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Circ_CHFR expedites cell growth, migration and inflammation in ox-LDL-treated human vascular smooth muscle cells via the miR-214-3p/Wnt3/β-catenin pathway

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Abstract. – OBJECTIVE: Atherosclerosis (AS) is a representative inflammatory vascular disease. This study explored the molecular pathogenesis of AS based on circular RNA (circRNA), the checkpoint with forkhead-associated and ring-finger domains (circ_CHFR).

PATIENTS AND METHODS: The cell model of AS *in vitro* was established by stimulating human vascular smooth muscle cells (VSMCs) with oxidized low-density lipoprotein (ox-LDL). The RNA expression was measured by quantitative Real-time polymerase chain reaction (qRT-PCR). Cell viability and colony formation ability were separately evaluated using 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyl tetrazolium bromide (MTT) and colony formation assay. Cell migration was assessed *via* the transwell assay. The inflammation injury was analyzed by enzyme-linked immunosorbent assay (ELISA). Associated proteins were determined through Western blot. The combination of hypothetic targets was ascertained using Dual-Luciferase reporter assay.

RESULTS: Circ_CHFR was up-regulated in AS serums and ox-LDL-stimulated VSMCs. Circ_CHFR depletion weakened the ox-LDL-induced promotion of cell growth, migration and inflammation in VSMCs. Circ_CHFR positively regulated Wnt3 expression and the downregulation of Wnt3 abrogated the ox-LDL-triggered injuries in VSMCs. Circ_CHFR functioned as the sponge of microRNA-214-3p (miR-214-3p) and miR-214-3p targeted Wnt3. Circ_CHFR regulated cell growth, migration and inflammation via regulating the expression of Wnt3 as a competitive endogenous RNA (ceRNA) of miR-214 in ox-LDL-treated VSMCs. Circ_CHFR/miR-214-3p axis mediated the Wnt3/β-catenin signal pathway.

CONCLUSIONS: Circ_CHFR contributed to the progression of AS through the miR-214-3p/Wnt3/β-catenin signals, which illuminated the molecular mechanism of AS and suggested circ_CHFR might be an index for AS treatment.

Key Words:
Circ_CHFR, Atherosclerosis, Inflammation, MiR-214-3p, Wnt3.

Introduction

Atherosclerosis (AS) is a chronic inflammatory disease characterized by the aggregation of lipids and fibrinogen in the wide arteries, which causes the formation of blood plaques and narrow arteries to further trigger the complications of cerebral infarction, coronary heart disease, stroke and so on[1,2]. The abnormal proliferation of vascular smooth muscle cells (VSMCs) and the phenotypic switching migrated from contractile to the proinflammatory phenotype are considered to drive the vascular remodeling[3,4]. Therefore, the molecular mechanism behind VSMCs is worthy of investigating to seek the novel effective therapy for AS.

Circular RNAs (circRNAs), a neotype subgroup of noncoding RNAs (ncRNAs) with the covalently closed-loop structures generated from back-splicing of precursor mRNAs (pre-mRNAs)[5,6], have been reported to be involved in the AS process as microRNA (miRNA) sponges[7,8]. Circ_ANRIL inhibited cell proliferation and motivated apoptosis in AS via the induction of nucleolar stress and p53 activation[9]. The checkpoint with forkhead-associated and ring-finger domains (circ_CHFR, hsa_circ_0029589), a circRNA symbolized by gene CHFR, was dysregulated in VSMCs and participated in the cell proliferation and migration[10]. But the functional mechanism of circ_CHFR in AS remains to be researched thoroughly.

MiRNAs are another kind of ncRNAs known as post-transcriptional mediators by inhibiting

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gene expression through binding to the 3' untranslated region (3'UTR) of the messenger RNA (mRNA)[11]. As for the involvement of miRNAs in AS, miRNA-27b could regulate the angiogenesis of endothelial cells via targeting Naa15 in AS[12]; miR-21 repressed VSMCs proliferation and AS formation by interaction with PTEN[13]. Liu et al[14] declared that long ncRNA LINC00341 facilitated the proliferation and migration of VSMCs by miR-214/FOXO4 axis, indicating miR-214-3p might also be relevant to the AS. Nevertheless, it is elusive whether miR-214-3p is correlated with circ_CHFR in AS.

Wnt3 is a member of Wnt family that is strongly associated with the Wnt/β-catenin signal pathway[15,16]. It has been proved that Wnt3 regulated cell apoptosis of cardiomyoblast[17] and cardiomyogenic differentiation[18]. Chen et al[19] testified that Cyclin D1 could modulate osteoarthritis chondrocyte apoptosis through the Wnt3/β-catenin signal pathway. And the Wnt/β-Catenin pathway also took participation in the regulation of VSMCs[20]. But less is known about the function of Wnt3 in AS.

This report underscored the association among circ_CHFR, miR-214-3p and Wnt3 in regulating cell growth, migration and inflammation of VSMCs, explaining the explicit molecular occurrence mechanism of AS based on VSMCs.

Patients and Methods

Serum Samples

Serum samples were collected after obtaining the authorization from the Institute Review Ethics Committee of Tianjin Third Central Hospital, Tianjin Medical University and the informed consent from all participants. Normal and AS serum samples were respectively acquired from healthy donors (n=32) in the Physical Examination Center and AS patients (n=32) at Tianjin Third Central Hospital, Tianjin Medical University, then saved in a container with enough liquid nitrogen to maintain the freshness of serums.

Cell Culture and ox-LDL Stimulation

Human vascular smooth muscle cells (VSMCs) were bought from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated in the mixed solution of F-12K basic medium (Gibco, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Gibco) and antibiotics (penicillin: 100 UI/mL, streptomycin: 100 microg/mL; Gibco) in a 37°C, 5% CO_2 humidified incubator. Oxidized low-density lipoprotein (ox-LDL) contributes to the generation of inflammation as a dominating risk factor of AS[21,22]. Herein, VSMCs were treated with ox-LDL (Solarbio, Beijing, China) to simulate the aberrant lipid environment, establishing the cell model of AS *in vitro*.

Transient Transfection

The oligonucleotides used in this study were purchased from RIOBIO (Guangzhou, China): small interfering RNA (siRNA) against circ_CHFR (si-circ_CHFR#1 and si-circ_CHFR#2) and Wnt3 (si-Wnt3), miR-214-3p mimic and inhibitor (miR-214-3p and anti-miR-214-3p) and their negative controls (si-NC, miR-NC and anti-miR-NC). The sequence of Wnt3 was inserted into the pcDNA empty vector (Invitrogen, Carlsbad, CA, USA) to construct the overexpression vector of Wnt3 (Wnt3). These oligonucleotides or vectors were transiently transfected into VSMCs using Lipofectamine 3000 (Invitrogen) in line with the operating procedures.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Refer to the previous report[23], we exploited the TransStart® Green qPCR SuperMix (Transgen, Beijing, China) to conduct the PCR reaction after the extraction of RNA and reverse transcription. The $2^{-\Delta\Delta Ct}$ method was applied for calculating the relative expression levels[24]. Thereinto, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for circ_CHFR, CHFR and Wnt3) and U6 (for miR-214-3p) were used as internal references. The primers sequences were listed as below: circ_CHFR (forward: 5'-CTTCCAGCCCATGCCCGACCGG-3', reverse: 5'-CAGAAGGCAGGCGGCGCAG-3'); CHFR (forward: 5'-GGCAACCAGAGGTTTGACAT-3', reverse: 5'-AGTCAGGACGGGATGTTACG-3'); miR-214-3p (forward: 5'-CAATACTGACAGCAGGCACA-3', reverse: 5'-TATGGTTGTTCACGACTCCTTCAC-3'); Wnt3 (forward: 5'-CCACAACACGAGGACGGAGA-3', reverse: 5'-CGCCCAGCCACACACTTC-3'); GAPDH (forward: 5'-AGAAGGCTGGGGCTCATTTG-3', reverse: 5'-AGGGGCCATCCACAGTCTTC-3'); U6 (forward: 5'-CTCGCTTCGGCAGCACATATACT-3', reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3').

Ribonuclease R (RNase R) Treatment

To compare the stability between circ_CHFR and linear CHFR, 3 U/μg of RNase R (Epicentre Technologies, Madison, WI, USA) was used to

incubate 2 μg RNA at 37°C. 30 min later, the circ_CHFR and linear CHFR expression levels were measured via the qRT-PCR analysis.

3-(4,5-Dimethylthiazol-2-y1)-2, 5-Diphenyl Tetrazolium Bromide (MTT) Assay

After treatment with 100 μg/mL ox-LDL for 24 h, VSMCs were transfected and cell viability was measured daily. MTT (Beyotime, Shanghai, China) was complemented to cells in 96-well plates with 10 μL/well, which were incubated with 100 μL dimethyl sulfoxide (DMSO; Beyotime) per well after 4 h. The optical density (OD) value that represented cell viability was examined at 490 nm via the microplate reader.

Colony Formation Assay

Firstly, the cell suspension was inoculated into the culture dish containing culture medium and cultivated for 2 weeks approximately. When macroscopic colonies were generated, the colony cells were counted using a microscope after fastening by Fixative Solution (Invitrogen) and dying with Giemsa (Thermo Fisher Scientific, Waltham, MA, USA).

Transwell Migration Assay

The upper chamber of transwell 12-wells chamber (Corning Life Sciences, Corning, NY, USA) was added with 100 μL cell suspension (4×10^5 cells/mL) and 600 μL F-12K culture medium was pipetted into the lower chamber. At 24 h post-incubation, migrated cells were fixed with 4% Paraformaldehyde Fix Solution (Beyotime) and stained with crystal violet (Thermo Fisher Scientific). Then the migrated cell number was analyzed through a microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

According to the instruction offered by the producer, ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA) was implemented for the detection of the relative concentrations of inflammatory cytokines Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α).

Western Blot

Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Thermo Fisher Scientific) was used for extracting total proteins. 40 μg proteins were used for performing the Western blot following the detailed description of the last report[20]. The antibodies used in our study included primary antibodies: anti-Wnt3 (Abcam, Cambridge, UK, ab32249, 1:1000), anti-β-catenin (CST, Danvers, MA, USA #8480, 1:1000), anti-phosphorylated-β-Catenin (anti-p-β-Catenin; CST, #4176, 1:1000) and anti-β-actin (CST, #4970, 1:1000), as well as anti-rabbit IgG/HRP-linked secondary antibody (CST, #7074, 1:3000). After the assaying of enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA), image collection and densitometric analysis were performed by ImageLab software version 4.1 (Bio-Rad Laboratories, Hercules, CA, USA)[25].

Dual-Luciferase Reporter Assay

The circ_CHFR and Wnt3 3'-UTR were severally amplificated and cloned into pGL3 luciferase basic vector (Promega, Madison, WI, USA), constructing wild-type (wt) reporters (circ_CHFR-wt and Wnt3-wt, containing the valid binding sites for miR-214-3p) and mutant-type controls (circ_CHFR-mut and Wnt3-mut, containing the mutant binding sites for miR-214-3p). After the co-transfection of these reporters and miR-214-3p or miR-NC respectively, the luciferase intensity of VSMCs was determined by the Dual-Luciferase reporter system (Promega). The relative luciferase activity was calculated following the formula: firefly luciferase intensity/renilla luciferase intensity.

Statistical Analysis

All experiments were carried out three times parallelly. Data were indicated as the mean ± standard deviation (SD). The statistical analyses and picture processing were executed by SPSS 19.0 (SPSS Inc., Armonk, NY, USA) and GraphPad Prism 7 (San Diego, CA, USA). Spearman's correlation coefficient was exploited for the analysis of the linear connection in AS serum samples. The difference analysis was performed by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ was regarded to be statistically significant.

Results

Circ_CHFR Expression was Markedly Promoted in AS Serums and ox-LDL-Stimulated VSMCs

We first ascertained the dysregulation of circ_CHFR in AS process by qRT-PCR. The AS serum samples manifested a relatively higher level of circ_CHFR by contrast to normal serum

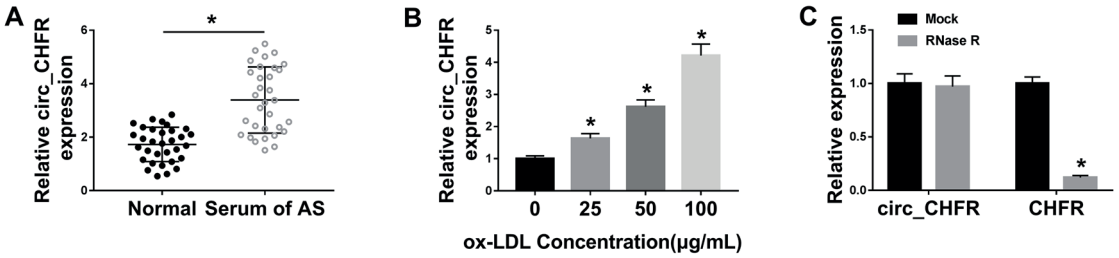

Figure 1. Circ_CHFR expression was markedly promoted in AS serums and ox-LDL-stimulated VSMCs. **(A-B)** The expression of circ_CHFR was examined by qRT-PCR in AS serum samples **(A)** and VSMCs treated with ox-LDL (0 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL) **(B)**. **(C)** The stability detection of circ-CHFR and linear CHFR after RNase R treatment was executed using qRT-PCR. *$p < 0.05$.

samples (Figure 1A). Also, circ_CHFR expression was boosted in VSMCs underwent ox-LDL stimulation and presented a gradually increased tendency with the rising of ox-LDL concentration (Figure 1B). After treatment with RNase R, circ_CHFR was more resistant to RNase R than linear CHFR, implicating the high stability of circ_CHFR (Figure 1C). These results showed the high aberrant expression of circ_CHFR in AS.

Knockdown of circ_CHFR Ameliorated the ox-LDL-Induced Promotion of Cell Growth, Migration and Inflammation in VSMCs

The exploration of circ_CHFR role was implemented by circ_CHFR loss-of-function via the transfection of si-circ_CHFR#1 and si-circ_CHFR#2. The qRT-PCR demonstrated that circ_CHFR expression was evidently declined in si-circ_CHFR#1 and si-circ_CHFR#2 groups compared with si-NC group, and the interference efficiency of si-circ_CHFR#1 was better (Figure 2A). Next, 100 μg/mL ox-LDL was exploited to treat VSMCs for 24 h, then si-circ_CHFR#1 or si-NC was transfected. After the analysis of MTT and colony formation assay, we found ox-LDL treatment induced the increase of cell viability (Figure 2B) and colony formation (Figure 2C) in VSMCs, whereas these effects were mitigated following knockdown of circ_CHFR. And the enhancement of cell migration was also shown in ox-LDL treatment group, which was alleviated by circ_CHFR inhibition (Figure 2D). In addition, the concentrations of inflammatory cytokines IL-6, IL-1β and TNF-α were significantly heightened after ox-LDL stimulation, while the downregulation of circ_CHFR weakened this increase (Figure 2E). All in all, the ox-LDL-induced aggravation of cell growth, migration and inflammation in VSMCs was abated with the low expression of circ_CHFR.

Circ_CHFR Positively Regulated Wnt3 Expression and Wnt3 Downregulation Abrogated the Injuries of VSMCs Caused by ox-LDL

Interestingly, circ_CHFR repression had an inhibitory effect on Wnt3 mRNA and protein expression levels after qRT-PCR and Western blot analyses (Figure 3A-B). Thus, we investigated the expression of Wnt3 in AS serums. As illustrated in Figure 3C, the Wnt3 mRNA expression was much higher in AS serum samples than that in normal serums. And Western blot validated the upregulation of Wnt3 protein level in AS serums again (Figure 3D). Prominently, circ_CHFR was positively related to Wnt3 (r=0.7405, p<0.0001) in AS serum samples (Figure 3E). To research the role of Wnt3 in AS, we used si-Wnt3 transfection to disturb the Wnt3 expression. Both mRNA and protein levels were decreased following the introduction of si-Wnt3 in VSMCs (Figure 3F-G). Next, experimental results indicated that transfection of si-Wnt3 rescued the ox-LDL-motivated acceleration of cell viability (Figure 3H) and colony formation ability (Figure 3I). Similarly, transwell assay testified that the augment of migrated cells number evoked by ox-LDL was abolished accompanying with the downregulation of Wnt3 (Figure 3J). Moreover, the concentrations of IL-6, IL-1β and TNF-α of ox-LDL+si-Wnt3 group were distinctly lower than that of ox-LDL+si-NC group, suggesting the inflammation injury in ox-LDL-treated VSMCs was reduced by Wnt3 knockdown (Figure 3K). Altogether, circ_CHFR could regulate Wnt3 level and Wnt3 depletion abrogated ox-LDL-induced injury of VSMCs.

Circ_CHFR Functioned as the Sponge of miR-214-3p and Wnt3 was a Target of miR-214-3p

It is explicit that circRNAs can be as the "sponges" of miRNAs[26,27]. We discovered the mutual binding sites between circ_CHFR and miR-214-3p using Starbase3.0 (Figure 4A). Furthermore, Dual-Luciferase reporter assay manifested that co-transfection of circ_CHFR-wt and miR-214-3p strikingly decreased the luciferase activity of VSMCs while circ_CHFR-mut and miR-214-3p co-transfection had no change, compared with miR-NC transfection groups (Figure 4B). And circ_CHFR knockdown resulted in a signal up-regulation of miR-214-3p expression (Figure 4C), implying that circ_CHFR could sponge miR-214-3p. Whereafter, the miR-214-3p level in AS was determined using qRT-PCR. As shown in Figure 4D-E, miR-214-3p was apparently down-regulated in AS serums and ox-LDL-treated VSMCs by comparison with normal serums and untreated VSMCs. And the liner relation between circ_CHFR and miR-214-3p levels in AS serums was obviously negative (r=-0.7961, p<0.0001) (Figure 4F). Meanwhile, we noticed that Wnt3 3'UTR had the complementary binding sites for miR-214-3p as well (Figure 4G). Then, Dual-Luciferase reporter assay affirmed the combination between miR-214-3p and Wnt3 (Figure 4H). QRT-PCR showed that the overexpression effect of miR-214-3p and repressive effect of anti-miR-214-3p on miR-214-3p expression in ox-LDL-treated VSMCs were great (Figure 4I). Overtly, miR-214-3p overexpression inhibited the Wnt3 mRNA and protein expression, but miR-214-3p inhibitor exhibited the opposite phenomenon (Figure 4J-K). Moreover, we found a negative relationship (r=-0.6368, p<0.0001) between miR-214-3p and Wnt3 expression in AS serum samples (Figure 4L). Thus, circ_CHFR acted as a sponge of miR-214-3p and miR-214-3p targeted Wnt3.

Circ_CHFR Modulated the ox-LDL-Motivated Cell Growth, Migration and Inflammation in VSMCs by Serving as a Competitive Endogenous RNA (ceRNA) of miR-241-3p to Affect Wnt3 Expression

We designed the groups si-circ_CHFR#1, si-circ_CHFR#1+anti-miR-214-3p, si-circ_CHFR#1+Wnt3 or corresponding controls to transfect after VSMCs were treated with 100 μg/mL ox-LDL for 24 h. QRT-PCR and Western blot analysis suggested that the inhibition of Wnt3 mRNA

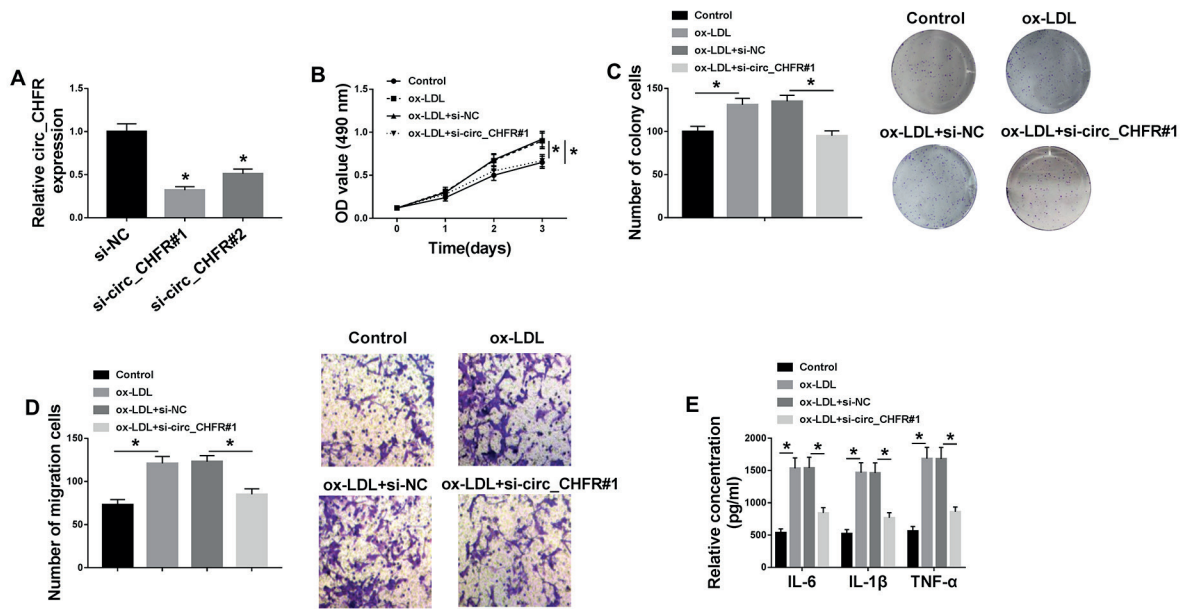

Figure 2. Knockdown of circ_CHFR ameliorated the ox-LDL-induced promotion of cell growth, migration and inflammation in VSMCs. **(A)** The interference effects of si-circ_CHFR#1 and si-circ_CHFR#2 were analyzed through qRT-PCR. **(B)** Cell viability was assessed by MTT assay in VSMCs treated with ox-LDL (100 μg/mL), ox-LDL+si-circ_CHFR#1 or matched controls. **(C)** The examination of colony formation in treated VSMCs was administrated by colony formation assay. **(D)** Cell migration ability was evaluated by transwell assay. **(E)** The concentrations of IL-6, IL-1β and TNF-α in treated VSMCs were determined via ELISA. *p < 0.05.

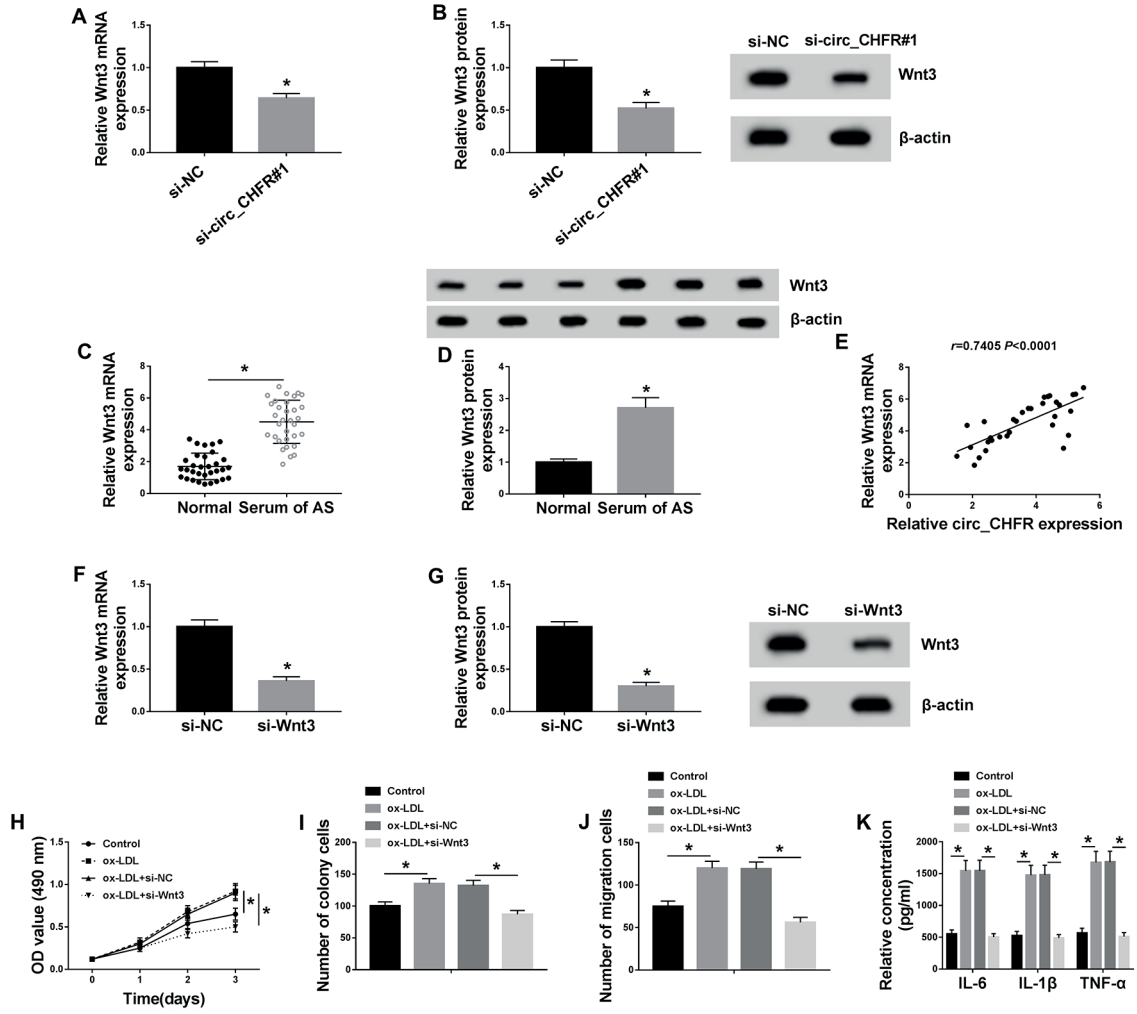

Figure 3. Circ_CHFR positively regulated Wnt3 expression and Wnt3 down-regulation abrogated the injuries of VSMCs caused by ox-LDL. **(A-B)** The mRNA and protein expression of Wnt3 was measured using qRT-PCR and Western blot after the transfection of si-circ_CHFR#1 or si-NC. **(C-D)** The Wnt3 mRNA and protein levels in AS or normal serum samples were assayed by qRT-PCR and Western blot. **(E)** The analysis of linear relation between circ_CHFR and Wnt3 was conducted by Spearman's correlation coefficient in AS serums. **(F-G)** The transfection efficiency of si-Wnt3 was evaluated through qRT-PCR and Western blot. **(H-J)** The measurement of cell viability (**H**), colony formation **(I)** and cell migration **(J)** was respectively performed by MTT, colony formation assay and transwell assay after treatment with ox-LDL and transfected with si-Wnt3 or si-NC. **(K)** The detection of inflammatory cytokines (IL-6, IL-1β and TNF-α) was carried out through ELISA. *$p < 0.05$.

and protein expression triggered by circ_CHFR knockdown in ox-LDL-treated VSMCs was reverted following miR-214-3p down-regulation or Wnt3 overexpression (Figure 5A-B), implying that circ_CHFR could regulate the expression of Wnt3 through repressing the combination between miR-214-3p and Wnt3 as a ceRNA for miR-214-3p. Simultaneously, the si-circ_CHFR transfection led to the suppressive effects on cell viability (Figure 5C) and colony formation (Figure 5D), whereas miR-214-3p inhibitor or up-regulation of Wnt3 returned these effects. Additionally, miR-214-3p depletion and Wnt3 promotion reversed the restraint of cell migration (Figure 5E) and inflammation (Figure 5F) by circ_CHFR depression after treatment with ox-LDL. Collectively, circ_CHFR could modulate cell growth, migration and inflammation by modulating the Wnt3 as a ceRNA of miR-214-3p in ox-LDL-treated VSMCs.

Circ_CHFR/miR-214-3p Axis Regulated the Wnt3/β-Catenin Signaling Pathway

To investigate the influence of circ_CHFR on Wnt/β-catenin signaling pathway, we examined

the expression of associated proteins. As Figure 6 depicted, the nuclear β-catenin level was refrained and cytoplasmic p-β-catenin expression was increased after the downregulation of circ_CHFR, but these effects were relieved through the repression of miR-214-3p or the rising of Wnt3 expression. This result clarified that circ_CHFR knockdown impeded the β-catenin signal via promoting miR-214-3p and inhibiting Wnt3 in ox-LDL-treated VSMCs, hinting that the regulatory axis of circ_CHFR/miR-214-3p could affect the Wnt3/β-catenin signaling pathway in AS process.

Discussion

AS can trigger multiple tanglesome complications to threaten the public health seriously[28], thus it is indispensable to understand the pathogenesis

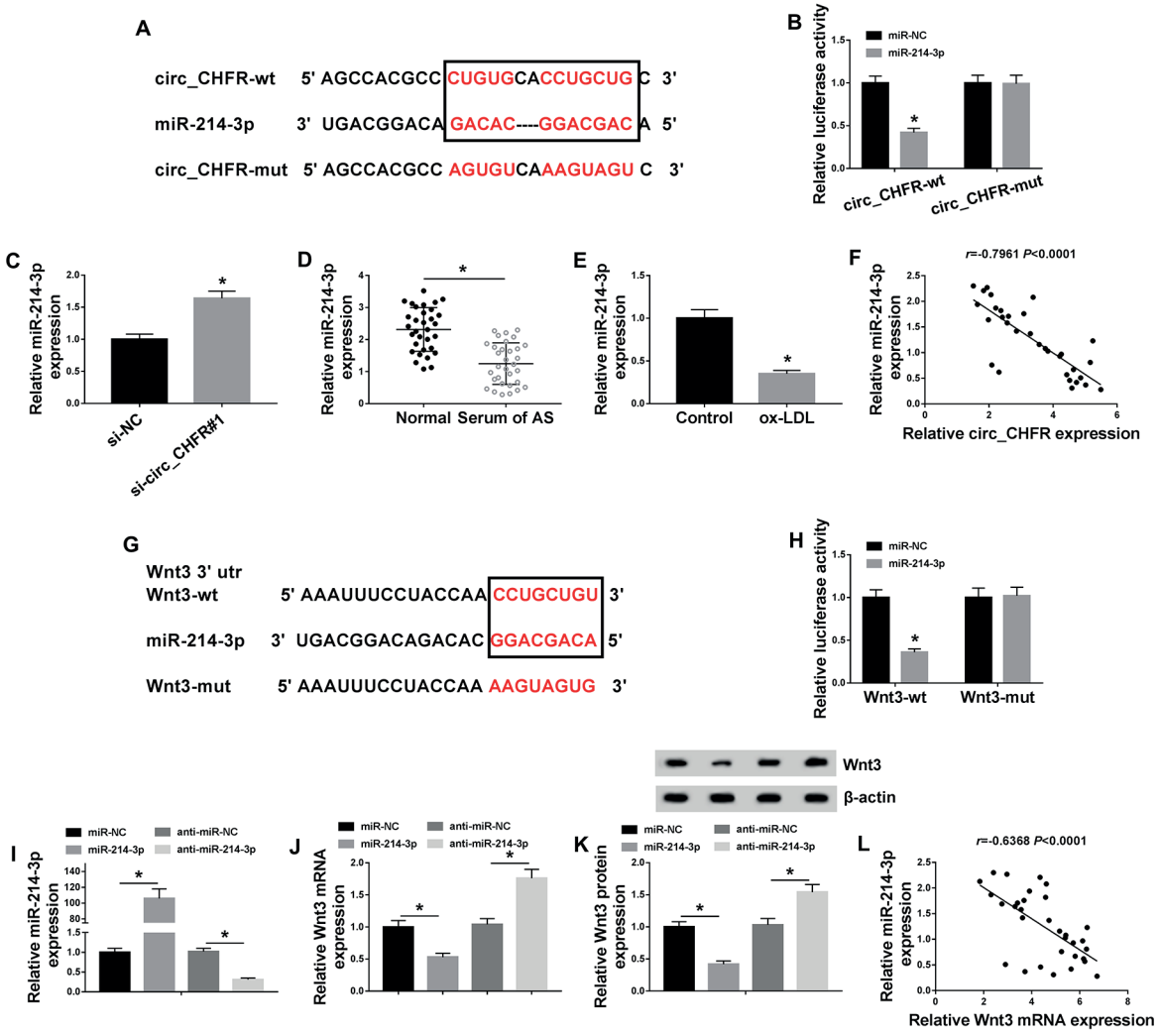

Figure 4. Circ_CHFR functioned as the sponge of miR-214-3p and Wnt3 was a target of miR-214-3p. **(A)** The binding sites between circ_CHFR and miR-214-3p were analyzed using Starbase3.0. **(B)** The combination between circ_CHFR and miR-214-3p was confirmed by dual-luciferase reporter assay. **(C)** The effect of circ_CHFR knockdown on miR-214-3p expression was assessed via qRT-PCR. **(D-E)** The miR-214-3p expression in AS serums and ox-LDL-treated VSMCs was assayed by qRT-PCR. **(F)** Spearman's correlation coefficient was exploited for analyzing the connection between circ_CHFR and miR-214-3p in AS serums. **(G)** The Starbase3.0 database was applied to conduct the bioinformatic analysis between miR-214-3p and Wnt3. **(H)** Dual-luciferase reporter assay was implemented to validate the interaction between miR-214-3p and Wnt3. **(I)** The overexpression effect of miR-214-3p and inhibitory efficiency of anti-miR-338-3p were evaluated by qRT-PCR. **(J-K)** QRT-PCR and Western blot were administrated to determine Wnt3 mRNA and protein expression after transfection of miR-214-3p, anti-miR-214-3p or relative controls. **(L)** The relation between miR-214-3p and Wnt3 in AS serums was analyzed using Spearman's correlation coefficient. *$p < 0.05$.

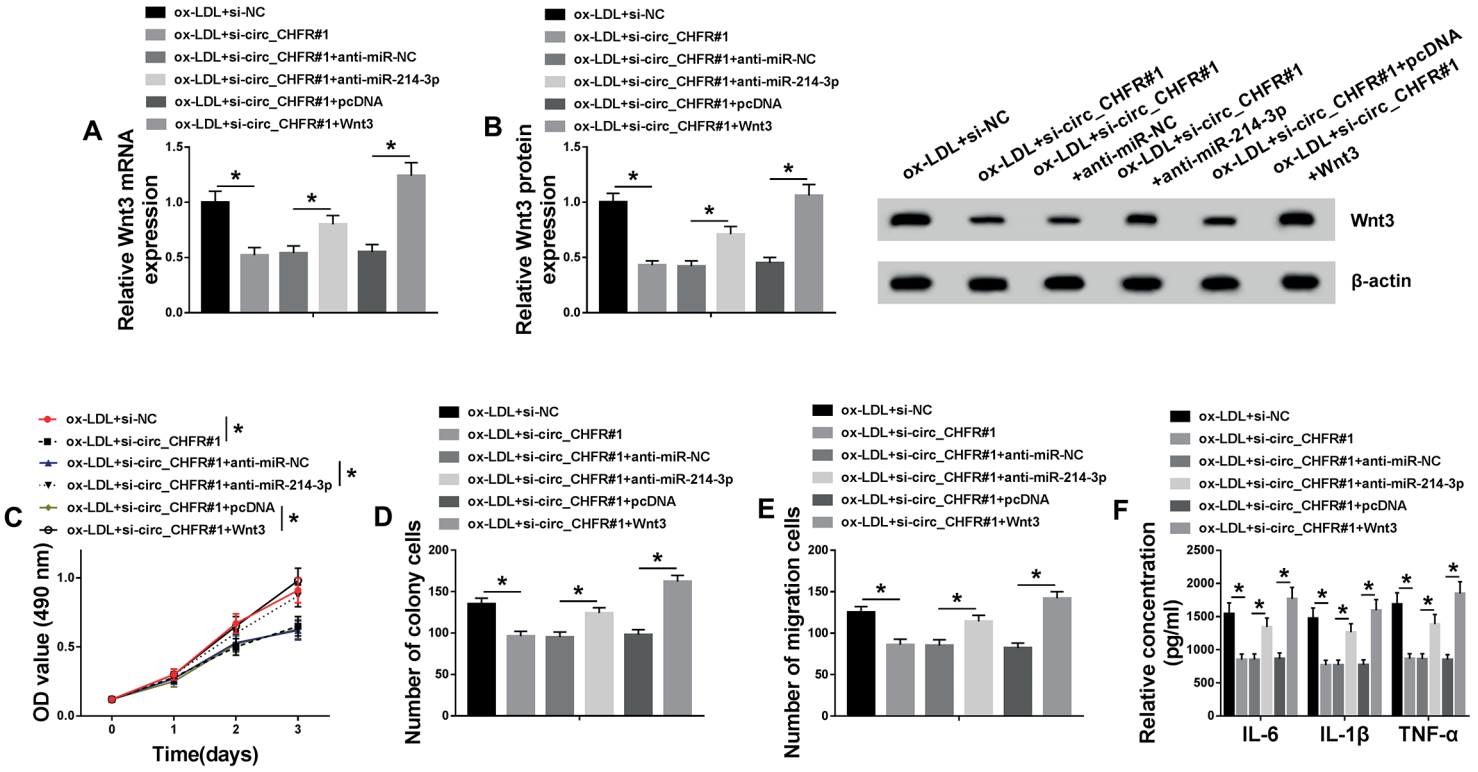

Figure 5. Circ_CHFR modulated the ox-LDL-motivated cell growth, migration and inflammation in VSMCs by serving as a competitive endogenous RNA (ceRNA) of miR-241-3p to affect Wnt3 expression. **(A-B)** The analysis of Wnt3 expression was conducted by qRT-PCR and Western blot in ox-LDL-treated VSMCs transfected with si-circ_CHFR#1, si-circ_CHFR#1+anti-miR-214-3p, si-circ_CHFR#1+Wnt3 or relative controls. **(C-D)** Cell viability **(C)** and colony formation (**D**) were severally measured through MTT and colony formation assay. **(E)** Transwell assay was applied for detecting the cell migration ability. (F) ELISA was performed to assay the concentrations of IL-6, IL-1β and TNF-α. *$p < 0.05$.

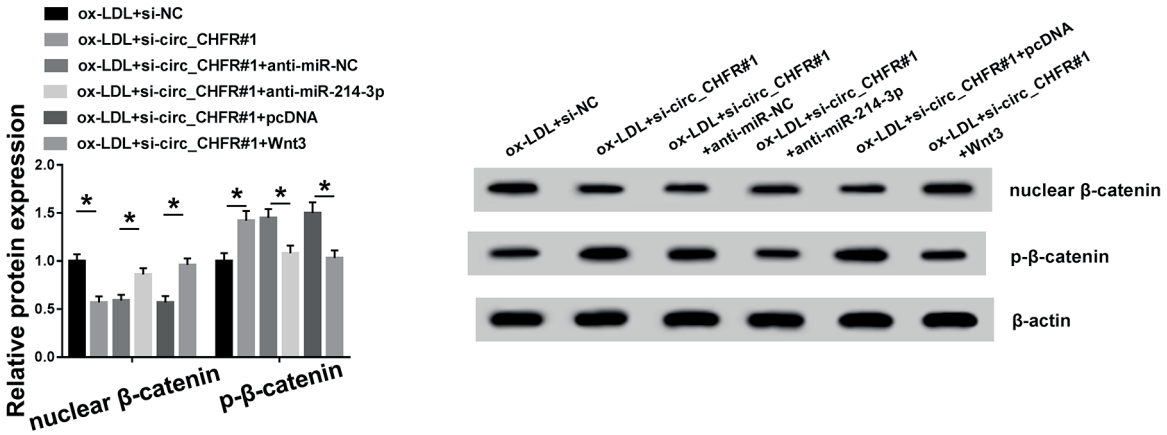

Figure 6. Circ_CHFR/miR-214-3p axis regulated the Wnt3/β-catenin signaling pathway. Western blot was exploited for measuring the protein expression of nuclear β-catenin and cytoplasmic p-β-catenin in ox-LDL-treated VSMCs transfected with si-circ_CHFR#1, si-circ_CHFR#1+anti-miR-214-3p, si-circ_CHFR#1+Wnt3 or matched controls. *$p < 0.05$.

of AS and search the molecular indicators. In our present study, the feedback loop of circ_CHFR/miR-214-3p/Wnt3/β-Catenin in regulating cell growth, migration and inflammation of VSMCs was unveiled.

Increasing researches put the highlight on the regulation of circRNAs in AS. Li et al[29] asserted the up-regulation of hsa_circ_0003575 in ox-LDL-treated human vascular endothelial cells (HUVECs) and its regulation in the proliferation and angiogenesis. Shen et al[30] claimed that circ_0044073 was highly expressed in AS and enhanced the proliferation and invasion of HUVECs via sponging miR-107. And Mao et al[31] showed that circ-SATB2 directly interacted with miR-939 to upregulate STIM1 expression, promoting the proliferation, migration and differentiation of VSMCs. Conformably, the circ_CHFR expression was memorably increased in serum samples of AS patients and ox-LDL-treated VSMCs during this study. After the circ_CHFR level was suppressed, ox-LDL-treated VSMCs exhibited the repression of cell growth and migration. Inflammation is ubiquitous in every stage of AS and several inflammatory cytokines, including IL-6, IL-1β and TNF-α, are the crucial indicators of AS generation[32, 33]. Thus, we detected the levels of these inflammatory cytokines and the results demonstrated that circ_CHFR down-regulation notably lightened the ox-LDL-induced inflammation in VSMCs, which provided the proof again for the pathogenic role of circ_CHFR in AS. After the role of circ-CHFR in AS was clarified, we found circ_CHFR could regulate the expression of Wnt3 in ox-LDL-treated VSMCs and speculated Wnt3 might also participate in the regulation of AS. As expected, Wnt3 had an up-regulation in AS serum samples, and Wnt3 depression exerted the similar inhibition of cell growth, migration as well as inflammation induced by ox-LDL in VSMCs, manifesting that Wnt3 was a pathogenic gene of AS.

As regards the molecular mechanism of circRNAs, it has been showed that circRNAs can function as a ceRNA for miRNA to regulate the expression of miRNA target to affect the inception and evolvement of human diseases, including AS[34-36]. We notarized that miR-214-3p acted as a miRNA target of circ_CHFR and circ_CHFR could regulate miR-214-3p expression as the miR-214-3p sponge. Interestingly, further experiments identified that miR-214-3p targeted Wnt3 and negatively modulated the level of Wnt3. The target relation between circ_CHFR and miR-214-3p as well as miR-214-3p and Wnt3 was uncovered for the first time. It was more innovative that circ_CHFR could act as a ceRNA of miR-214 to alleviate the repressive effect of miR-214 on Wnt3, consequently exerting the regulation on Wnt3 and forming the regulatory network of circ_CHFR/miR-214-3p/Wnt3 in VSMCs. Therefore, the function of circ_CHFR in regulating VSMCs growth, migration and inflammation relied on the miR-214-3p/Wnt3 axis.

Wnt/β-catenin signaling pathway is a canonical Wnt pathway involved in the disease regulation[37]. In the presence of Wnt ligands, β-catenin can avoid the phosphorylation to accumulate in

cytoplasmic and be translocated into the nucleus to promote the expression of downstream genes[38]. In the activated status of Wnt/β-catenin signaling pathway, the p-β-catenin level is down-regulated along with the increase of nuclear β-catenin level[39]. Herein, circ_CHFR inhibition could elevate the cytoplasmic p-β-catenin level and repress the nuclear β-catenin by regulating the miR-214-3p/Wnt3 axis, suggesting that circ_CHFR enhanced Wnt3/β-catenin signal pathway through inhibiting miR-214-3p.

Conclusions

The current research elucidated that circ_CHFR functioned as a ceRNA of miR-214 to modulate Wnt3 level and it improved the cell growth, migration and inflammation in ox-LDL-treated VSMCs via miR-214-3p/Wnt3/β-catenin signal pathway. These findings might afford a deep-going expounding for the progression of AS or other cardiovascular diseases at the molecular level.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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