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Original Article

Atorvastatin inhibits proliferation and calcification of rat heart valve interstitial cells via activating the mTOR signaling pathway

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Abstract: Accumulating evidence suggests that atorvastatin can activate the mTOR signaling pathways in a variety of cells, thereby inducing downstream response, but the mechanism is still unclear. Here, in order to investigate whether atorvastatin can inhibit proliferation and calcification of rats heart valve interstitial cells and the signaling mechanisms responsible for this effect. Rats heart valve interstitial cells were isolated and identified *in vitro* in the research and the cells were induced by calcified medium for calcification. SiRNA was used to inhibit the expression of mTOR protein and flow cytometry 6 was used to detect the cell cycle and apoptosis rate. The changes of calcium deposition were observed by Alizarin red S staining, and the numbers of calcified nodules in cells were calculated. Protein expression levels of S6K, p70S6K, osteocalcin and osteopontin in cells was detected by Western blot. Our results indicated that rat heart valve interstitial cells were isolated successfully. After the treatment, the cells grew slowly and apoptosis rate increased. There was no significant effect in the cell cycle after atorvastatin treatment. And atorvastatin treatment can significantly inhibit the cell from calcification. Knockdown of mTOR could significantly counteract the effect of atorvastatin. The atorvastatin can suppress the protein expression of S6K and the phosphorylation level of S6K protein significantly. The atorvastatin can inhibit the calcification which induced by calcified medium, but the knockdown of mTOR can reverse this trend. Our study showed that atorvastatin could slow down the proliferation of rat heart valve interstitial cells through activating mTOR, down-regulating levels of S6K, p70S6K. What's more, atorvastatin could inhibit rat heart valve interstitial cells from calcification through inhibiting the expression levels of osteocalcin and osteopontin.

Keywords: Atorvastatin, rat heart valve interstitial cells, cell calcification, cell proliferation, apoptosis

Introduction

Calcific aortic valve stenosis (CAVS) is a kind of multiple senile degenerative disease, and its incidence rate increases with the increase of age [1]. Calcification is one of the most common and important pathologic features of CAVS. In recent years, clinical studies have found that the specific gene expression related with osteoblast increased in the area of valve interstitial calcification in patients with degenerative aortic valve [2, 3]. Valve interstitial cells are activated in various pathogenesis of CAVS, and abnormal differentiation occurs, which indicates that calcification plays an important role in the occurrence and development of CAVS [4]. Thus, inhibiting its differentiation may become a new breakthrough point for the treatment of CAVS. Statins lipid regulating drugs can reduce the concentration of low density lipoprotein and increase the concentration of high density lipoprotein to bring beneficial effects to the patients [5]. In addition, its protective effect on the heart and other tissues is increasingly valued by people. Studies showed that atorvastatin can activate the mTOR signaling pathway in a variety of cells, thereby inducing downstream response.

mTOR (mammalian target of RAPA) is a highly conserved protein kinase in eukaryotic cells, and it is also a target material of immunosuppressive drugs and anticancer drugs [6]. mTOR is a central control factor of cell growth.

It makes the corresponding response according to the nutritional condition of the cell environment and involves in regulating the activities of protein kinase and protein phosphatase, so as to control the expression of protein synthesis and related transcription gene [7]. Hence, it plays a key role in the regulation of cell growth and proliferation, and it is closely related to the cell cycle [8]. mTOR affects gene transcription and protein synthesis by modulators such as p70S6K and 4E-BP1 to regulate cell growth [9]. The occurrence of most diseases is closely related to the abnormal regulation of mTOR signaling pathway. mTOR signaling pathway can make the corresponding response according to the nutritional conditions of the cell environment and involves in regulating the activities of protein kinase and protein phosphatase, so as to control the expression of genes related to protein synthesis [10]. Hence, it plays a key role in the regulation of cell growth and differentiation. The occurrence of most diseases is closely related to the abnormal regulation of mTOR signaling pathway [11]. In terms of tumor, closely-related physiological processes such as cell proliferation, differentiation and so on are regulated by mTOR. Inhibition of mTOR pathway can effectively block the transduction of abnormal signal of various growth factors and inhibit the occurrence and development of cancer [12]. Recent studies have indicated that mTOR activation is closely related to atherosclerosis, myocardial fibrosis, myocardial hypertrophy, myocardial ischemia and other cardiovascular diseases [13-17]. However, there are few reports in the study of CAVD. In this study, the mechanism of action of atorvastatin in the proliferation and calcification process of rat heart valve interstitial cells was studied by culturing rat heart valve interstitial cells in vitro and inducing cell calcification. It provides a theoretical basis for the development of new drugs of relevant diseases like senile degenerative diseases.

Materials and methods

Reagents

Atorvastatin was purchased from American Sigma Company. The primary antibodies (osteocalcin, osteopontin and GAPDH) in the Western Blot detection were purchased from Abcam (Abcam, USA). Alizarin red S staining reagents were purchased from American Sigma Company. The reagent kits used in BCA protein concentration detection were purchased from Nanjing KeyGEN Bio TECH Corp. Ltd. Calcified culture medium compositions (10 mmol/Lβ-glycerophosphate, 50 μg/ml vitamin C, and standard culture medium of 100 nmol/L dexamethasone) were purchased from American Sigma Company. DMEM culture medium and fetal bovine serum were purchased from GIBCO of the United States. PBS buffer solution: NaCl 8.0 g, KCl 0.2 g, KH_2PO_4 0.24 g, $Na_2HPO_4 \cdot 12H_2O$ 3.628 g were dissolved in 800 ml distilled water, adjust the pH value to 7.4 with hydrochloric acid, set the constant volume of distilled water to 1,000 ml with high-pressure sterilization and preservation in room temperature. TBS (Tris•HCl buffer salt solution): add 8.8 g NaCl to 10 ml of 1 mol/LTris•HCl, adjust the pH value to 7.4 with hydrochloric acid, set the constant volume of distilled water to 1,000 ml.

Cell culture and treatment

Rat heart valve interstitial cells were isolated in vitro by collagenase digestion and passage cultured in DMEM culture medium supplemented with 10% of fetal bovine serum, 1% of L-glutamine, 100 mg. L-1 streptomycin, 1×10^5 U. L-1 penicillin. The cells were maintained at 37°C in the incubator of 5% CO_2. After the cells reached sufficient numbers, detect the expression of α-SMA (α-smooth muscle actin) and vimentin by immunofluorescence method for cell identification. After identification, the cells were divided into 4 groups: the normal control group without any treatment, atorvastatin group (cells were cultured with 10 μmol/L atorvastatin), mTOR group (cells were treated by siRNA mTOR), mTOR + atorvastatin group (cells were treated by siRNA mTOR, and then were cultured with 10 μmol/L atorvastatin). For calcification study, cells were induced and cultured for calcification with calcified culture medium. The mTOR siRNA were purchased from Ribobio (Guangzhou, China). TurboFect transfection reagent (Thermo scientific, USA) were used for cell transfection according to the manufacturer's instruction. Cells were harvested 24 h after for the experiments as mentioned above, and transfection efficiency was evaluated with Western Blot analysis, cells exposed to the calcification and atorvastatin treatment.

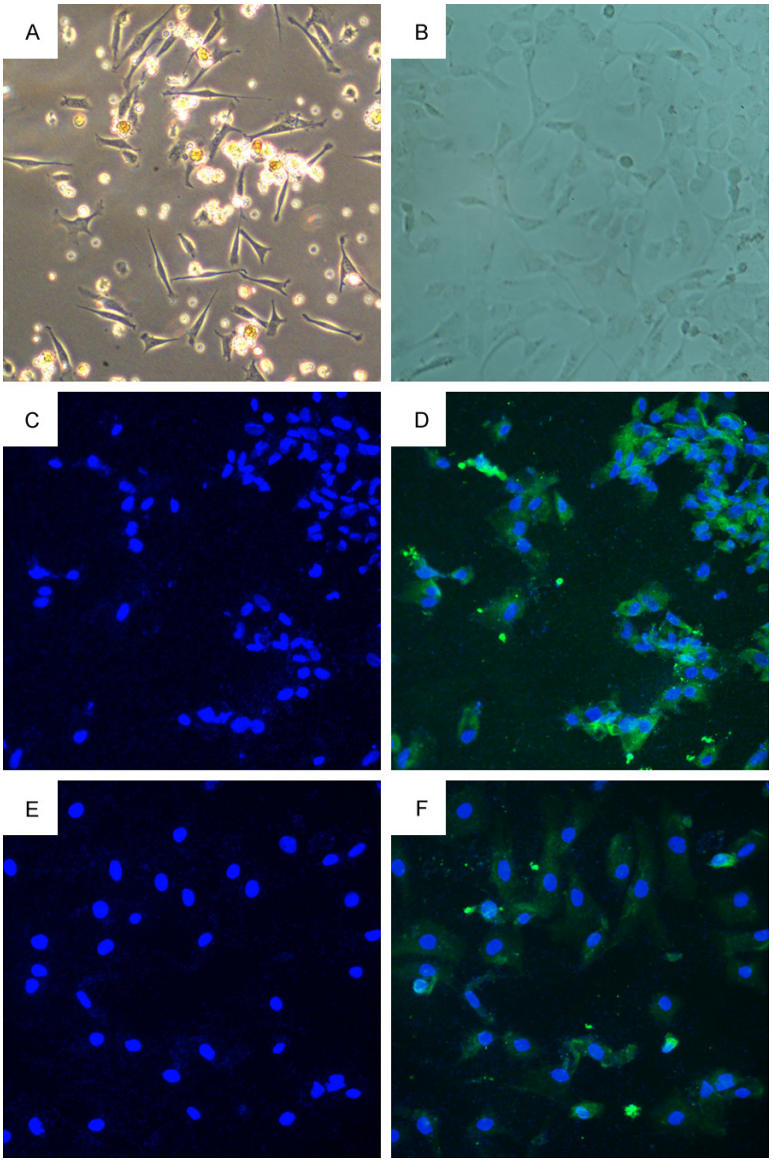

Figure 1. Isolation and identification of ratcardiac valve interstitial cells. A: Primary rat cardiac valve interstitial cells. B: The third generation of rat cardiac valve interstitial cells. C, D: The marker protein of rat cardiac valve interstitial cell. Green indicate the protein expression of vimentin. E, F: The marker protein of rat cardiac valve interstitial cell. Green indicate the protein expression of α-SMA.

Flow-cytometric analysis of apoptosis

The change of apoptosis rate was detected by flow cytometry to verify whether atorvastatin inhibiting cell calcification is related to the change of apoptosis rate. Cells were treated with atorvastatin for 24 h. Each group took 3 holes with 0.25% trypsin digestion. The cells were collected 5 min after 1,000 r/min centrifugation. Wash 2 times with PBS washing liquor. The cells were suspended in the buffer solution in reagent kits of PI-Annexin v apoptosis double staining with the concentration of 1×10^6 cells/ml. 400 mesh sieves could accurately draw 0.5 ml cell suspension solution into the sample tube. Then 5 μL Annexin v-FITC and PI dye liquor were added. After dark staining at room temperature for 10 min, conduct the detection. Obtain 1×10^4 cells by CellQuest software, and analyze the percentage of living cells, early apoptotic cells and dead cells.

MTT assay

Cells growth was in good condition, and the cells fusion rate reached 85%. According to the detection time, MTT mother solution was added into the cells in each group (to a final concentration of 0.5 mg/ml). Continue to culture the cells for 4 h and then discard the culture solution. 150 μL DMSO was added to each hole and shocked until the MTT crystals dissolved. The absorbance at 450 nm was measured with enzyme marker for 7 consecutive days.

Alizarin red S staining

According to the above group, rat heart valve interstitial cells were cultured for 7 days with calcified culture medium. And the cells were fixed for 1 h with 70% ethanol and washed for 3 times with double distilled water. Then the cells were stained for 30 min with Alizarin red solution. After being stained, the cells were washed for 3 times with double distilled water, and were induced by calcification for 7 days. It was found the formation of calcium nodules in rat heart valve interstitial cells by Alizarin red S staining. The 5 holes were randomly selected for comparison from each experimental group, and

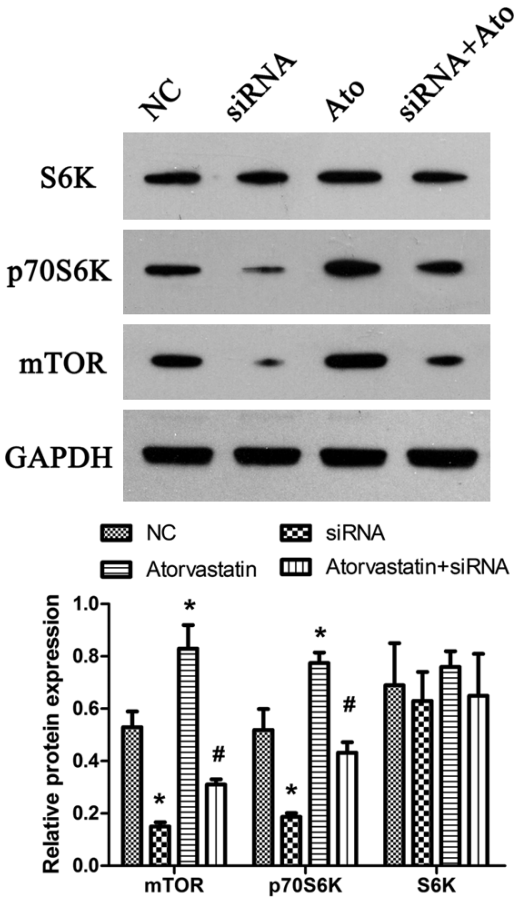

Figure 2. The protein expression of mTOR, S6K and p70S6K in rat cardiac valve interstitial cells. NC, the normal control group without any treatment; siRNA, cells were treated by siRNA mTOR; Ato, cells were cultured with 10 μmol/L atorvastatin; siRNA + Ato, cells were treated by siRNA mTOR, and then were cultured with 10 μmol/L atorvastatin. Compared with NC group, *P<0.05; compared with atorvastatin group, #P<0.05.

they were observed under the microscope. Meanwhile, the calcium nodules were counted.

Western blot analysis

Rat heart valve interstitial cells were cultured for 7 days, and the expression levels of S6K, p70S6K, osteocalcin, osteopontin in cultured cells were detected. According to the operation method of reagent kits for the protein extraction provided by TAKARA, the total protein of rat heart valve interstitial cells was extracted and quantified. After SDS-PAGE electrophoresis, transfer with PVDF film and incubate with antibody. Block with 5% skim milk powder-TBS sealing and incubate with first antibody (1:1,500) at room temperature for 2 h. Wash PVDF film with TBS for 5 min*5 times, and incubate with second antibody (1:5,000) at room temperature for 1 h. Then wash PVDF film with TBS for 5 min*5 times. Conduct the chemiluminescence, developing and fixing, and Quantity One v4.4.0 software was used to analyze the light density of the corresponding bands of the target protein and GAPDH.

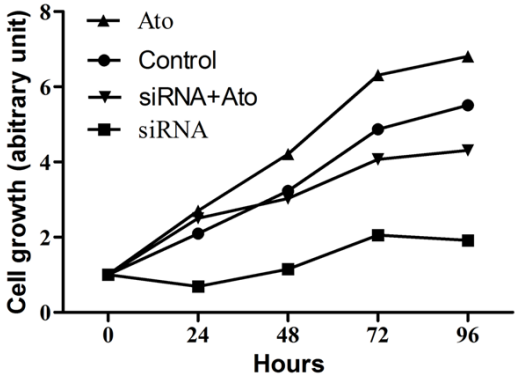

Figure 3. Effect of mTOR and atorvastatin on proliferation of rat cardiac valve interstitial cells. MTT assays were performed to determine the proliferation in rat cardiac valve interstitial cells. Experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 and GraphPad Prism 5 software. Statistical data represent mean ± sem and were determined using single factor analysis of variance. Comparisons between the two groups were performed using a student-test. P<0.05 was considered statistically significant.

Results

Rat heart valve interstitial cells were isolated successfully

The primary culture of cardiac valve tissues from rats was extracted and then passage cultures to verify whether the passage cells were the target cells. In **Figure 1A**, the cells were spindle shaped, fibroblast-like, and no special growth pattern, which indicated that rat heart valve interstitial cells were isolated and cultured successfully. In **Figure 1B**, **1C**, Vimentin and α-SMA (marker for smooth muscle cells) were both positive expressions, which proved

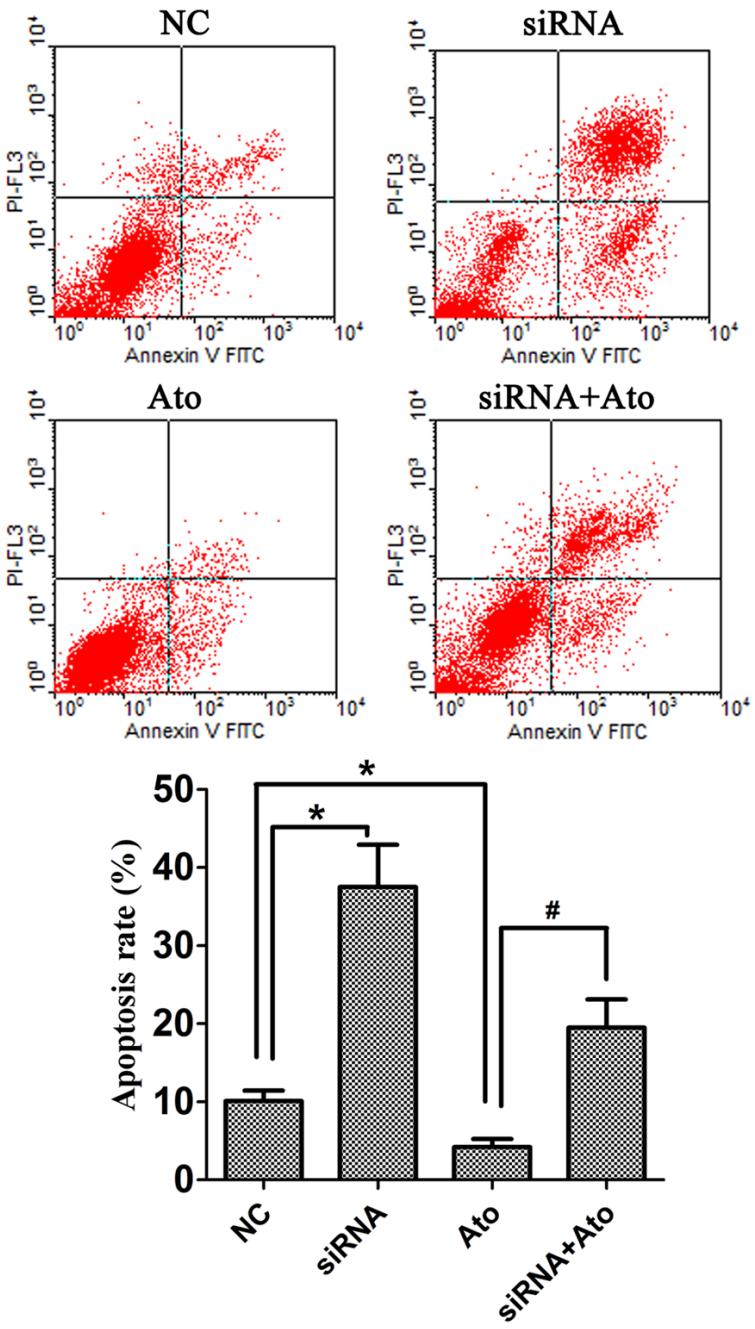

Figure 4. Effect of mTOR and atorvastatin on apoptosis of rat cardiac valve interstitial cells. Apoptotic rates were detected through flow cytometry in rat cardiac valve interstitial cells. Experiments were performed in triplicate. Compared with NC group, $^{*}P<0.05$; compared with atorvastatin group, $^{\#}P<0.05$.

that the isolated and cultured cells were the target cells.

Atorvastatin activate mTOR signaling pathways

p70S6K is an important downstream node in the mTOR pathway, and the expression levels of mTOR and p70S6K were detected with Western Blot (**Figure 2**). Compared with normal group, the expression of mTOR and p70S6K were significantly increased in atorvastatin treatment group ($P<0.01$). However, there was no significant difference between normal group and mTOR knockdown group. This results indicated that atorvastatin activated the mTOR signaling pathway.

Atorvastatin induce the growth of rat heart valve interstitial cells

The effect of atorvastatin on cell activity was discussed in cell growth curve of MTT detection. In **Figure 3**, cell activity in atorvastatin treatment group was significantly higher than that of normal group. However, the knockdown of mTOR counteracted the effect of atorvastatin in rat heart valve interstitial cells proliferation. These results showed that atorvastatin significantly induced the activity of rat heart valve interstitial cells.

Atorvastatin suppress the apoptosis of rat heart valve interstitial cells

The apoptosis of rat heart valve interstitial cells was detected with flow cytometry, and the results showed that atorvastatin inhibited the apoptosis of rat heart valve interstitial cells significantly. However, there was no significant difference between normal group and mTOR knockdown group (**Figure 4**). This further indicated that the atorvastatin can affect the differentiation process of rat heart valve interstitial cells by targeting mTOR signaling pathway. For the more, our results showed that atorvastatin had no significant effect on the cell cycle.

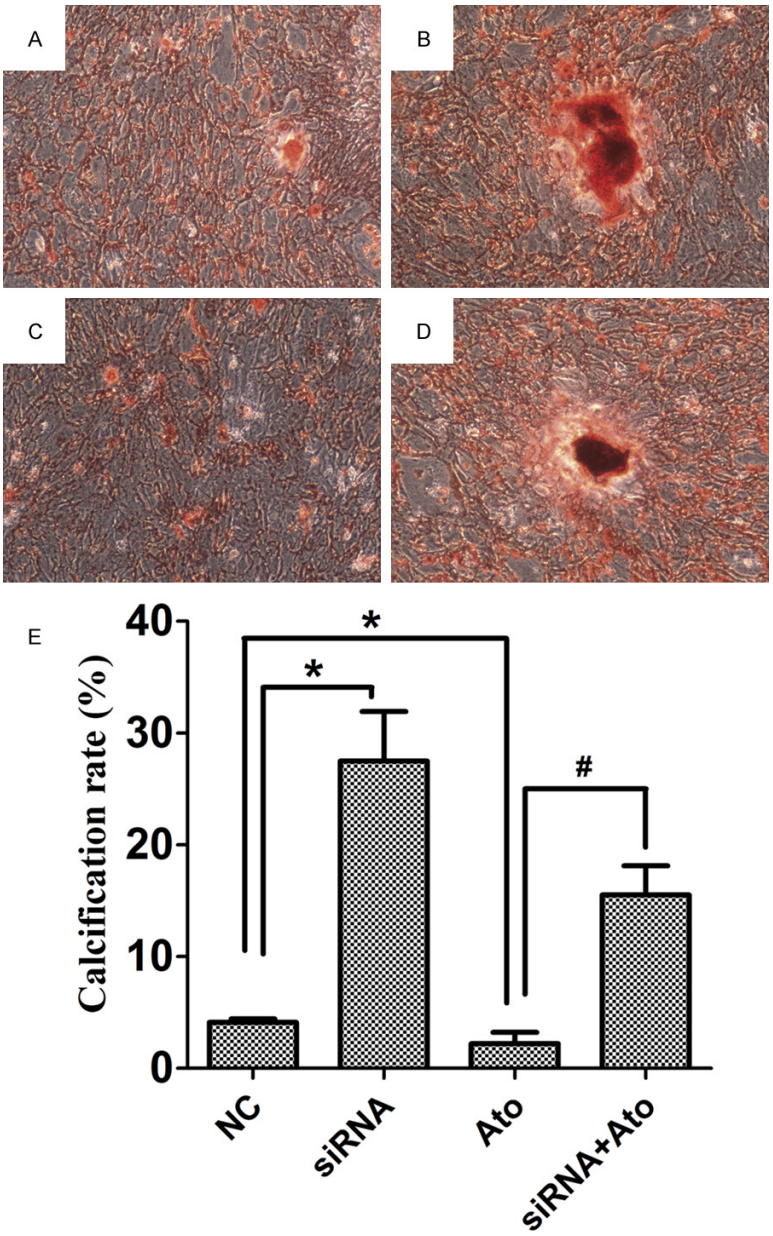

Figure 5. Effect of mTOR and atorvastatin on calcification of rat cardiac valve interstitial cells.The formation of calcium nodules in rat cardiac valve interstitial cells by Alizarin red S staining. A. NC, the normal control group without any treatment; B. siRNA, cells were treated by siRNA mTOR; C. Ato, cells were cultured with 10 μmol/L atorvastatin; D. siRNA + Ato, cells were treated by siRNA mTOR, and then were cultured with 10 μmol/L atorvastatin. E. Compared with NC group, $^{*}P<0.05$; compared with atorvastatin group, $^{\#}P<0.05$.

Atorvastatin inhibit the calcification of rat heart valve interstitial cells

Alizarin red S staining assay showed that the count of calcified nodules in calcified group was significantly higher than that in normal control group ($P<0.05$). After treatment with atorvastatin, the nodule count was significantly less than that in the calcification group. However, the knockdown of mTOR counteracted the effect of atorvastatin in rat heart valve interstitial cells calcification (**Figure 5**). This result indicated that atorvastatin could inhibit the calcification of rat heart valve interstitial cells by activating mTOR signaling pathway.

Atorvastatin inhibit the expression of osteogenesis related protein in rat valvular interstitial cells

In order to explore the relationship between atorvastatin and mTOR in rat heart valve interstitial cells calcification, the expression of osteogenesis related protein in rat valvular interstitial cells were detected with Western Blot. The results showed that the expression of osteocalcin and osteopontin in calcification group were significantly higher than those in normal control group ($P<0.05$). After atorvastatin treatment, the expression levels were significantly lower ($P<0.05$). However, and the expression of osteocalcin and osteopontin increased significantly after mTOR knockdown (**Figure 6**). This result indicated that atorvastatin could down-regulate the expression of osteocalcin and osteopontin by activating mTOR signaling pathway.

Discussion

Aortic valve calcification can cause aortic stenosis and aortic insufficiency, which is an important pathological change in senile degenerative valvular disease [18]. Valve calcification was widely thought to be a passive process of calcium phosphate deposition, and it is now clear that the process is a widespread and

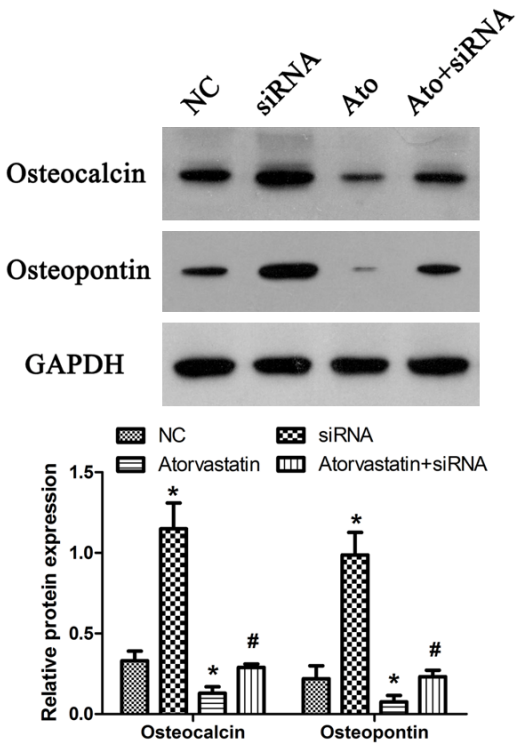

Figure 6. Effect of mTOR and atorvastatin on the expression level of osteogenesis related protein. The relative protein expression of osteocalcin and osteopontin were detected in rat cardiac valve interstitial cells. Compared with NC group, $^{*}P<0.05$; compared with atorvastatin group, $^{\#}P<0.05$.

highly conserved cellular pathway mediated by the active process [19]. In the previous studies, it was found that the abnormal proliferation and calcification of valve interstitial cells play a key role in the development of CAVD [20, 21]. Therefore, inhibition of proliferation and calcification of valve interstitial cells has become a new breakthrough point for the treatment of CAVD [22]. In this study, valve interstitial cells were isolated and cultured from rat aortic valve by collagenase digestion method. β-glycerol phosphate culture medium was used to induce calcification of rat heart valve interstitial cells *in vitro*.

Under physiological and pathological conditions, the function of interstitial cells of aortic valve is regulated by many factors, among which the mTOR family is an important signal pathway to regulate cell growth [23-25]. MTOR is the target material of atorvastatin (an immune inhibitor) in mammalian cells. It is a highly conserved protein kinase and central control factor of cell growth [26]. It can regulate the expression of genes related to protein synthesis by regulating the activity of phosphatase protein and kinase protein, and make the corresponding response according to the nutritional conditions of the surrounding environment of the cell growth [27-29]. Our early experimental results showed that, in the valve tissues of patients with Aortic calcification stenosis valvular disease, the phosphorylation of S6K (mTOR downstream substrate ribosomal protein) was significantly higher than that in normal valve tissues, indicating the abnormal activation of mTOR in CAVS valve tissues. Therefore, it can be inferred that the activation of mTOR signaling pathway involves in the occurrence and development of calcified aortic valve stenosis.

Early clinical studies have indicated that statin drugs have a unique therapeutic effect on patients with concomitant coronary artery calcification and osteoporosis [30, 31]. Statin drugs not only inhibit the formation of osteoclasts and bone resorption, but also promote the formation of new bone. Studies have also indicated that the pharmacological effects of atorvastatin are closely related to the mTOR signaling pathway [32, 33]. In the present study, we found that the effect of atorvastatin on the inhibition of calcification was significantly affected by mTOR expression (siRNA inhibition) in aortic valve interstitial cells. Thus, we conclude that atorvastatin inhibits the calcification of rat heart valve interstitial cells by activating the mTOR signaling pathway. Whether mTOR is a direct target material of atorvastatin is still unknown and needs to be explored in the future.

In conclusion, our study showed that atorvastatin inhibited the activation of mTOR signaling pathway in the mechanism of calcification of rat heart valve interstitial cells.

Acknowledgements

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Disclosure of conflict of interest

None.

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