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Original Article

Baicaleininhibits VSMCs proliferation via regulating LncRNAAK021954 gene expression

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Abstract: Atherosclerosis is responsible for the global medical burden of cardiovascular diseases, of which the proliferation of vascular smooth muscle cells (VSMCs) plays a key role in the development. Recent evidences demonstrated that baicalein could attenuate the proliferation of VSMCs and had no influence on VSMCs migration. However, the precise molecular mechanisms of baicalein inhibiting the proliferation of VSMCs were not clear. In this study, we investigated the viability and apoptosis behaviour of VSMCs and its downstream molecular changes with exposed to different dose of baicalein. Firstly, we observed significant reducing in the VSMCs proliferation and decreasing of FGF18 expression in a dose dependent manner after addition of baicalein for 24 h and 72 h. Moreover, the mRNA expression profile of VSMCs after treatments was evaluated by microarray analysis. Microarray analysis showed that long non-coding RNA (lncRNA) AK021954 gene expression was significantly increased in the baicalein treated group compared with the control group. Inversely, the VSMCs proliferation showed a notable increase after small silent RNA of lncRNAAK021954 treatment. These results indicated that lncRNAAK021954 gene and FGF18 involved in baicalein inhibiting the proliferation of VSMCs. It may provide a promising method in treatment of atherosclerosis.

Keywords: Atherosclerosis, baicalein, proliferation, VSMCs, FGF18, LncRNAAK021954

Introduction

Classically, there are bound to be many other factors at work for atherosclerotic plaques, complex pathologies of medium to large blood vessels, which the bio-mechanism vascular [1], metabolic, inflammatory and immune systems are involved in [2]. And the majority of cardiovascular diseases are the outcome of vascular remodeling triggered by vascular injury [3], which often initiate many kinds of vessel pathologies including monocyte accumulation, foam cell formation and role of lipoproteins and immune mechanisms occurred in accelerated atherosclerosis. Specifically, VSMCs proliferation is also a key etiological factor in the development of atherosclerosis [4, 5]. The last few years had witnessed many molecular regulators contributing to VSMCs proliferation within the atherosclerotic-prone vessel wall and the atherosclerotic plaque itself [6, 7]. The late study elucidated the effect of lipoxygenase on proliferation in spontaneously hypertensive rats VSMCs [8]. Lipoxygenase also plays a major role in the role of inflammation of atherogenesis [9]. Its resolution would offer novel approaches to interfere atherogenesis.

Baicalein, a major flavonoid extracted from the root of scutellaria baicalensisgeorgi, it is also known as a lipoxygenase inhibitor, which can influence VSMCs proliferation [10-14]. Moreover, baicalein is linked to PPAR-gamma, a member of the nuclear receptor family that implicates in cell differentiation and proliferation [15, 16]. According to these reports, baicalein may has effectively potential capacity to treat atherosclerotic. Fibroblast growth factor 18 (FGF18) involves in a variety of biological processes [17, 18], and it also expresses in human cardiovascular tissues and induces them igration of endothelial cells [19].

FGFRs cantransfer ex-nucleus signal to transcription factor through ERK, p38MAPK signaling pathway [20]. Although the signaling mole-

cules which involve in the embryonic and postnatal development have been studied extensively, the signaling leading to atherosclerosisis yet less understood. The expression of several secreted FGF18 by endothelial cells drawn more focus on their paracrine effects on neighboring cells during tissue regeneration or tumor formation [19, 21]. Therefore, it is meaningful to explore the association between baicalein and FGF18 expression in VSMCs proliferation. Moreover, lots of considerable evidence indicate ncRNAs, particularly lncRNAs, have an important role in atherosclerosis [22]. LncRNAs as important potential regulators of the progression of atherosclerosis, may have a chance to become anti-atherosclerosis target genes [23].

Current evidences suggest that baicalein has a prominent role in attenuating vascular disorder genesis during atherosclerosis development, and has no influence in VSMCs migration [24]. Given the benefits of associating with baicaein and atherosclerosis, we attempt to delineate the mechanisms of baicalein regulating VSMCs proliferation. We report here for the first time that decreasing of FGF18, through the addition of baicaein, inhibited VSMCs proliferation. We had further screened out lncRNAsAK021954 as a novel downstream target of baicaein on restraining VSMCs proliferation by microarray analysis. Consistent with this, the silencing of lncRNAsAK021954 resulted in elevated proliferation of VSMCs.

Methods and materials

Reagents

Human aortic VSMCs were purchased from Lonza (Allendale, NJ). Baicalein, anti-FGF18 antibody, anti-GADPH antibody, anti-Mouse IgG, Alexa Fluor-568 goat anti-rabbit antibody, FITC-phalloidine, and 4,6-diamino-2-phenylindole (DAPI) were obtained from Sigma Aldrich Chemical Company (St.Louis, MO, USA). A cell count kit-8 and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime biotech company, (Nantong, China). Fluoromount-G was from Southern Biotech. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and all other cell culture reagents were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA). In the experiments, baicalein was prepared by dissolving with DMSO. Dilutions were made in phosphate-buffered saline (PBS) and filtered through a 0.22 μM syringe filter.

Cell culture and treatments

VSMCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Cells were detached with 0.25% trypsin/EDTA and seeded into 10 cm tissue culture plates at a density of 10^5 cells per milliliter. All experiments were conducted during cell passages 3 to 7. Prior to addition of baicalein, 95% confluent VSMCs were serum-starved overnight by incubating in DMEM with 0.1% FBS. Cells were incubated in a humidified incubator at 37°C and 5% CO_2 in the presence or absence of baicalein for the indicated times.

VSMCs proliferation

VSMCs were plated in24-well plates and then exposed to baicalein (50 μg/mL) or not for 24 h or 72 h. Before collection cells, the cells were subsequently washed three times with cold PBS. A cell count kit-8 was employed to quantitatively evaluate the cells' viability. Briefly, approximately 1×10^5 cells were seeded on each film in the 24 wells plates for 24 h and 72 h, then the culture medium was removed and washed with PBS for twice. Approximately 900 μL serum-free DMEM medium and 100 μL CCK-8 solution were added to each sample, followed by incubation at 37°C for 3 h. Supernatant was transferred to 96-well plate, the optical density (OD) at 450 nm was determined using a microplate reader (MultiskanMK33, Thermolabsystems, Finland). Six parallel experiments in each sample were used to assess the cell proliferation.

FITC-phalloidine staining

In this study, cells were counted by FITC-phalloidine staining. After fixed with 4% paraformaldehyde in PBS for 10 minutes at 37°C, Cells were permeabilized with 0.1% Triton X-100 in PBS for 7 min and blocked with 5% BSA in PBS for 1 h at 37°C. Between each step described above, cells were washed three times with PBS for 5 minutes at 37°C. Followed blocking cells were stained with 5 mg/ml FITC-phalloidine (1:100) for 1 h at 37°C in the dark. Images were obtained by a laser scanning confocal microscope (TCS SP5, Leica, Germany).

Flow cytometry

To detect the apoptosis of VSMCs after addition of baicalein [25], the Annexin V-FITC Apoptosis Detection Kit was used. Briefly, VSMCs were try psinized and washed once with PBS. Then the cells were stained with Annexin V/FITC for 30 min at 4°C and propidium iodide for 15 min in the dark according to manufacturer protocol. The rate of cell apoptosis was determined by flow cytometry.

Preparation of total RNA and quantitative real time RT-PCR

The mRNA expression was assessed by real-time PCR. Total RNA from cultured cells wereisolated with Trizol according to manufacturer's protocol (Invitrogen, Carlsbad, CA) and reverse-transcribed using PrimeScript™ RT Master Mix (Takara Biotechnology Co. Ltd, Dalian, China). The cDNA was amplified in 96 wells reaction plates with KAPA SYBR FAST qpcr kit Master Mix (2×) Universal (KapaBiosystems, Wilmington, US) on an ABI 7900 real-time PCR thermocycler. PCR cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 10 sec, 56°C for 20 sec, and 72°C for 10 sec. The primer sequences were for FGF18 forward primer: 5-TGAACACGCACTCCTTGCAGT-3; reverse primer: 5-GAATTCTACCTGTGTATGAACCGAAA-3 [26], GAPDH forward primer: 5-GCACCGTCAAGGCTGAGAAC-3; reverse primer: 5-TGGTGAAGACGC CAGTGGA-3. GADPH was a internal reference of levels. The ΔΔCt-method (change in ΔCt [=Ct of the target gene minus Ct of the house keeping gene]) was used for relative quantification. Fold changes in expression were calculated according to the transformation: fold increase $=2^{(-\Delta\Delta Ct)}$. PCR efficiency was tested and ensured to be similar for both the target gene and GADPH control gene.

Western blotting

After treatment with the indicated agents (0 μg/ml, 50 μg/ml, 100 μg/ml) for 24 h and 72 h, cells were washed twice with cold PBS and then cells were harvested using RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF). The protein was centrifuged at 14,000 g for 10 min. The concentration was measured by Bicinchoninic Acid (BCA) Protein Assays (Thermofisher). The 25 micrograms of protein supernatants were separated by 10% SDS-PAGE (PAGE) gels and transferred to nitrocellulose membrane. Membranes were blocked for 1 h in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: anti-FGF18 antibody at 1:3000. The protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a FluorChem® image analyzer (ProteinSimple).

Microarray analysis

VSMCs were cultured in differentiation medium overnight followed by a further 72 h with or without 50 μg/ml Baicalein. Total RNA was isolated using TRIzol reagent (Life). RNA was column-purified using the RNeasy MidiKit (Qiagen, Valencia, CA) as the manufacturer's handbook, ethanol-precipitated overnight at 20°C, and resuspended in RNase-free water. RNA purity was assessed using the Agilent RNA 6000 Nano reagents and 2100 Bioanalyzer (Agilent, Santa Clara, CA). Microarray analysis was performed by keygen biotech, China, as per their standardized techniques, using a one-color system and the Agilent SurePrint G3 human gene expression array (44,000 gene array). Following co-hybridization, spots were scanned numerous times and signal intensities and the data from multiple scans, normalization, and background correction which were determined by GeneSpring GX 10 software (Silicon Genetics, Redwood City, CA). Differentially expressed genes were identified and considered significant with a fold change threshold of 10 using volcano plot analysis ($P \leq 0.001$ as determined by ANOVA).

siRNA transfection

VSMCs were plated in 0.5% FBS DMEM for 24 h and 72 h followed by incubation with 33 nM small interfering RNA (siRNA) for 12 h. Media was changed the next day and then the addition of the same concentration of baicalein (50 μg/ml) to different groups for 24 h and 72 h was performed. Scrambled siRNA: 5-CUUCCUCUCUUUCUCUCCCUUGUGA-3, was used as a control. The sequence for lncRNAAK021954 siRNA was 5-ACAUAGAAGCUAGGACUA-3 (Integrated DNA technologies). Lipofectamine 2000-mediated transfection was performed as per merchant's instructions (Invitrogen). Then we used qPCR to assess the target gene of siRNA,

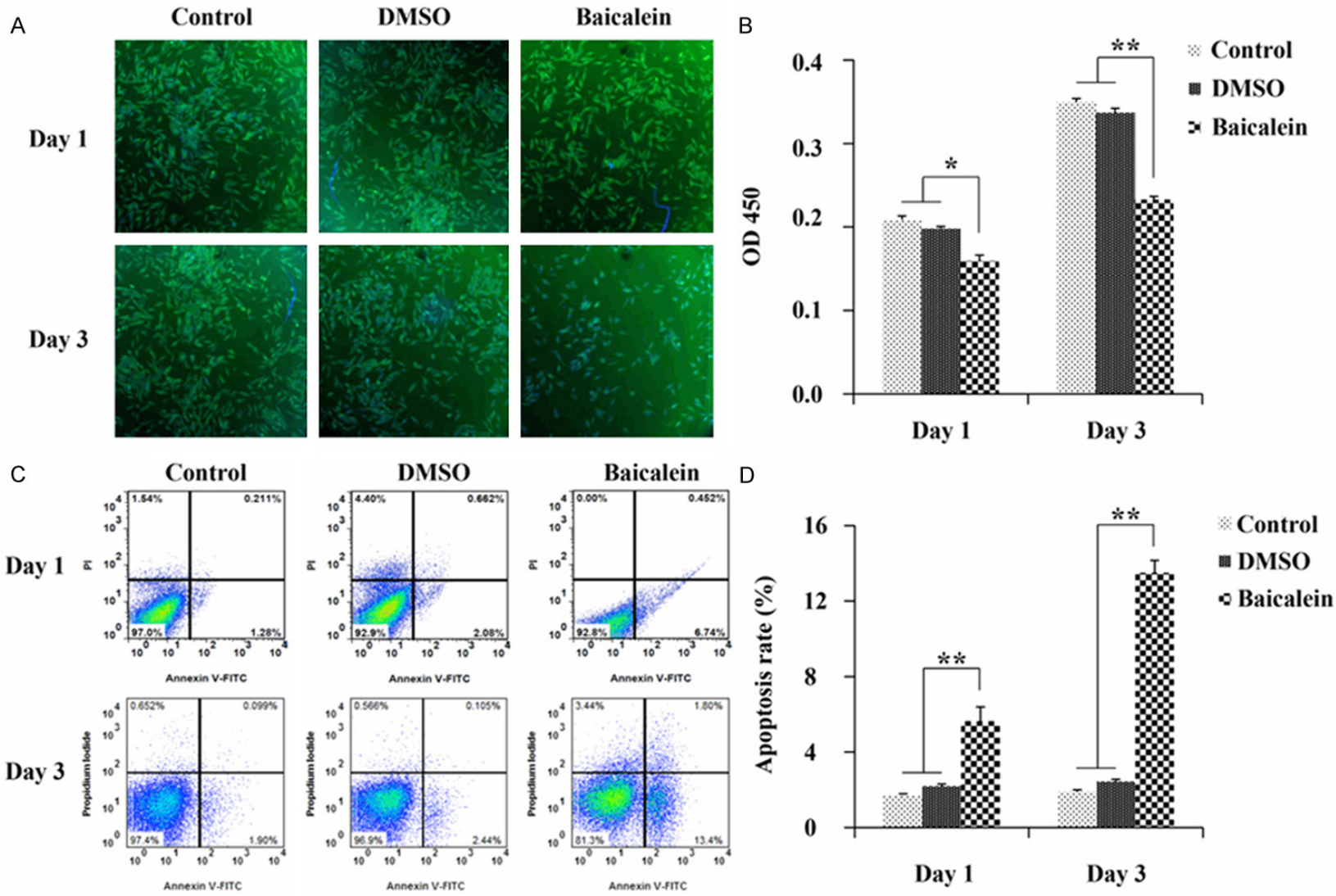

Figure 1. The proliferation and apoptosis behavior of VSMCs when treated with baicalein. A. The proliferation of VSMCs after treated with baicalein for 24 h and 72 h by FITC-phalloidine staining. B. The quantity analysis of VSMCs proliferation on Day 1 and Day 3 by CCK-8 test. C and D. The apoptosis behavior of VSMCs with baicalein treatment on Day 1 and Day 3 using flow cytometry.

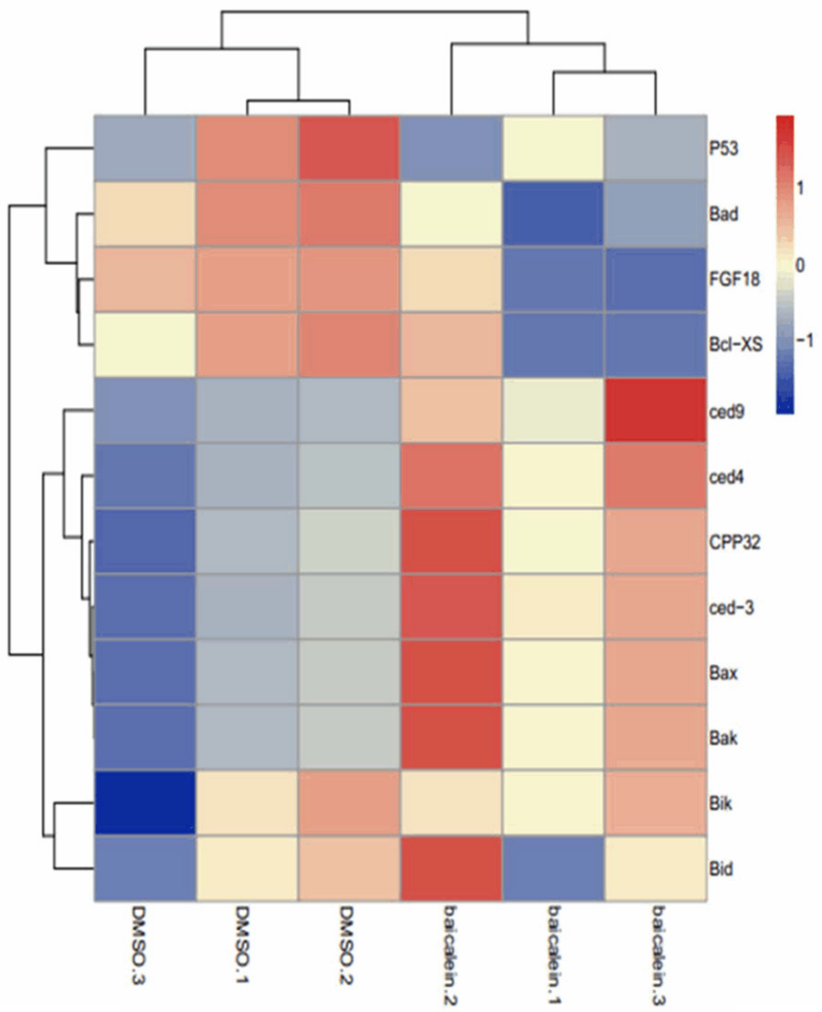

Figure 2. Data validation, and identification of FGF18 as a novel baicalein downstream target gene. Gene expression changes after VSMCs treated with baicalein (50 μg/ml) for 72 h.

siRNA was able to effectively suppressed lncRNAAK021954 expression.

Statistical analysis

The results are expressed as the mean ± SD of at least three independent experiments. For comparisons among multiple groups, statistical significance was determined using the one way analysis of variance (ANOVA) using SPSS version 12.0. Significance levels were set at: *P<0.05, **P<0.01.

Results

Baicalein suppressed VSMCs proliferation

We firstly observed the general inhibition effects of baicalein via immunofluorescent staining, as **Figure 1A** showed, the proliferation of VSMCs was distinct prohibited by baicalein on day 3 when compared with control and DMSO treated group. And CCK-8 assays results also demonstrated that baicalein significantly reduced cell proliferation compared with other two groups as shown in **Figure 1B**. At the end of the 24 h and 72 h growth study, cell viability in baicalein treated group was 33% and 31% lower than that in control and DMSO, respectively (P<0.05), (P< 0.01). Moreover, to determine whether baicalein induced apoptosis in VSMCs, flow cytometry analyses was performed as shown in **Figure 1C**. Corresponding graphs show fold change ± S.E in **Figure 1D**, the rate of apoptosis for 24 h was 6.74 ± 0.97% in baicalein, which was significantly higher than those of control (1.28 ± 1.26%) and dmso (2.08 ± 0.80%). Similarly, the rates for 72 h were 13.4 ± 0.97%, 1.90 ± 1.26%, 2.44 ± 1.26% in baicalein, control, DMSO (P<0.05). These results indicated that baicalein obviously suppressed VSMCs proliferation.

FGF18 as a downstream target gene involved inbaicalein inhibiting the proliferation of VSMCs

To further confirm genes expression, we treated VSMCs with baicalein for 72 h. In **Figure 2**, four genes, P53, Bad, Bcl-xs and FGF18 (top of the heat map), were down-regulated in the microarray data with baicalein treatment when compared with the control all time course, in which FGF18 was of the most obvious reduction. Otherwise another 8 genes (bottom of the heatmap) up-regulated (P<0.05).

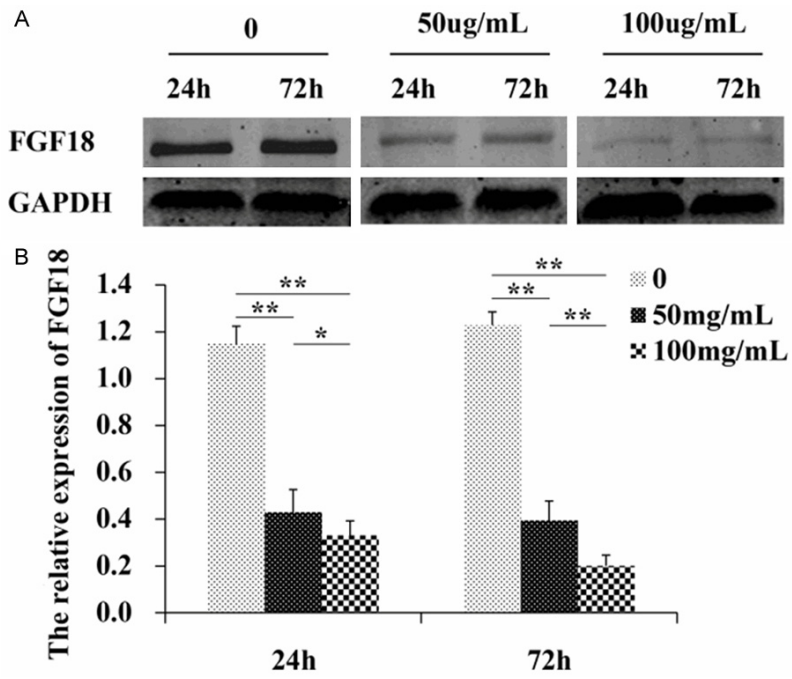

Figure 3. Effects of baicalein concentration on FGF18 protein expression in VSMCs. A. Expression levels of FGF18 protein were determined by Western blot analysis. B. qPCR analysis of FGF18 mRNA expression in VSMCs during proliferation course. Corresponding graphs show fold change ± S.E normalized to GAPDH from three independent experiments (*P<0.05, **P<0.01).

Baicalein inhibited VSMCs proliferation via regulating FGF18 protein expression in dose dependent manner

Attenuated FGF18 protein levels were detected at both proliferation time points (24-72 h) respectively following treatment with baicalein at 50 μg/mL and 100 μg/mL. The expression of FGF18 protein was significantly lower in the cells treated with 100 μg/mL than with 50 μg/mL baicalein. GADPH expression was analyzed to ensure equal loading of samples (**Figure 3A**). Subsequent qPCR results further confirmed the fact that baicalein induced a significant reduction in FGF18 mRNA expression in VSMCs in dose dependent manner (**Figure 3B**).

LncRNAAK021954 involved in the process of proliferation inhibition of VSMCs

To determine the molecular mechanisms of anti-proliferation of baicalein, we performed microarray analysis on RNA level collected from treatment and control groups across a proliferation time course. As shown in **Figure 4A**, Genes by more than 10-fold were selected for FDR at 0.01. The results of the microarray are summarized in a heat map (**Figure 4B**). Importantly, LncRNAAK021954 gene increased by the largest margin as target of baicalein in VSMCs (P<0.05) and subsequent confirmation through qPCR revealed the novel lncRNAAK021954 gene. Therefore, we proposed that baicalein-induced proliferation of VSMCs through a mechanism that lncRNAAK021954 involved in the inhibition of proliferation.

Interference LncRNAAK-021954 expression promoted VSMCs viability

To further confirm the role of lncRNAAK021954 in VSMCs proliferation, we next designed siRNA to silence target gene, subsequent analysis revealed that proliferation was enhanced following siRNA transfection in when compared with both scrambled siRNA plus baicalein and baicalein groups. As shown in **Figure 5A**, we found that the number of VSMCs was higher than the other two groups for 24 h by FITC-phalloidine staining and VSMCs were incubated with cck-8 solution for 72 h to assess the optical density (OD). The proliferation in the siRNA group was higher than the other two groups for 72 h (P<0.01). The change for 24 h, however, was not significant in the cell count program followed by transfection with siRNA (**Figure 5B**). Moreover we detected the apoptosis ratio with siRNA treatment for 72 h. The rate of apoptosis was 4.56 ± 0.97% in siRNA plus baicalein was lower than scrambled siRNA plus baicalein (10.1 ± 1.26%) and baicalein (12.6 ± 0.80%) (P<0.05) (**Figure 5C**). These results together indicated that lncRNAAK021954 silencing could rescue the effect of baicalein on apoptosis of VSMCs.

Discussion

The proliferation of VSMCs is a process subject to dynamic cardiovascular injury, and is closely associated with atherosclerosis. It had previ-

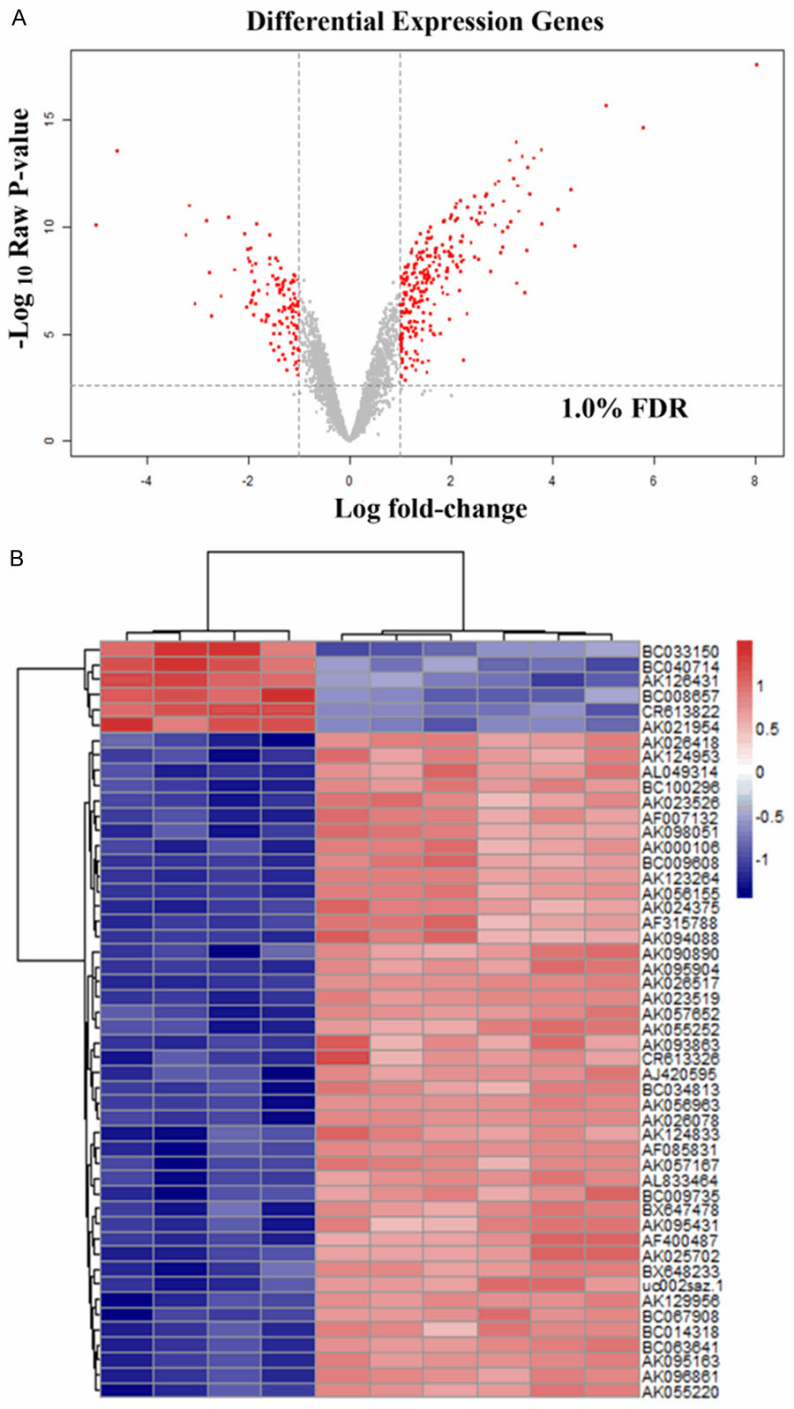

Figure 4. Identification of novel downstream targets of baicalein in VSMCs using volcano plot (A) and heat map analysis (B). 6 genes were significantly up-regulated after addition of baicalein. Each conditionis representative of data from three samples; the color scheme, which represents the -fold change, is indicated at the right side of heat map.

ously been investigated that baicalein had the anti-proliferative effect to VSMCs in vivo [11], which was involved in many kinds of signal paths and molecular behaviors such as restraining canonical transient receptor, cytochrome p-450 arachidonic acid and lipoxygenase (LOX) metabolites. Previous studies demonstrated that 12/15-LOX played a critical role in the development of atherosclerosis, hypertension, heart failure [27]. It was essential to explore the mechanisms underlying that baicalein, 12/15-LOX inhibitor, baicalein, significantly attenuated atherosclerosis development.

In the present study, we elucidated the association between FGF18 and proliferation of VSMCs with addition of baicalein. Importantly, microarray analysis was used to screen the long noncoding RNA after treatment of baicalein in inhibition of VSMCs proliferation.

Fibroblast growth factor 18 (FGF18) belongs to the FGF family and is most homologous to FGF-8 and FGF-17 among the FGF family. Many studies have extensively reported that FGFs have functions such as cell proliferation, migration, differentiation, and angiogenesis in various cells and tissues [28]. Studies have identified that FGF18 is an important signaling molecule in the regulation of articular cartilage, embryonic development, cell growth, morphogenesis, bone-cartilage tissue repair, tumor growth, and invasion [17], but its biological impact on VSMCs remain unknown. In addition, FGF18 is wildly expressed in the cardiovascular tissue [19], it has no direct evidence to support the function to VSMCs proliferation. In our study, with the pro-

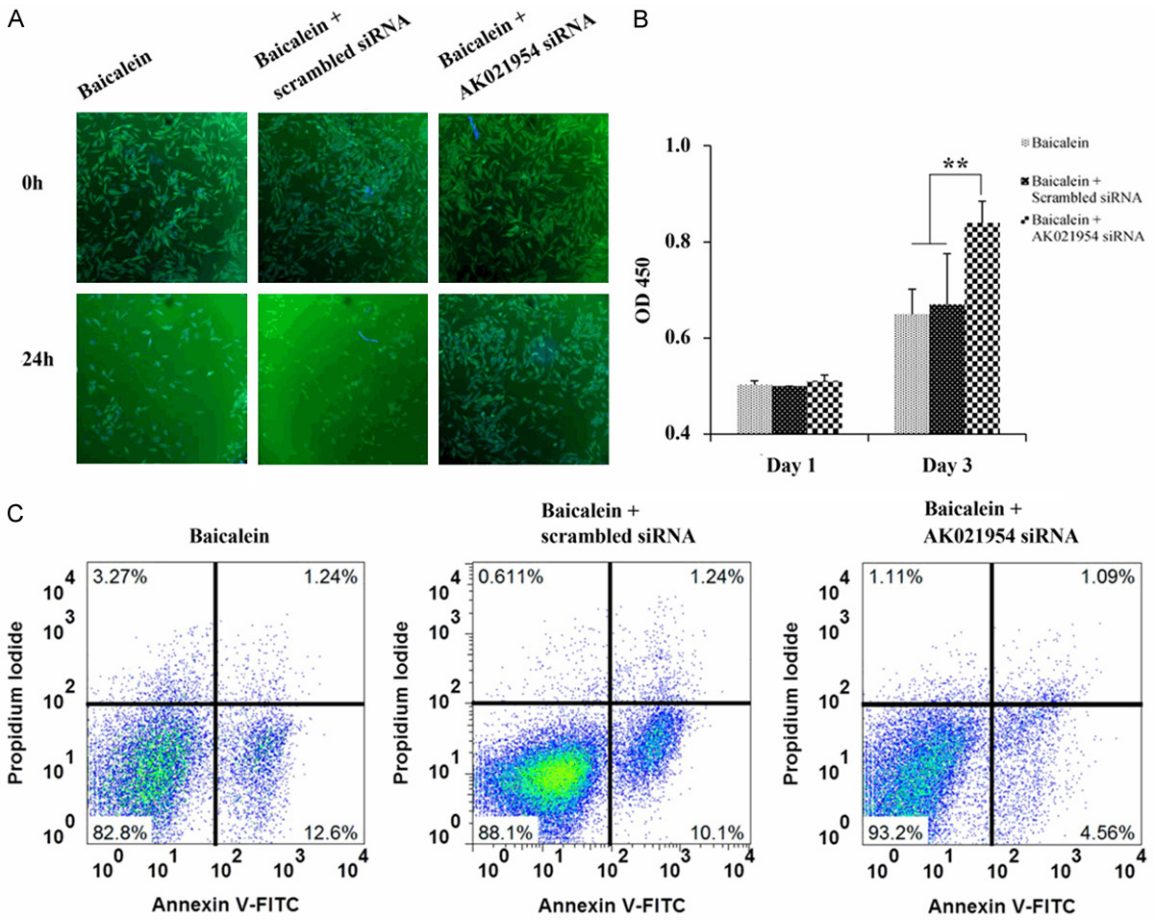

Figure 5. Interference LncRNA AK021954 expression promoted VSMCs viability. A and B. The effect of LncRNA AK021954 siRNA on the proliferation of VSMCs for 24 h or 72 h respectively, through FITC-phalloidine staining and cell count kit-8. C. To determine the effect of LncRNA AK021954 siRNA on apoptotic of VSMCs by flow cytometry.

liferation after treatment of baicalein was significantly decreased, FGF18 expression was down-regulated in a dosage dependent manner, which might be associated with cell proliferation.

LncRNA plays an important potential regulator of the progression of in several physiological and pathological conditions such as cancer and cardiovascular diseases, including atherosclerosis [23]. Wu G et al. reported that LncRNA-p21 as a novel regulator of cell proliferation and apoptosis in atherosclerosis mice model [29]. In our study, microarray analysis revealed lncRNAAK021954, a novel and significantly up-regulated gene, following addition of baicalein, whose function had been less reported and was involved in VSMCs proliferation. Subsequent verification by siRNA confirmed the hypothesis that baicalein regulated the proliferation of VSMCs through gene lncRNA-AK021954, the over-expression of lncRNAAK-021954 might become a considerable approach to treat atherosclerosis.

Conclusion

We propose that upon stimulation with baicalein, up-expressed target gene lncRNAAK-021954 inhibited the proliferation of VSMCs, whereas attenuated FGF18 level in dosage dependent manner. Although the association between FGF18 and lncRNAAK021954 was not investigated in the study, these data together suggest that baicalein may have therapeutic potential in anti-proliferative effect to atherosclerosis and may provide new scientific evidence to facilitate the clinical treatment of atherosclerosis.

Disclosure of conflict of interest

None.

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