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Original Article

Astragalus improve aging bone marrow mesenchymal stem cells (BMSCs) vitality and osteogenesis through VD-FGF23-Klotho axis

Xiang Pu*, Yihui Chai*, Liancheng Guan, Wen Li, Jie Gao, Zhibin Jiang, Qian Li, Yongzhen Wu, Yunzhi Chen

*Department of Preclinical Medicine, Guizhou University of Traditional Chinese Medicine, Guiyang 550025, Guizhou, China. *Equal contributors and co-first authors.*

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Abstract: To clarify the regulation of astragalus on the aging BMSCs model and the effect of astragalus on Vitamin D (VD)-FGF23-Klotho axis. siRNA was used to interfere the expression of VDR gene in aging BMSCs. Serum containing astragalus in different concentrations was added to the cultured cells. The expression of osteocalcin and alkaline phosphatase were detected by alizarin red staining and ELISA. Cell vitality was detected by flow cytometry, CCK-8 test, and *β*-galactosidase staining. The expression of FGF23, Klotho, CYP27B1, and CYP24A1 was detected by qRT-PCR and western blot. The results showed that after reducing VDR gene expression, the aging BMSCs model showed decreased activity and osteogenic ability, increased expression of FGF23, Klotho and CYP24A1, and decreased expression of CYP27B1. After adding serum-containing astragalus, the activity of cells and the osteogenic ability was increased; the expression levels of FGF23, Klotho and CYP24A1 were decreased, the expression levels of CYP27B1 were increased, and the trend was more obvious with the increase of astragalus concentration. This study confirmed that astragalus could inhibit the aging of BMSCs and improve the osteogenesis ability by regulating the VD-FGF23-Klotho pathway. This study provided a certain research basis for the therapeutic of traditional Chinese medicine (TCM) on primary osteoporosis.

Keywords: Astragalus, BMSCs, VD-FGF23-Klotho axis, aging, osteogenesis differentiation

Introduction

Primary osteoporosis is a degenerative disease characterized by low bone mass, bone microstructure destruction, and increased bone brittleness that causes bones to easily break with the continuous growth of age. Osteoporotic fractures cause great harm, high disability rates, and high mortality [1]. The number of people suffering from osteoporosis in mainland China has reached about 110 million. Osteoporosis has become a serious social and medical problem [2]. New prevention and treatment strategies are needed to improve the situation.

BMSCs are undifferentiated mesenchymal stem cells derived from mesoderm with infinite expansion and differentiation into nerve, muscle, and bone. It plays an important role in bone formation and repair. BMSCs differentiation ability of osteoporosis rats was significantly reduced after ovariectomy. The degree of femoral osteoporosis was improved after transplanting normal BMSCs [3]. In patients with osteogenesis insufficiency, BMSCs transplantation would promote bone growth, improve bone content, and reduce the incidence of breaks [4]. BMSCs of aging rats were blocked at G1 phase and the β-galactosidase staining positive cells were increased. The proliferation ability of cells was significantly decreased [5]. The multidirectional differentiation ability of BMSCs decreases with aging and this change is consistent with the phenotype of osteoporosis, which suggested that the biological function of BMSCS accelerates the generation of osteoporosis.

The VD-FGF23-Klotho axis regulates calcium and phosphorus metabolism in the body. VD precursor $25(OH)_2D_3$ generates $1,25(OH)_2D_3$

under the action of CYP27B in the kidney. $1,25(OH)_2D_3$ regulates VDR in bone, myocardium, and other tissues. CYP24A breaks down $1,25(OH)_2D_3$. VD, VDR, CYP27B, and CYP24A together constitute the VD axis (referred as VD/VDR). VD may directly stimulate calcium and phosphorus absorption, provide raw materials for bone mineralization, promote intestinal calcium absorption, and improve bone mineralization and osteoblast formation [6]. VD deficiency is an important factor in primary osteoporosis. VD plays an important role in treating primary osteoporosis [7]. Klotho is secreted by the kidney [8] and closely related to aging, while Klotho is also expressed in bone and other tissues. Knockdown of *Klotho* gene in mice showed decreased bone density, hypogonadism, and other aging manifestations [9]. Up-regulated Klotho gene expression can significantly improve bone metabolism and bone microstructure in castrated rats. FGF23 is an endocrine protein synthesized by bone cells and osteoblasts and is closely related to many bone metabolic diseases. FGF23 gene knockout mice also showed reduced bone density, shortened life span, and other aging manifestations [10, 11]. As a cofactor of fibroblast growth factor receptor in FGF23 signal transduction, Klotho binds to FGF receptor (FGFRs) and regulates phosphorus and calcium metabolism together with FGF23. FGF23 combined with FGFRs-Klotho down-regulated CYP27B gene, inhibited the synthesis of $1,25(OH)_2D_3$, up-regulated CYP24A, and promoted the decomposition of $1,25(OH)_2D_3$ [12].

Clinical and experimental studies have shown that kidney deficiency is an important pathogenesis of the occurrence and development of primary osteoporosis. Astragalus is an important traditional Chinese medicine. Astragalus has a good anti-aging effect. Clinical studies show that astragalus can effectively improve osteoporosis, but its molecular regulation still needs further study. Astragalus promotes bone formation and inhibits bone resorption in postmenopausal women. It is speculated that astragalus can regulate the aging process of BMSCS and play an anti-osteoporosis role, but its molecular mechanism needs to be further clarified. Our previous studies have shown that astragalus can regulate the contents of VD, FGF23, and Klotho in the serum of rats with osteoporosis model. Therefore, it is suggested that astragalus can regulate the FGF23-Klotho axis through VD/VDR to improve the aging BMSCs vitality and bone formation.

Materials and methods

Preparation of drug-containing serum

Fifteen male SPF SD rats were purchased from the Animal Center of Chongqing (Third Military Medical University), weight (200±50) g. The rats were on a caged free diet. The experiment was carried out after observing the general physiological conditions such as eating, activity, and urine were not abnormal.

Astragalus membranaceus: 36 g Astragalus membranaceus in 800 mL liquid medicine.

The rats were fed with Astragalus membranaceus for 7 d according to the dose of 10 g/kg. Two hours after the last gavage, the rats were anesthetized with abdominal aorta and incubated at 37°C for 30 min. The serum was separated by centrifugation and filtered through a 0.22 μm microporous membrane filter and stored at -20°C for use.

SiRNA screening and synthesis

Starting from the initiation code (AUG) of the coding region of VDR mRNA (NCBI Reference Sequence: NM_009504.4), three lines starting with the "AA" sequence, 21 nucleosides in length and GC content ranging from 45% to 55% were searched. The left and right base sequences serve as potential interference targets. The three siRNAs are $siRNA_1$, 5'-ACTTTGACCGGAATGTGCCTCGGAT-3'; $siRNA_2$, 5'-CCCTTCAATGGAGATTGCCGCATCA-3'; and siRNA3, 5'-GATCTTGTCAGTTACAGCATCCAAA-3'. The siRNAs were synthesized by Chongqing Biomedicine biotechnology co. LTD.

Isolation and inducing aging of BMSCs

The investigation was permitted by the Law of the People's Republic of China on Protecting Wildlife, and the protocol was approved by Guizhou University of Traditional Chinese Medicine, China. The femur and tibia of SD rats were taken, and the bone marrow was flushed out of the bone with 10 ml PBS and 100 u/ml heparin injectors. The cells were centrifuged at 1,000 rpm for 8 min. Cells were added to

DMEM (Gibco, USA) with 15% FBS (Gibco, USA), resuspended and placed in a 25 cm^2 plastic bottle (Corning, USA) to make MSCs adhere. The culture medium was changed after 3 days and non-adherent cells were discarded. The medium was replaced every 3 days. All experiments described below were conducted using MSCs from the third generation to the fifth generation. 0.03 g/L D-gal (Sigma, USA) was added to induce aging BMSCs according to previous studies [13, 14].

CCK8 test

Cells (1×10^4 cells/well) were seeded in 96-well plates and incubated at 37°C for 24 h, 100 μL per well, and 3 replicate wells per cell were set up. The 96-well plate was transferred to an incubator for cultivation (37°C, 5% CO_2). We added 10 μL of CCK-8 solution (Beyotime, China) to each well and incubated in the incubator for 1-4 h. The absorbance at 450 nm was measured with a microplate reader.

Flow cytometry

The cells were cultured in a 6-well plate to 60%-70%. After 14 d culture, the cells were aspirated into a 1.5 mL centrifuge tube and centrifuged at 2000 rpm for 5 min. The cells were washed twice with PBS, centrifuged at 2000 rpm for 5 min, and collected $1\text{-}5\times10^5$ cells. We added 1 mL of ice bath to pre-cool 70% ethanol, then mixed them gently by pipetting, and fixed at 4°C for 2 h or longer. Cells were centrifuged for 3-5 min at 1000 rpm to pellet the cells. We added 1 mL ice bath pre-chilled PBS and resuspended the cells. 0.5 mL of propidium iodide staining solution was added to each tube, and incubated at 37°C for 30 min in dark. This was detected by a flow cytometer at 488 nm.

Real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, USA) from cells according to the manufacturer's instructions. Then qRT-PCR was performed using a Real-time PCR System (Bio-rad, USA) with the SYBR Green PCR Kit (Takara, Otsu, Japan). The expression levels of nrf1 were normalized to β-actin.

Western blot analysis

Total proteins were extracted from cells using ice-cold lysis buffer, the extracted protein was quantified by BCA kit (Beyotime, China). Briefly, 30 ng/well proteins were separated by SDS-PAGE gels, then transferred into polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat powdered milk for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. After washing the PVDF membranes with TBST 3 times, the membranes were incubated with the secondary antibodies for 1.5-2 h at room temperature. The membranes were then washed 3 times again with TBST and washed with TBS for the last time. Protein expression levels were visualized using an enhanced chemiluminescence detection system.

β-galactosidase staining

After 14 days of culture, senescence-associated β-galactosidase (SA-β-gal) staining was performed according to the β-galactosidase staining kit (Beyotime, China). The cell culture solution was aspirated, washed with PBS 1 time, and added 1 mL of β-galactosylase staining fixative, fixed at room temperature for 15 minutes. The cell fixative solution was aspirated and the cells were washed with PBS 3 times, each time for 3 minutes. PBS was sucked out and 1 mL of staining solution was added to each hole. They were incubated overnight at 37°C, and the 6-hole plate was wrapped to prevent evaporation. Observations and photographs were completed under a microscope.

Alizarin red staining

After 14 days of culture, the cells were fixed with 70% ethanol for 1 h, washed 3 times with double steam, and dyed with alizarin red solution (Sigma, USA) for 30 min. After staining, it was washed 3 times with double steaming water and observed under a microscope.

Statistical analyses

Data are expressed as mean with standard deviation (SD). Analysis of variance (ANOVA) using Tukey's test was applied to compare the mean of each group with that of the control group. $P<0.05$ was considered to be statistically significant.

Result

Screen the optimal siRNA fragment

To reduce the expression of VDR gene, we synthesized 3 siRNAs to transfect BMSCS, and the

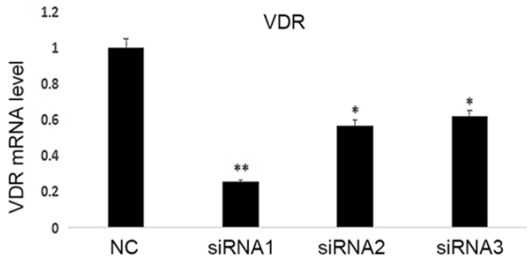

Figure 1. Interference effect of qRT-PCR detection of 3 siRNAs groups. NC: empty vector transfection group. * indicate P<0.05, ** indicate P<0.01.

expression of VDR gene was detected by qRT-PCR. The results showed that the VDR expression in three siRNAs treatment groups were 0.25, 0.57, and 0.62, respectively (**Figure 1**). Compared with the NC group, siRNAs treatment groups showed a decrease in mRNA expression. We selected siRNA1 as the best interference to carry out follow-up experiments.

Cell viability test

To investigate the effect of astragalus membranaceus on the viability of senile bone marrow mesenchymal stem cell model after VDR gene silencing, low dose (20%), medium dose (40%), and high dose (60%) drug-containing serum were added to the cells, respectively. $1,25(OH)_2D_3$, which is thought to delay the ageing of BMSCS, was also added. Cell viability was measured by β-galactosidase staining, flow cytometry, and CCK-8 assays. After 14 days of culture, the results of β-galactosidase staining showed that the SA-β-Gal positive rate of the negative fragment control (si-NC), positive interference (si-VDR), positive interference fragment plus 20% drug-containing serum (si-VDR/20%), positive interference fragment plus 40% drug-containing serum (si-VDR/40%), positive interference fragment plus 60% drug-containing serum (si-VDR/60%), and positive interference fragment plus $1,25(OH)_2D_3$ group were 33.50%, 55.21%, 41.22%, 34.41%, 18.13%, and 36.46%, respectively (**Figure 2A**, **2C**). After 14 days of culture, the percentage of the S phase cells of the six groups were 50.58%, 37.07%, 37.68%, 40.68%, 41.05%, and 45.32%, respectively (**Figure 2B**, **2D**). The viability of each group was detected by CCK-8 method at 12 h, 24 h, 48 h, 72 h, 7 d, and 14 d, respectively. Compared with the si-VDR group, the cell viability was increased after the addition of drug-containing serum. It increased with the dose (**Figure 2E**). Taken together, the above results indicate that cell viability is reduced after reducing VDR expression, and cell viability can be restored after the addition of drug-containing serum and increases with the dose.

Detection of osteogenic differentiation ability of cells

The osteogenic capacity of the five groups was measured. After induction of differentiation for 14 days, alizarine red staining showed that the osteogenic differentiation ability of cells was lost after reducing VDR. Several calcium nodules were formed after adding low-dose serum-containing drugs, and the number of calcium nodules was increased as the dose of serum-containing drugs increased (**Figure 3A**). ELISA was used to detect the contents of osteocalcin and alkaline phosphatase. It was found that their expression trend was consistent with that of alizarin red staining (**Figure 3B**, **3C**). However, western blot results showed no significant trend of osteocalcin expression in each group (**Figure 3D**), and qRT-PCR results were consistent with ELISA and alizarin red staining results (**Figure 3E**). The results showed that after reducing the VDR gene expression, the osteogenic ability of cells was decreased, and astragalus restored the osteogenic ability of cells, and the degree of recovery increased with the increase of astragalus dose.

Deteting the expression of FGF23, Klotho, CYP27B and CYP24A

Subsequently, FGF23, Klotho, CYP27B1, and CYP24A1 were detected. qRT-PCR results showed that after reducing VDR, the expressions of FGF23 and CYP24A1 were increased, while the expressions of Klotho and CYP27B1 were decreased. The expression levels of FGF23 and CYP24A1 were gradually decreased, and Klotho and CYP27B1 were gradually increased after adding different doses of drug-containing serum (**Figure 4A-D**). The expression levels of FGF23 and Klotho were detected by ELISA, and the trend was consistent with the results of qRT-PCR (**Figure 4E**, **4F**). In addition, western blot detected the protein expressions of FGF23, Klotho, CYP27B1 and CYP24A1, and the results showed that the

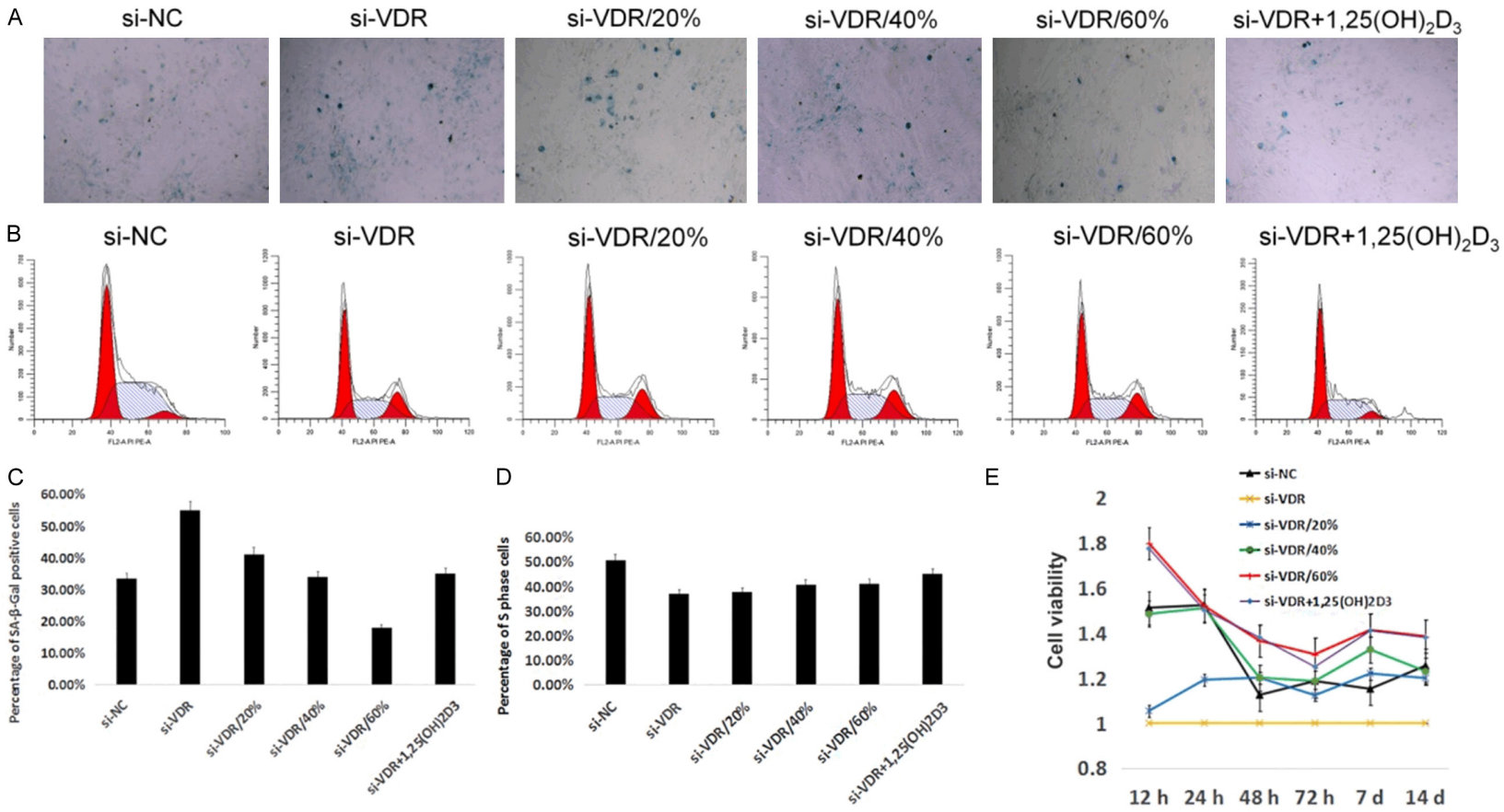

Figure 2. Cell viability detection. A. β-galactosidase staining. B. Cell cycle was detected by flow cytometry. C. Statistical results of β-galactosidase staining. D. Streaming results statistics. E. CCK-8 test results.

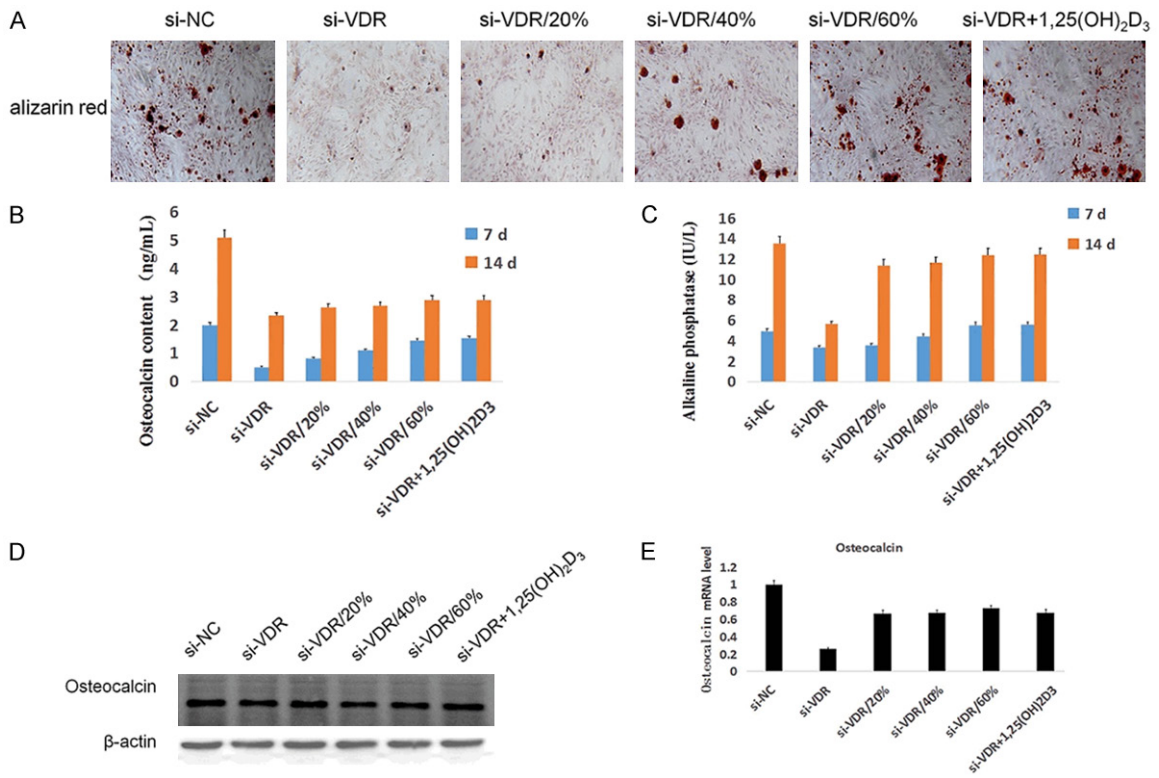

Figure 3. Detection of osteogenic differentiation ability of cells. A. Alizarin red staining. B. The content of osteocalcin was detected by ELISA. C. Alkaline phosphatase content was detected by ELISA. D. WB was used to detect the expression of osteocalcin. E. qRT-PCRr was used to detect the expression of osteocalcin mRNA.

protein expressions were consistent with the mRNA expression trend (**Figure 4G**, **4H**). Through the above experiments, it can be found that after VDR expression decreased, the FGF23 and CYP24A1 expression increased, the Klotho and CYP27B1 expression decreased, while astragalus could recover the gene and protein expression changes caused by VDR decrease.

Discussion

Cell aging is the cumulative result of changes in cell structure and function. It is functionally manifested as reduced oxidative phosphorylation, slowed respiratory rate, reduced enzyme activity and receptor protein, which leads to decreased cell function and inhibited cell proliferation. Its growth stops in the G1 phase, cannot enter the S phase, or stops in anaphase of mitosis [15]. In our research, it shows that the aging cells and the system of VDR gene expression interference will reduce the cell vitality, cell proliferation, and osteogenesis ability of differentiation. Osteocalcin and significantly reduce alkaline phosphatase expression, SA-β-Gal expression power increases. After adding serum-containing astragalus, with the increase of astragalus dose, cell proliferation differentiation viability was improved. Astragalus increased Klotho and CYP27B1 expression. In addition to regulating the balance of calcium and phosphorus in the body through circulation and maintaining the integrity of bones, VD, and FGF23-Klotho axis regulates the form of autocrine [16]. Studies have shown that the levels of VD and Klotho in the blood gradually decrease with age [17]. In vitro studies have shown that $1,25(OH)_2D_3$ can delay the aging of BMSCs, inducing osteogenic differentiation of BMSCs and the expression of Klotho and FGF23mRNA of bone aging genes, which has a certain anti-aging effect [18]. It is suggested that VD and VDR are closely related to the FGF23-Klotho axis and the aging of the body. VD regulates phosphate homeostasis and may be related to delaying aging and intervening the occurrence and development of age-related diseases [19]. Therefore, the FGF23-Klotho axis regulated by

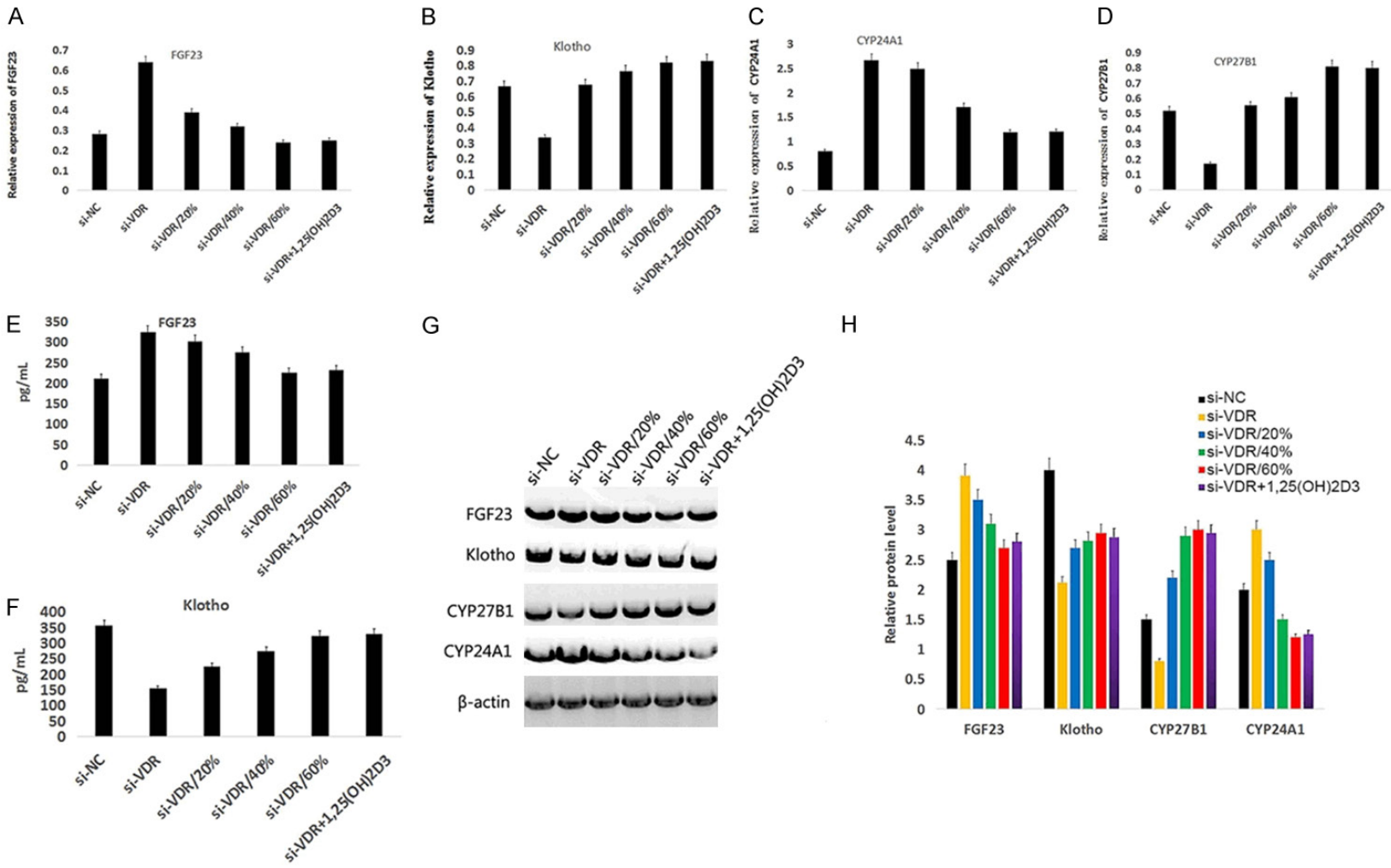

Figure 4. Deteting the expression of FGF23, Klotho, CYP27B1 and CYP24A1. (A-D) The expressions of FGF23, Klotho, CYP27B1 and CYP24A1 genes were detected by qRT-PCR. (E) FGF23 expression was detected by ELISA. (F) ELISA detected Klotho expression. (G) The protein expressions of FGF23, Klotho, CYP27B1 and CYP24A1 were detected by western blot. (H) The relative density of western blot in (G).

VD/VDR can be used as a therapeutic target for primary osteoporosis [20].

Cytological experiments showed that astragalus activate AKP, an important marker of osteoblast differentiation and maturation [21]. It can promote the metabolism and protein synthesis of BMSCs induced by culture, promote the proliferation of BMSCs and osteoblast differentiation, enhance the activity of ALP, and inhibit the loss of bone collagen and bone phosphorus to prevent osteoporosis [22]. Studies have shown that astragalus can delay aging by regulating cell cycle-related proteins and improving the expression level of Klotho gene [23]. The mechanism of astragalus on the VD axis was examined in this study. The experiment showed that astragalus could regulate the VD axis to affect the rat model of osteoporosis. The metabolic activity of VD is closely related to FGF23 and Klotho. We proposed that astragalus could regulate the bone formation process through VD-FGF23-Klotho. Experiments based on the above understanding, the efficacy of astragalus intervention was verified. The molecular mechanism of astragalus inhibiting the aging process of BMSCs and treating primary osteoporosis was clarified, providing a new research approach for the therapeutic mechanism of TCM on primary osteoporosis.

In our research, after reducing VDR gene expression, the aging BMSCs model showed reduced viability, decreased osteogenic capacity, increased expression of FGF23 and CYP24A1, and decreased expression of Klotho and CYP27B1. This study clarified that astragalus can inhibit the aging process of BMSCs by regulating the VD-FGF23-Klotho pathway and improve osteogenesis ability. Astragalus can restore the reduced function of BMSC caused by VDR gene silencing, and has a protective effect on BMSCs. It provides a basis for the research of traditional Chinese medicine on the treatment of primary osteoporosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yunzhi Chen, Department of Preclinical Medicine, GuiZhou University of Traditional Chinese Medicine, Guiyang 550025, Guizhou, China. Tel: +86-18798602065; E-mail: 1239638026@qq.com

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