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Original Article

Honokiol promoted apoptosis by targeting FOXL2/lncRNA AK001884 in vascular smooth muscle cells

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Abstract: Pathological contribution of vascular smooth muscle cells (VSMCs) in plaque to the progression of atherosclerosis has been proved. Honokiol is a small-molecule polyphenol isolated from the genus Magnolia, which has been found to have antiangiogenic in preclinical models. But the effect of honokiol on VSMCs and its specific mechanism is not clear. Therefore, the objective of this study is to explore the mechanism of honokiol on VSMCs function, hoping to find new therapeutic targets for atherosclerosis. We discovered that cell viability of VSMCs was significantly inhibited, and apoptosis rate of VSMCs was significantly increased with the treatment of honokiol via CCK-8 assay and flow cytometry, respectively. By screening potential proliferation and apoptosis associated genes from VSMCs treated with honokiol or DMSO, we found that FOXL2, a transcriptional factor, was significantly decreased after treatment of honokiol *in vitro*, suggesting that FOXL2 exerts important roles in promoting cell proliferation and suppressing cell apoptosis in VSMCs. Furthermore, through performing LncRNA chip assay, we identified that LncRNA (AK001884) was the potential biomolecule which is essential for inhibiting cell proliferation and promoting cell apoptosis induced by honokiol on VSMCs via downregulating FOXL2. The results of this study indicate that the effects of honokiol on inhibition of VSMCs proliferation and induction of VSMCs apoptosis via down-regulation of FOXL2 and subsequently increased expression of LncRNA (AK001884). Our studies provide a theoretical basis for applying honokiol for clinical atherosclerosis treatment.

Keywords: Honokiol, LncRNA, VSMC, FOXL2, AK001884, proliferation, apoptosis

Introduction

Atherosclerosis is the leading cause of death and disability in the developed world [1]. So far, statins was proved to be major effective drug available to treat atherosclerosis [2] mainly through two mechanisms. At first the relationship was simple: statins inhibited synthesis of the cholesterol that contributed to atheroma, which means less cholesterol, less atheroma [3]. Recent studies have revealed atherosclerosis is a notable inflammatory disease, and in parallel, statins appear to inhibit inflammatory processes directly [3]. Vascular smooth muscle cells (VSMCs) proliferation and accumulation trigger vascular atherosclerosis. This phenomenon is linked to an increased risk of coronary heart disease (CHD) [4]. The lesions of atherosclerosis represent a series of highly specific cellular and molecular responses, which could lead to persistent inflammatory reaction such as recruiting inflammatory cells and secreting inflammatory cytokine [5], finally induce the changes of VSMCs from the quiescent state to active state [6], and form a vicious cycle.

Honokiol is a pharmacologically active small molecule isolated from the Chinese traditional medicinal herb-Houpu [7]. It's reported that honokiol can be served as an agent with multiple medicinal applications, and plays pivotal roles in anti-angiogenic, anti-tumor and anti-inflammatory properties [8]. For pro-apoptosis properties, honokiol has two distinct mecha-

nisms: inhibition of Ras signaling [9] and activation of the mitochondrial transition pore [10]. For anti-angiogenic properties, honokiol blocks the phosphorylation and activation of Rac signal pathway due to VEGF-VEGFR2 interactions [11]. These data suggest the inhibition of cell proliferation and pro-apoptosis effect of honokiol.

FOXL2, as a transcriptional factor, belongs to Human Forkhead-box (FOX) gene family which consists of at least 43 members [12]. FOXL2 initially found to be responsible for the blepharophimosis ptosis epicanthus inversus syndrome [13]. Recent years, it has been proved that FOXL2 have potent pro-apoptosis and proliferation inhibition effects [12]. By regulation of the expression of FOXL2, it may exert pro-apoptosis effects in VSMCs.

Recent studies have begun to elucidate the role of noncoding RNAs in atherosclerosis [14]. It has recently become apparent that, in addition to small RNAs, many long noncoding RNA (LncRNAs) play important roles in biological processes such as cell differentiation, cell cycle, maintenance of stem-cell-like phenotypes, and the expression patterns of LncRNAs are different depending on cell-type [15, 16]. Yet, there is rare study investigate the role of LncRNAs in VSMC. RNA sequencing (RNA-seq) has recently been applied to assess the transcriptome of normal and failing murine hearts of mice to discover that LncRNAs may be involved [17]. It has also been proved that LncRNAs play a role in Ang II reaction in VSMCs [18]. Therefore, a thorough investigation of transcripts regulated by FOXL2 in honokiol administrated VSMCs is essential for further exploration.

Huge efforts have been made to inhibit the accumulation of VSMCs, honokiol has raised to be a promising drug in atherosclerosis therapy. To explore the specific mechanisms of honokiol treatment on VSMCs may provide strong evidence for its clinical application.

Materials and methods

Cell culture and honokiol treatment of VSMCs

VSMCs were purchased from Saisi biotech company, China. Honokiol (50,100 μg/ml) treated cells were processed for RNA extraction or chromatin-immunoprecipitation experiments. The culture media was Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and antibiotics 1% (v/v). For siRNA transfection, VSMCs were incubated in DMEM supplemented with 10% FBS only, as recommended by the transfection agent manufacturer.

siRNA transfections

Small interfering RNAs (siRNAs) targeting LncRNA (AK001884) were used to downregulate the expression of AK001884. 1×10^6 cells were transfected siRNAs targeting to AK001884 or control (Non-Targeting Control) at a final concentration of 50 nmol/L, according to manufacturer protocol (Amaxa). The algorithm GPboost was used to design siRNAs for targeting LncAK001884. The sequences of siRNAs-AK001884 were as follows: siRNA-1: CATTCCTTGTCCAACTGAATGTGAA; siRNA-2: TCCCTGTGAAGAGCCTGCCTGTTAT; siRNA-3: GAGTGGTGTGAAGGTTCGTTCTAAA, siRNA-NC: GACCTACAACTACCTATCA. And the sequences were synthesized by GenePharma (China). SiRNA targeting human FOXL2 (No. sc106837) was purchased by Santa Cruz biotechnology (USA).

Western blot analysis

Protein was isolated from cultured VSMCs with Trizol reagent (Invitrogen, Carlsbad, Calif) and standard Invitrogen protocols. After protein concentration quantitation with the modified Bradford assay (Bio-Rad Laboratories, Hercules, Calif), protein was then separated by 10% SDS-PAGE and transferred to PVDF membranes, incubated with primary antibodies against FOXL2, (Santa Cruz Biotechnology, Santa Cruz, Calif). The blots were visualized with a chemiluminescene system (Amersham Bioscience, Buchinghamshire, UK). The signals were quantified by Image Pro Plus software (Media Cybernetics).

Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA from VSMCs was extracted with TRIzol reagent (Life Technologies, USA) according to the manufacturer's instruction. Accumulation of the PCR product was monitored in real time, and the threshold cycle (Ct) was determined with the 7900HT Fast Real-Time

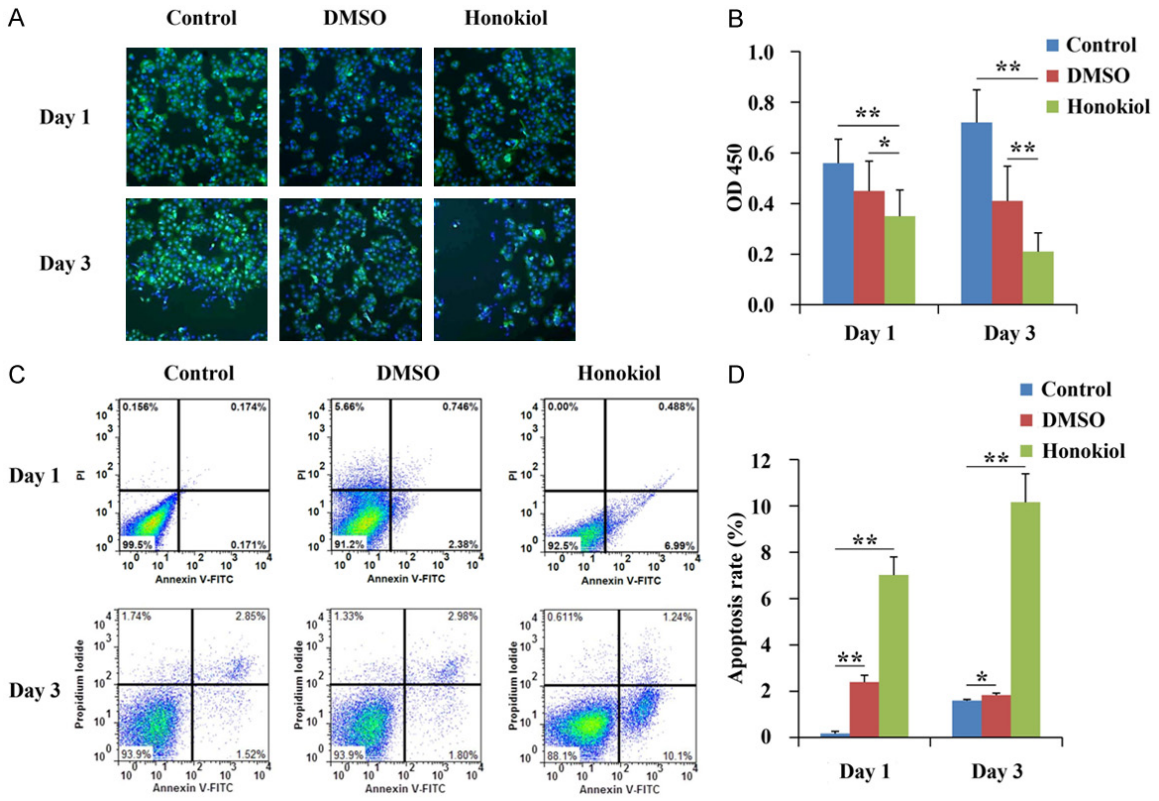

Figure 1. Honokiol inhibited proliferation and promote apoptosis of VSMCs. A, B. After treated with honokiol, the proliferation rate of VSMCs was detected by phalloidine staining and CCK-8 at day 1 and day 3. C. The apoptosis of VSMCs was measured by flow cytometry at day 1 and day 3. D. Apoptosis rate of VSMCs hasd been calculated. Experiments were repeated three times. There were significant differences between control and honikiol group cells and the other groups. *P<0.05, **P<0.01.

System (Applied Biosystems, Fostercity, CA). The relative change in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method with normalization to β-actin. FOXL2-forward: 5'-CAAGTACCTGCAGTCTGGCT-3', FOXL2-reverse: 5'-TCCCAGGCCATTGTACGAGT-3'; AK001884-forward: 5'-CTGGGAATGGCACAGTGGAA-3', AK001884-reverse: 5'-GAACAGCAGGGGGAGTTCAT-3'; β-actin-forward: 5'-AGGATTCCTATGTGGGCGAC-3', β-actin-reverse: 5'-ATAGCACAGCCTGGATAGCAA-3'.

Apoptosis detection with Annexin V-FITC/PI Staining

Living cells and apoptotic cells could be detected using the Annexin V-FITC/PI apoptosis kit by flow cytometry. The VSMCs cells were treated with the indicated concentrations of honokiol in 6-well plates. Then the cells were harvested and washed twice with ice-cold PBS. After discarding the supernatant, the cells were resuspended in 500 μL binding buffer and stained with 5 μL Annexin V-FITC and 10 μL PI for 5 min in the dark at room temperature. Then, the samples were detected by flow cytometry and the apoptotic population was analyzed using FlowJo Software (Treestar, USA).

Construction of the LncRNA microarray (LncRNA CHIP) and PCR array

We pooled three replicate samples of cells with different treatments (siRNA-FOXL2, siRNA-NC) or VSMCs in different groups (blank exposure, DMSO exposure and honokiol exposure) on day 1 and day 3 respectively, to perform LncRNA microarray analysis or PCR array. Total RNA was isolated from the two samples as above and was quantified using a NanoDrop spectrophotometer (NanoDrop). RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Each sample was then amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias, using a random priming method. The labeled cRNAs were hybridized onto the Mouse LncRNA Array v2.0 (8 × 60 K, Arraystar).

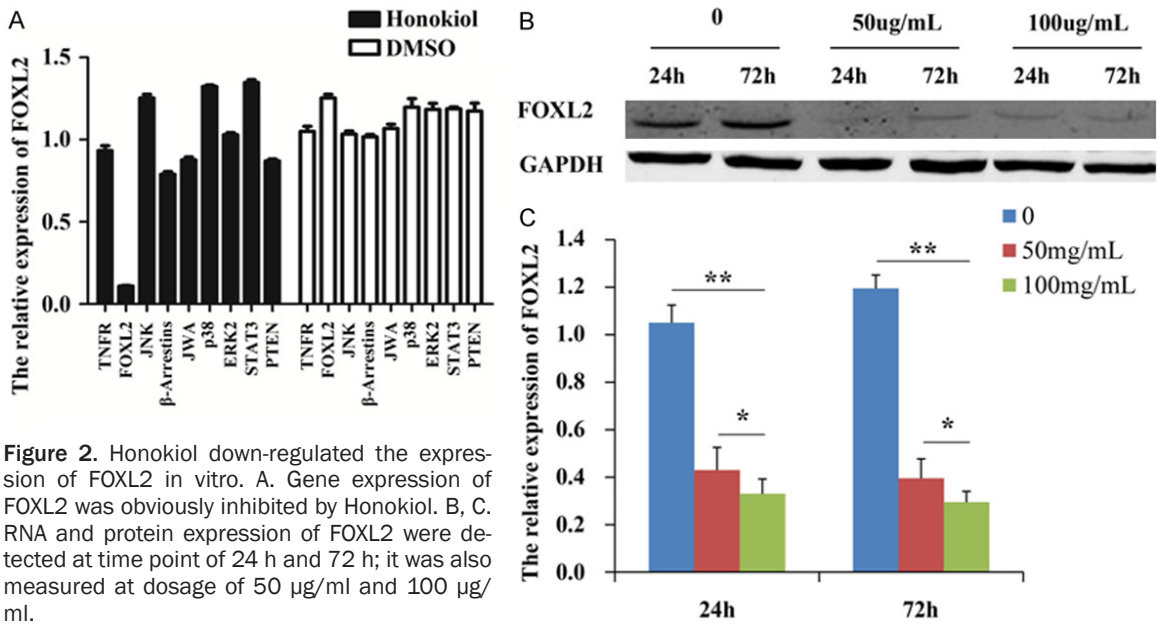

Figure 2. Honokiol down-regulated the expression of FOXL2 in vitro. A. Gene expression of FOXL2 was obviously inhibited by Honokiol. B, C. RNA and protein expression of FOXL2 were detected at time point of 24 h and 72 h; it was also measured at dosage of 50 μg/ml and 100 μg/ml.

The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (part number G2505C). Data were extracted using Agilent Feature Extraction software (version 11.0.1.1).

Cytoskeleton, cell morphology and cell proliferation examination

VSMCs were seeded on cover slips in 24-well plates for 24 h and fixed by 4% paraform for 20 min, then blocked with BSA for 20 min, the cells were treated with 1% Phalloidine-FITC (Sigma Chemical Corp., USA) for 20 min and washed 3 × 5 min with PBS, sealed with 50% glycerin. Finally, the cells were observed and photographed under Confocal Microscope.

For cell proliferation, aorta abdominalis VSMCs were cultured in 96-well plates and cell proliferation was measured using a cell proliferation colorimetric assay (CCK-8) according to the manufacturer's instructions. Absorbance was measured 72 h after treatment with siRNA using a microplate reader (Multi-Spectrophotometer, Viento, Dainippon) at a wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software for Windows. Data from experiments were reported as mean ± S.D. Significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A value of $P<0.05$ was considered statistically.

Results

Honokiol inhibits proliferation and promotes apoptosis of VSMCs

Primary cultures of VSMCs from different treating group were subjected to proliferation and apoptosis evaluation. As shown in **Figure 1**, VSMCs were incubated with honokiol for 24 h and 72 h, and cells were incubated with DMSO as its control. The cell cytoskeleton and morphology of VSMCs was visualized by phalloidine staining, cells in blank control group or DMSO group had more abundant microfilaments at day 1 and day 3, compared with honokiol group. And the honokiol group cells displayed unordered actin fibers and mal-morphology at day 1 and day 3 dependently (**Figure 1A**). We also detected the proliferation ability of VSMCs with honokiol treatment by CCK8 assay (**Figure 1B**). The proliferation of VSMCs was obviously decreased in honokiol treated group compared with control group at day 1 and day 3 ($p<0.01$), and the differences between honokiol treated group and DMSO group at day 1 ($p<0.05$) and day 3 ($p<0.01$) were statistically significant. These results confirmed proliferation inhibition ability of honokiol in VSMCs. Apoptotic VSMCs after administration of honokiol were detected by the Annexin V-FITC/PI apoptosis kit by flow cytometry at day 1 and day 3. As shown in **Figure 1C**, after treated by honokiol, apoptotic

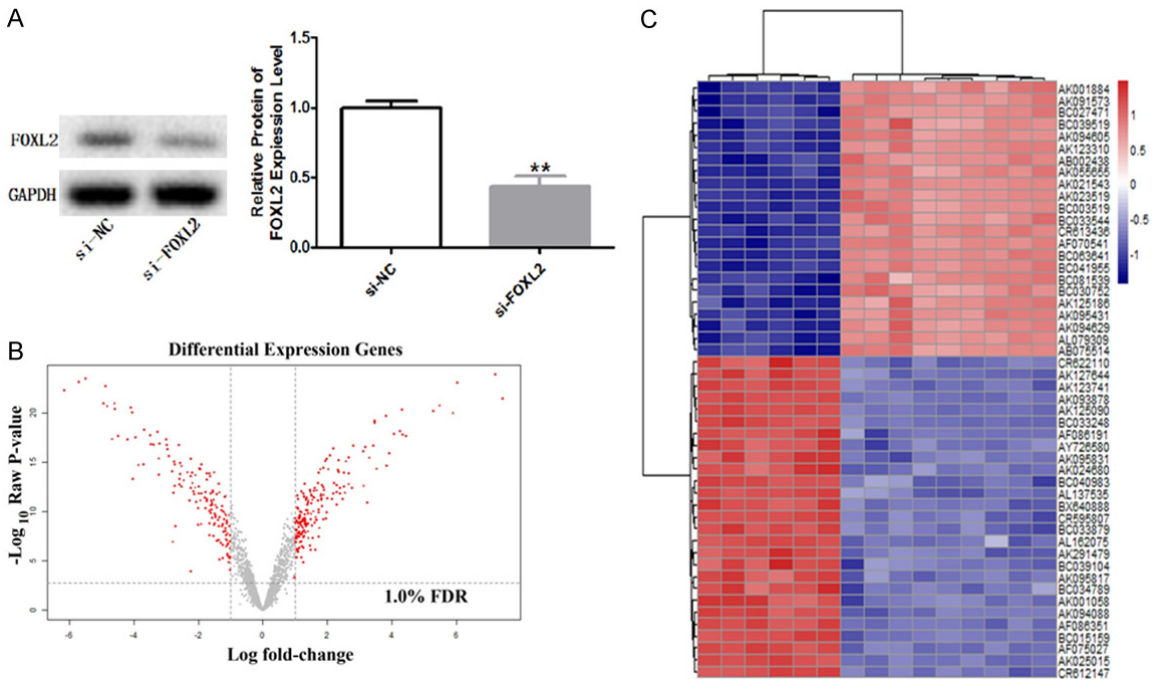

Figure 3. LncRNA (AK001884) expression increased in FOXL2 silenced VSMCs. A. The knockdown efficiency of FOXL2-siRNA was determined by Western Blot. B, C. The volcano plot and heat map shows lncRNA (AK001884) expression was upregulated significantly in FOXL2 silenced VSMCs by lncRNA microarray.

VSMCs reach to approximate 7% compared with 0% and 2% in control and DMSO treated group at day 1 ($p<0.01$). At day 3, the apoptosis rate increased to almost 10% after treatment by honokiol compared with approximate 1% in control and DMSO treated group ($p<0.01$). These data showed that honokiol could effectively promote apoptosis of VSMCs.

Screening and validation of the suppressed gene associated with cell proliferation and apoptosis

To further confirm the most suppressed gene by honokiol, the PCR-array was adapted to screen from gene clusters associated with cell proliferation. As we can see from (**Figure 2A**), after screened from JNK, β-Arrestins, p38, FOXL2, STAT3, PTEN and ERK2, the FOXL2 expression showed the greatest suppression among genes. To further validate the regulation of FOXL2 by honokiol, we analyzed the expression of FOXL2 by Western-Blot and qPCR. Firstly, it was confirmed by Western-Blot, concentration of FOXL2 was decreased significantly in a dose-dependent manner but with a unclear time-dependent manner (**Figure 2B**). In addition, we explored the FOXL2 expression in VSMCs from RNA level, as shown in **Figure 2C**. There was a significant difference in FOXL2 expression between non-treated group and honokiol (50,100 mg/mL) treated group ($p< 0.01$). Moreover, the expression of FOXL2 was obviously reduced in 100 mg/mL honokiol treated group than 50 mg/mL honokiol treated group ($p<0.05$), however, there was no significant difference between 24 h honokiol treated groups and 72 h treated group at corresponding dosage. From these data, we proposed that honokiol performed its function via down-regulation of FOXL2.

Silence of FOXL2 promote LncRNA (AK001884) expression in VSMCs

Then, we silenced FOXL2 by adopting siRNA targeting to human FOXL2 gene, the knockdown efficiency was about 70% (**Figure 3A**). As shown in **Figure 3B** and **3C**, after transfected FOXL2 siRNA in VSMCs, we selected the differentially expressed LncRNA compared with scramble group. After removed the redundant and unannoated sequences, with FDR<1%, 27 genes were found to be significantly down-regulated and 23 genes to be significantly up-regulated ($p<0.0001$) in FOXL2 silenced group compared to scramble group. We found that the expression of LncRNA (AK001884) at the highest le-

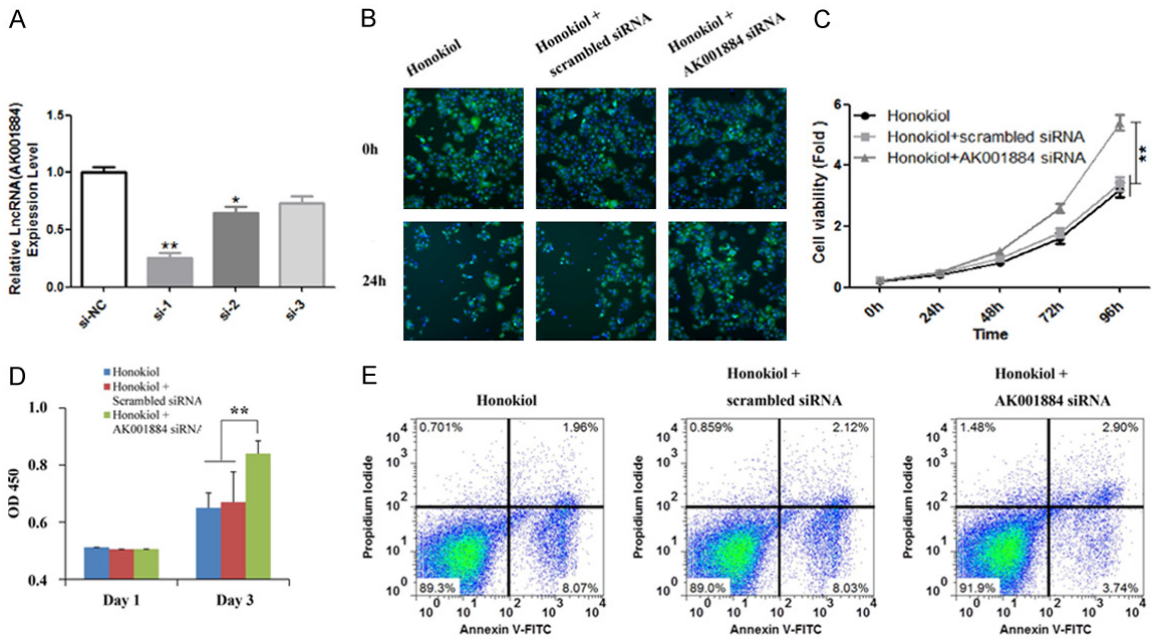

Figure 4. SiRNA silence of lncRNA(AK001884) reverse the effect of honokiol on proliferation and apoptosis. A. The knockdown efficiencies of AK001884-siRNAs were determined by qPCR. B. Morphology and cell proliferation examination of VSMCs by phalloidine staining. C. The proliferation of VSMCs was determined by CCK-8. D. After lncRNA (AK001884) was silenced by target siRNA, honokiol inhibition on proliferation of VSMCs was reversed significantly. E. Honokiol promoted the apoptosis of VSMCs, siRNA silence of lncRNA (AK001884) reverse this effect.

vel in FOXL2 silenced group. There results suggested that FOXL2 was able to regulate the expression of LncRNA (AK001884).

LncRNA (AK001884) was indispensible in Honokiol-FOXL2-lncRNA axis

To further determine the exact role of LncRNA (AK001884) involved in honokiol-FOXL2-lncRNA axis, we silenced LncRNA (AK001884) using siRNA. As shown in **Figure 4A**, the knockdown efficiency was approximately 70%. After treated by honokiol for 24 h, VSMCs were observed reduced significantly, knocking down of lncRNA (AK001884) abolished the effect of honokio l (**Figure 4B**). We further evaluated VSMCs proliferation by CCK8 assay (**Figure 4C**, **4D**). At day 1, it had no change in cell proliferation of AK001884 silenced and honokiol treating group compared with honokiol group. However, at day 3, the proliferation ability of VSMCs was significantly higher in AK001884 silenced and honokiol treating group than that in scramble and honokiol treating group ($p<0.01$). Additionally, we performed CCK-8 curve to determine the proliferation of VSMCs, results showed that the VSMCs growth were increased when cells were treated with siRNA-AK001884. Following we silenced AK001884 by siRNA, apoptosis induction effect of honokiol was attenuated (**Figure 4E**). These results indicated that LncRNA (AK001884) was indispensible in honokiol-FOXL2-lncRNA axis and should be considered as a core function-exertion factor of honokiol.

Discussion

As a major part of atherosclerotic plaques, VSMCs have been viewed as directly responsible for generating the atherosclerotic plaque [6]. Until now, there is no effective drug, even statins which proved to be major effective drug available to treat atherosclerosis [2], in treating proliferation and accumulation of VSMCs. Therefore, development of new drugs for treatment of VSMCs proliferation and accumulation is urgently needed. Honokiol is a small-molecule polyphenol isolated from the genus Magnolia. Recently, honokiol has been found to have antiangiogenic, anti-inflammatory, and antitumor properties in preclinical models, without appreciable toxicity [8]. These findings have increased interest in bringing honokiol to the clinic as a novel chemotherapeutic agent for

inhibiting proliferation and inducting apoptosis of VSMCs.

It's reported that honokiol has two major mechanisms of action; it has both direct proliferation inhibition and apoptosis induction properties. For proliferation inhibition activity, honokiol blocks the phosphorylation and rac activation due to VEGF-VEGFR2 interactions [12]. For apoptosis induction property, honokiol blocks signaling in tumors with defective p53 function and activated Ras by directly blocking the activation of phospholipase D by activated ras [9].

Recent studies demonstrated that noncoding RNAs could play pivotal roles in VSMC biology [18]. Amy Leung, etc. provided the first identification of Ang II-regulated lncRNAs, which suggests functional roles for these lncRNAs in mediating cellular responses to Ang II. And they proved that these lncRNAs was indispensable to regulate the proliferation of VSMCs. These newly identified noncoding transcripts could be exploited as novel therapeutic targets for Ang II-associated cardiovascular diseases [18].

In the present study, we first investigate the profile of the expression of transcription-associated gene, combined with high-throughput sequencing methods to elucidate the specific mechanism of VSMCs response to honokiol. Additionally, our group screen out a transcriptional factor FOXL2 was significantly reduced in honokiol treated VSMCs.

As a transcriptional factor, FOXL2 regulate a series of transcriptional products (including mRNA, LncRNA and miRNA) [19]. High-throughput sequencing method is a best method available to elucidate the functional properties of FOXL2. Subsequently, we did LncRNA chip to screen the transcriptome of FOXL2 after treated with honokiol. Intriguingly, we identified that LncRNA (AK001884) was the potential biomolecule which is essential for the biological function of honokiol on VSMCs. After silence LncRNA (AK001884) with siRNA, the proliferation inhibition and apoptosis induction of VSMCs by honokiol treatment was reversed, this result indicated that LncRNA (AK001884) played a crucial role in biological function of honokiol on VSMCs.

These transcripts represent potential new avenues of research into how VSMCs respond to honokiol. To further demonstrate that LncRNA (AK001884) involves in regulation of proliferation and apoptosis, it should began with specific mechanism of LncRNA in regulation of proliferation and apoptosis associated factors. LncRNAs could function as host transcripts for miRNAs, LncRNAs may function as enhancers and regulate the expression of proximal genes, which could be proliferation and apoptosis associated genes [12]. Studies investigating coregulation of LncRNAs with their neighbors and whether this occurs via alterations of enhancer chromatin modifications will be informative in understanding the role of LncRNAs in honokiol effects. LncRNAs could also function as a cofactor of transcriptional factor and signaling pathway (such as VEGR2 and p53 etc.) which were crucial for cell proliferation and apoptosis [20-22]. Subsequent research will contribute to clarify the mechanism of LncRNA (AK001884) in regulating the proliferation and apoptosis in VSMCs, to provide a theoretical basis for the treatment of honokiol.

Taken together, this study proved that the effect of honokiol on inhibiting cell proliferation- and inducting cell apoptosis- by suppressing the expression of FOXL2. Transcriptome of VSMCs by FOXL2 silence indicated that LncRNA (AK001884) is responsible for biological effects of honokiol. This FOXL2/LncRNA (AK001884) axis represent potential mechanism of how VSMCs respond to honokiol treatment, ultimately, providing effective therapeutic target for CHD.

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Disclosure of conflict of interest

None.

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