by *P < 0.05, **P < 0.01, and ****P < 0.0001. The red arrow indicates the target knocked down in subsequent steps.

Subsequently, we validated the impact of circ_0002198 silencing on KFs cell apoptosis. Flow cytometry analysis using Annexin V/PI double staining revealed a significantly higher apoptosis rate in the si-circ group compared to the control group (Figure 3C). Additionally, TUNEL analysis indicated that the downregulation of circ_0002198

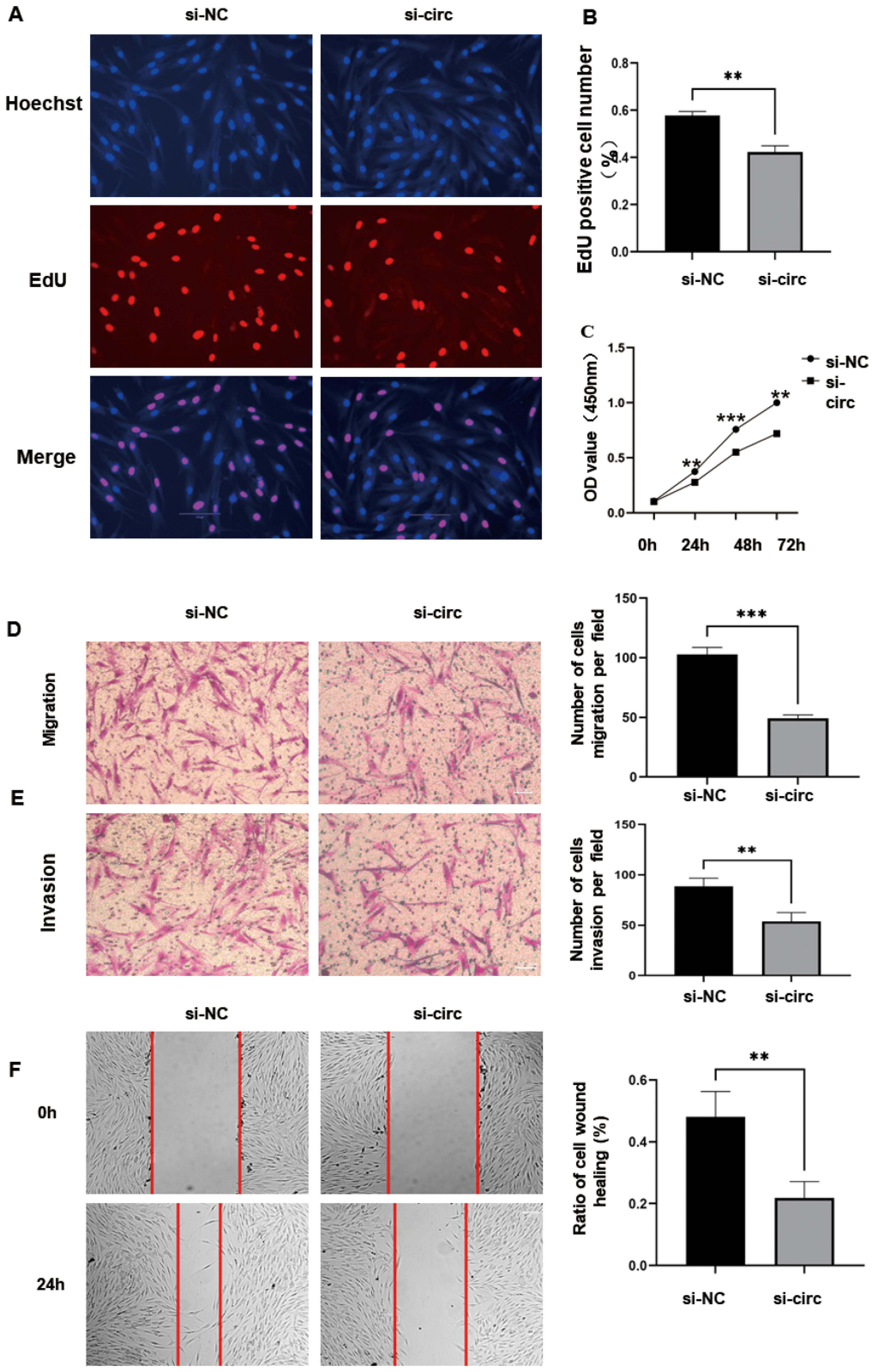

Figure 2 Effect of circ_0002198 Silencing on Keloid Fibroblast Proliferation, Migration, and Invasion. (**A–C**): The downregulation of circ_0002198 was assessed through EdU and CCK8 assays to evaluate the proliferation of keloid fibroblasts. Scale bars = 125 μm. (**D–F**): The migration and invasion abilities of keloid fibroblasts were analyzed following circ_0002198 silencing using wound healing and transwell assays. Scale bars = 100 μm. Data are presented as mean ± SD from three independent experiments. **P < 0.01, ***P < 0.001.

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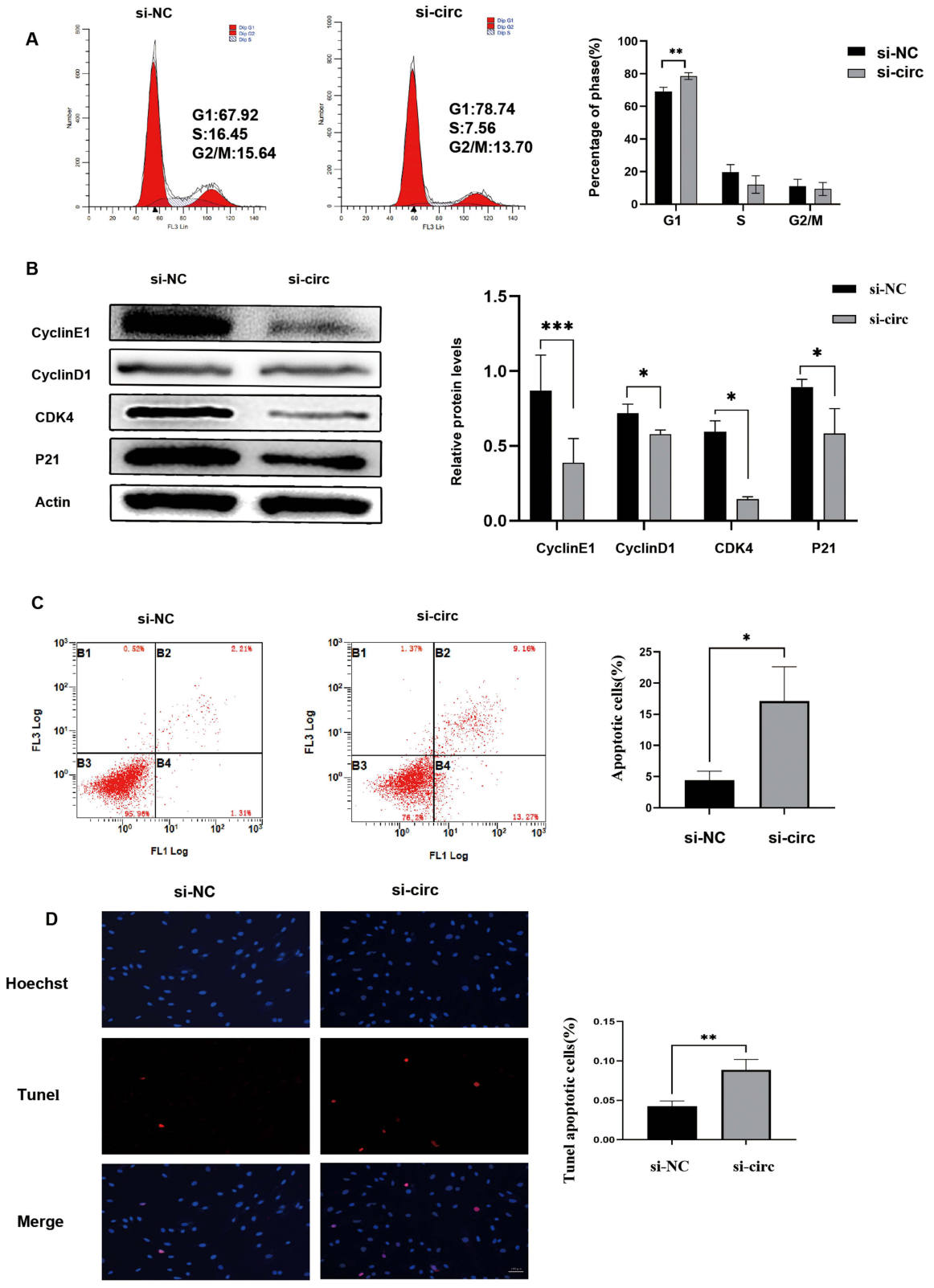

Figure 3 Circ_0002198 promotes keloid fibroblast proliferation by regulating cyclin and accelerating cell cycle progression. (**A**). Flow cytometric cell cycle analysis of keloid fibroblasts treated with si-circ and si-nc for 48 hours. The bar graph shows the percentage of cells in the G1-G0, S, and G2-M phases. (**B**). Protein expression levels of cell cycle-regulating genes in keloid fibroblasts treated with si-circ and si-nc for 48 hours. (**C** and **D**). Apoptosis rate of keloid fibroblasts treated with si-circ and si-nc for 48 hours, assessed by flow cytometry and TUNEL assay for Terminal Deoxynucleotidyl Transferase activity. Data are expressed as mean ± SD from three independent experiments. Scale bars = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001.

significantly increased the number of TUNEL-positive cells compared to the control group (Figure 3D). These findings suggest that circ_0002198 promotes KFs proliferation and inhibits apoptosis by regulating gene expression, thereby accelerating cell cycle progression.

circ_0002198 Binds to Eukaryotic Translation Initiation Factor 4A3 (EIF4A3)

Several recent studies have reported that many circRNAs regulate cell functions by interacting with proteins.[12,13] To investigate whether circ_0002198 acts in this manner, we used the Circinteractome[14] and CircFunBase[15] databases to predict the proteins that might bind to circ_0002198 (Figure 4A). Circinteractome database forecast results showed that

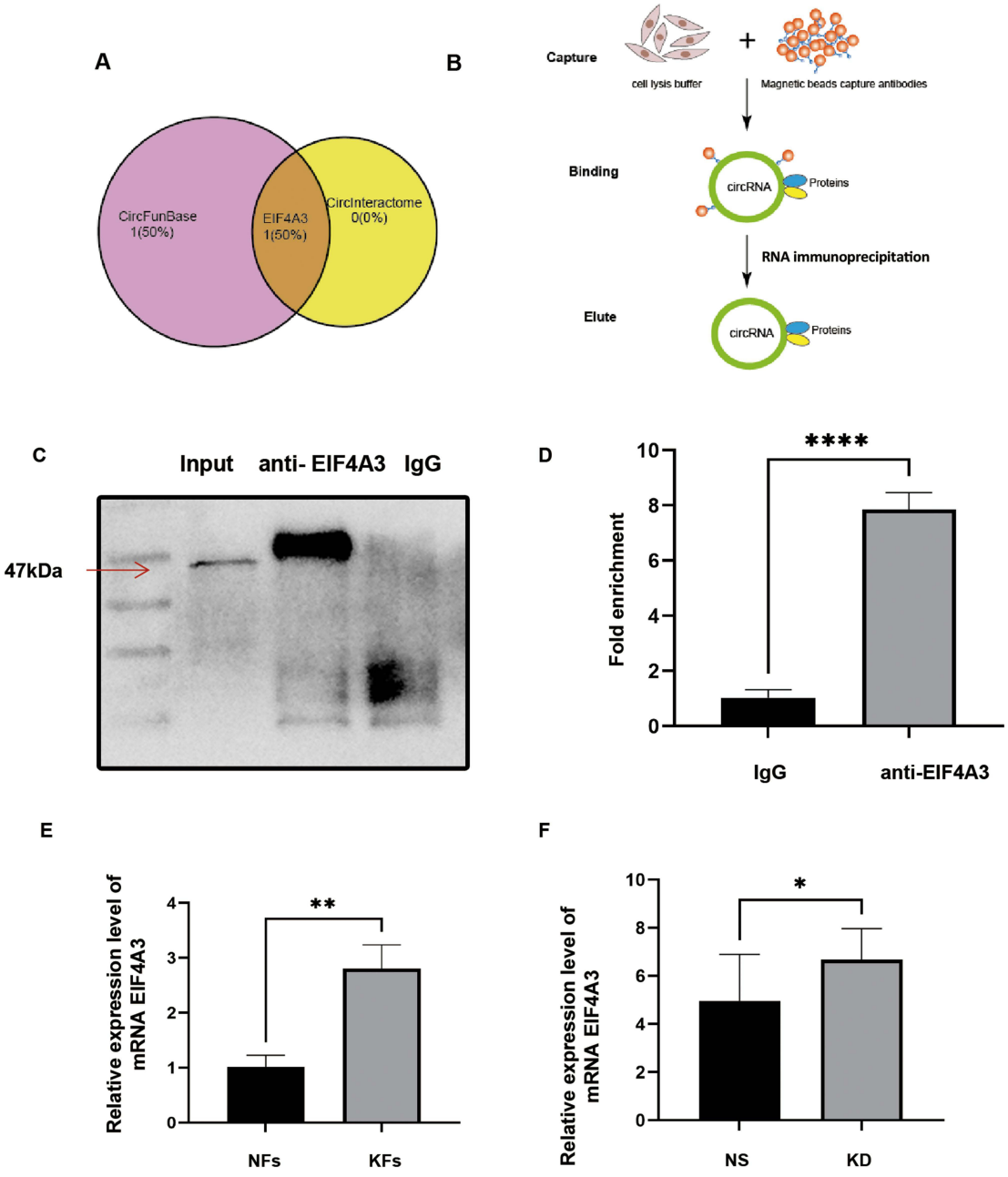

Figure 4 Circ_0002198 interacts with EIF4A3. (**A**). The figure illustrates the overlap of specific proteins bound by circ_0002198, as predicted by CircInteractome and CircFunBase. (**B**). Schematic representation of circ_0002198 RNA immunoprecipitation (RIP). (**C** and **D**). RIP was conducted in keloid fibroblasts, followed by Western blotting and qRT-PCR on the co-precipitated protein and RNA. Using IgG as a positive control, EIF4A3 was highly enriched in the input group; the enrichment factor for EIF4A3 binding to circ_0002198 flanking regions in qRT-PCR was 7.86-fold. (**E** and **F**). EIF4A3 expression was quantified by qRT-PCR in keloid fibroblasts and normal skin fibroblasts, as well as in nine keloid and normal skin tissue samples. GAPDH was used as an internal reference. Data are shown as mean ± SD. Results are from three independent experiments; *P < 0.05, **P < 0.01, ****P < 0.0001.

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EIF4A3 and circ_0002198 flanks had two binding sites (Tags=2) (Figure 4B), suggesting that EIF4A3 is more likely to bind to the circ_0002198 flanks. An RNA-binding protein immunoprecipitation (RIP) assay was used to verify whether EIF4A3 was bound to circ_0002198, and IgG was used as a positive control (Figure 4C and D). Western blotting and qRT-PCR were performed on the KF protein and RNA samples (Figure 4E and F), and these data results suggest that EIF4A3 can bind to circ_0002198 and may be involved in regulating the function of KFs.

EIF4A3 Promotes KFs Growth Through Induction of circ_0002198

To investigate the interplay between circ_0002198 and EIF4A3 further, we employed overexpression plasmids and siRNA to modulate the expression of EIF4A3 in KFs. The results demonstrated that silencing EIF4A3 significantly suppressed the expression of circ_0002198 in KFs, while EIF4A3 overexpression markedly elevated circ_0002198 expression in KFs (Figure 5B-E). Similarly, circ_0002198 siRNA led to a notable decrease in EIF4A3 levels (Figure 5A). These findings suggest that EIF4A3 may play a role in mediating circ_0002198 biogenesis.

To validate that EIF4A3 can influence cell cycle progression by promoting circ_0002198 expression, Western blot analysis was conducted to assess the expression of Cyclin D1, Cyclin E1, CDK4, and p21 proteins in KFs subjected to bidirectional interference with si-circ alone or si-circ in conjunction with EIF4A3 overexpression. The results indicated that the inhibitory effect of si-circ on the expression of cyclin D1, cyclin E1, CDK4, and p21 proteins could be reversed by the induction of the EIF4A3 overexpression plasmid (Figure 5F and G). In summary, these results suggest that EIF4A3 may participate in regulating cell cycle progression and KFs proliferation by promoting circ_0002198 expression.

Discussion

CircRNAs, as non-coding RNAs, are emerging as novel biomarkers in various diseases due to their high abundance, conservation, and specific expression including hypertrophic scars.[16,17] These covalently closed RNA molecules play key roles in regulating gene expression and disease pathogenesis.[6,7,18] CircRNAs are more suitable as diagnostic markers or therapeutic targets for diseases than linear transcripts because of their high abundance, high sequence conservation, and specific expression.[19,20] Our study focuses on circ_0002198's role in KF behavior, but factors like the cellular micro-environment, cytokines, growth factors, and ECM properties may also influence these processes.[21] Previous study revealed circ_0002198 role with other non-coding RNA role immune regulation.[22] In fibrotic diseases, including keloids, circRNAs have been underexplored. A prior study suggested that circ_0002198 could be a keloid-specific marker.[10] In our study, keloid and normal skin tissues were obtained from nine patients to validate its expression, showing significant upregulation in both keloid tissue and KFs. Suppression of circ_0002198 in KFs inhibited proliferation, migration, invasion, induced apoptosis, and blocked the cell cycle. Furthermore, EIF4A3 was found to enhance circ_0002198 expression and influence KF cell cycle regulation. These findings support circ_0002198's potential role in keloid progression and its promise as a novel diagnostic or therapeutic target.

An increasing amount of data indicates that circRNAs can play a role in fibrotic diseases of the liver, kidney, and heart through different regulatory mechanisms.[23,24] As a typical fibrotic disease, some studies have revealed different mechanisms of action of circRNAs in KFs and NFs. Studies have shown that circ_0008259 overexpression can inhibit the expression of COL1A1 and COL3A1 proteins in KFs. Another study showed that silencing of circCOL3A1-859267 significantly inhibits the expression of type I collagen in UVA-irradiated NFs, whereas circCOL3A1-859267 regulates the expression of type I collagen in NFs by sponge absorption and isolation of Mir-29c.[25] Our experimental results confirm the role of circRNAs in keloid progression; we found that downregulation of circ_0002198 inhibited the biological functions of KFs and significantly inhibited the cell cycle of KFs in the G1 phase. The G1 phase, a checkpoint of the cell cycle, is crucial for determining the cell's fate.[26,27] For diseases with excessive cell proliferation, continuous cell division is regulated by a variety of strictly developed cell cycle regulation mechanisms, and cell cycle checkpoints can delay cell cycle progression or induce cell cycle withdrawal or cell death as a response to irrecoverable DNA damage.[28] Our findings support the idea that cell cycle checkpoints regulate cell cycle progression by inhibiting cyclin-dependent kinase (CDK) activity, which is consistent with our observation that downregulation of circ_0002198 significantly reduced the expression of cyclins (Cyclin D1/E1) and CDK4 in keloid fibroblasts (KFs), aligning with G1-

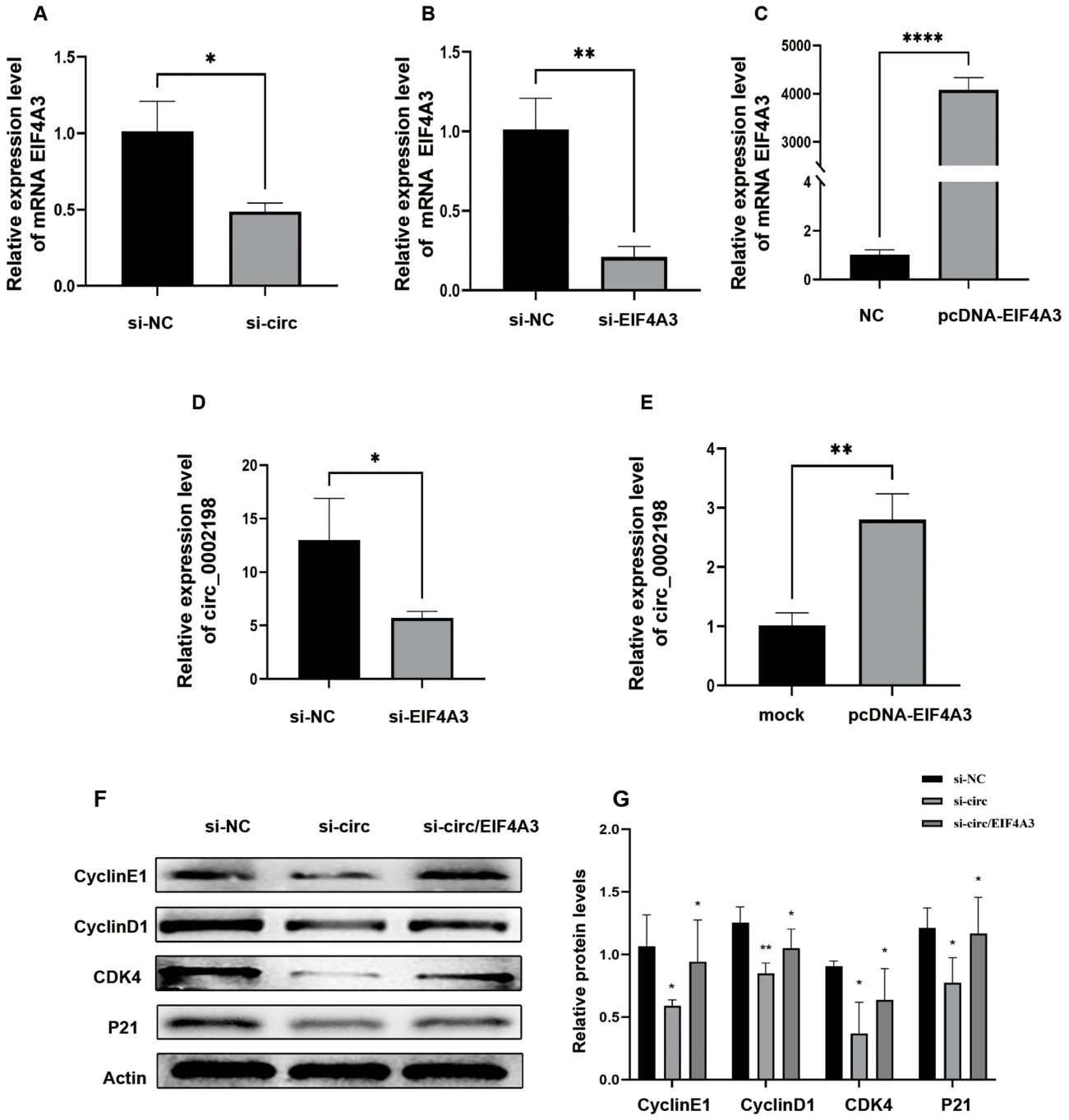

Figure 5 EIF4A3 can participate in the regulation of the KFs cell cycle by promoting circ_0002198 expression. (**A**–**C**) keloid fibroblasts were transfected with EIF4A3 expression vector, si-EIF4A3, and si-circ and the relative expression of EIF4A3 in each group was measured by qRT-PCR. (**D** and **E**) The relative expression of circ_0002198 in keloid fibroblasts transfected with EIF4A3 expression vector and si-EIF4A3 was assessed. **(F** and **G)** Keloid fibroblasts were transfected with si-circ and si-circ + EIF4A3, and the relative protein levels of downstream cell cycle-related molecules were determined by Western blot. Data are presented as mean ± SD, and results represent three independent experiments. *P < 0.05, **P < 0.01, ****P < 0.0001.

phase cell cycle arrest.[28] Notably, we also observed a decrease in the expression of P21, a well-known cell cycle inhibitor that regulates CDK4/6-Cyclin D and CDK2-Cyclin E complexes.[29] P21 has been shown to have dual roles in cell division and apoptosis, with its expression levels influencing these processes.[30] Our data suggest that reduced P21 expression following circ_0002198 downregulation may promote apoptosis in KFs and inhibit cell division.

CircRNA-protein interactions affect protein expression, biogenesis, and pathophysiological processes.[31,32] Bioinformatic predictions were therefore made to identify proteins that might bind to circ_0002198. EIF4A3 was

identified as a circ_0002198 binding protein. The DEAD-box protein EIF4A3 is the core of the exon binding complex (EJC) and, together with MLN51, constitutes the main component of RNA binding.[31,33] Notably, the molecular mechanisms through which EIF4A3 promotes circ_0002198 cyclization remain a critical aspect of this study. The DEAD-box protein EIF4A3 is known to be involved in RNA metabolism, particularly by facilitating RNA circularization through its RNA-binding capabilities.[34] It has been reported that the binding of EIF4A3 with circRNAs can promote the expression of circRNAs and regulate cell function in various diseases.[12,35,36] By interacting with the flanking regions of circ_0002198, EIF4A3 potentially stabilizes the circular form of this RNA, making it resistant to degradation by exonucleases. This stabilization is crucial for circ_0002198's biological functions, including its regulation of the cell cycle.Zheng et al found that the combination of EIF4A3 and circSEPT9 promoted the expression of circSEPT9 and reversed the expression of cyclin D1, cyclin E1, and other cyclin proteins in triple-negative breast cancer cells.[12] EIF4A3's promotion of circ_0002198 cyclization could also lead to the efficient recruitment of associated proteins and microRNAs, which may enhance the biological activity of circ_0002198 in regulating cell cycle checkpoints and apoptosis. Another study reported that in glioma, EIF4A3 positively regulates the expression of circCCNB1 by binding to circCCNB1, and promotes glioma cells' growth by regulating cyclin D1 expression.[13] Further supporting this, our study demonstrated that EIF4A3, which is significantly upregulated in keloid tissues and KFs, can bind to the flanking regions of circ_0002198. EIF4A3 overexpression in KFs promoted circ_0002198 expression, while its downregulation led to reduced circ_0002198 levels. Additionally, we observed bidirectional effects on cyclin E1, cyclin D1, CDK4, and P21 expression when circ_0002198 was interfered with via siRNA alone or in combination with EIF4A3 overexpression. EIF4A3 may also enhance the recruitment of other regulatory proteins or microRNAs to circ_0002198, influencing its interaction with the P21/CDK4/Cyclin D/E signaling pathway. This suggests that EIF4A3 stabilizes circ_0002198, potentially acting as a scaffold for key cell cycle regulators and promoting the transition from the G1 to S phase. Moreover, EIF4A3 knockdown has been shown to induce p53 and P21 expression in a manner similar to chemical inhibition of ribosome biogenesis, further supporting our observations.[37] The interaction between circ_0002198, P21, and other key proteins may therefore regulate cell cycle checkpoints, with reduced P21 expression following circ_0002198 downregulation promoting apoptosis, while its increased expression under EIF4A3 overexpression conditions may protect cells from cell cycle arrest and promote cellular survival.

One limitation of this study is the small sample size (nine patients), which may affect the statistical power and generalizability of the results. A larger, more diverse cohort is needed to confirm circ_0002198's role as a biomarker and therapeutic target for keloid treatment. Additionally, while siRNA knockdown was used to investigate circ_0002198's function, overexpression studies would provide a more comprehensive understanding of its regulatory effect in scar tissue. Future research should explore the molecular pathway through which EIF4A3 stabilizes circ_0002198, particularly its role in G1-phase cell cycle regulation and fibroblast proliferation. Further investigation into its clinical utility as a diagnostic marker and therapeutic target for fibrosis-related disorders is also necessary.

Conclusion

In brief, our study revealed a novel circ_0002198-mediated KFs cell cycle regulation mechanism. Although the specific mechanism of promoting cyclization and regulating the cell cycle of circ_0002198 by EIF4A3 needs further research, circ_0002198 regulates the biological behavior of KFs and the expression of cyclin E1/Cyclin D1/CDK4/P21 by binding to EIF4A3, which has been proven to play a role in the occurrence and development of keloids. We will further explore the specific mechanism by which circ_0002198 regulates the KFs cell cycle and its role in keloid models in vivo and preclinical trials.

Data Sharing Statement

The data that support the findings of this study are included within manuscript or supplementary materials. More detailed data available from the corresponding author upon reasonable request.

Ethics Approval

All experimental procedures were conducted in accordance with a protocol approved by Harbin Medical University's Ethics Committee, and patients agreed to participate in this investigation.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared no competing interests.

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