Rock2 Regulates Proliferation and Differentiation of Chondrocytes in Osteoarthritis through β-catenin Signaling

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Research Article

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Abstract

Background

Osteoarthritis (OA) adversely affects quality of life of elderly patients and is among hotspots and challenges of current research efforts. However, mechanism of occurrence and development of OA has not been fully elucidated.

Methods

Through qRT-PCR and Western blot assays, the current study established that levels of Rock2, a key protein in Rho signaling pathway, were significantly higher in OA cartilage. Furthermore, the current study explored effect of down-regulating Rock2 expression on growth and apoptosis of cartilage cells using CCK-8, Edu and Flow cytometry assays. Alkaline phosphatase (ALP) and Alizarin Red S (ARS) assays were then used to determine differentiation effects.

Results

Findings showed that expression of Rock2 is closely related to proliferation, apoptosis and differentiation of chondrocytes. Furthermore, the current study confirmed that Rock2 affects growth and differentiation of chondrocytes by activating β-catenin signaling pathway.

Conclusion

the current study provided novel insights to targeted therapy of OA.

Introduction

Previous studies aver that osteoarthritis (OA) is the most common degenerative joint disease[1], which is characterized by degradation of extracellular matrix (ECM) or progressive loss of cartilage[2, 3]. With rising number of elderly population, number of patients with osteoarthritis is increasing, which adversely affect quality of life of elderly patients. In addition, more young people suffer from serious knee injuries due to excessive pressure caused by increasing antagonistic physical exercise[4]. However, mechanism of regulating OA pathogenesis remains unclear due to complicated combination of metabolic, mechanical, inflammatory and genetic factors. Therefore, in addition to palliative pain control, there is urgent need for similar physical therapy or prosthetic joint replacement. However, there is no effective drug therapy target, necessitating search for effective molecular targets to provide more theoretical basis for targeted treatment of OA. Cartilage tissue and cells play important roles in progress of osteoarthritis[5, 6]. Several previous studies and clinical data show that cartilage cells are broken in patients with osteoarthritis, and synthesis of extracellular matrix leads to dysfunction of articular chondrocytes, which, in turn, promotes degradation of ECM. This eventually leads to loss of ECM and cartilage degradation[7]. Therefore, elucidating molecular mechanisms of articular chondrocytes in progress of OA is key to progress of OA treatment.

Previous studies reported that Rho family related genes act as protein molecular switch and play key roles in cellular signal transduction[8]. Rho kinase (Rock) is among most important downstream target effector proteins of Rho subfamily. Previous studies reported that Rock2 is involved in many cellular biological activities, including cell morphogenesis, motility, cell division, proliferation, migration and cell adhesion. Rock2 is activated by interaction with Rho /GTPase and regulates actin cytoskeleton through some molecular signaling pathways, thereby affecting cell growth and differentiation[9]. Previous studies have also established that Rock2 plays important roles in smooth muscle and nerve regeneration as well as in neuronal development[10]. Furthermore, recent studies have established that Rho signaling pathway protein plays important roles in progress of osteoarthritis[11], but its mechanism is still unclear. Previous studies aver that Wnt/ β-catenin signaling pathway plays important roles in osteoblast maturation and bone formation[12, 13], which is the focus of OA research. Studies have shown that Wnt pathway is inhibited in normal cartilage, and its activation promotes OA and Wnt family to regulate bone mass. In addition, some previous studies have established that Wnt/β-catenin signal-induced chondrocyte hypertrophy and differentiation, followed by OA aggravation and apoptosis due to β- catenin inactivation may complicate normal bone development[14, 15]. However, mechanism of Wnt/β-catenin signaling pathway regulation in OA needs to be explored further.

The current study explored Rock2 expression in OA chondrocytes and its effects on proliferation and apoptosis of chondrocytes. In addition, the current study demonstrated role of Rock2 in OA and mechanism of Wnt/β-catenin signaling pathway in regulation of OA chondrocyte proliferation and apoptosis. The current study, therefore, provided new insights for OA treatment.

Materials And Methods

Clinical samples

A total of 15 specimens were collected from patients who underwent total knee arthroplasty (TKA), with age distribution ranging between 58-75 years. In addition, a total of 15 normal cartilage specimens were collected after traumatic fracture. All patients signed informed consent before sample collection. All specimens were stored at -80^{o}C immediately after collection.

qRT-PCR

Total RNA from human OA cartilage and normal cartilage tissues were extracted based on TRIzol^{TM} reagent (Invitrogen, USA). TRT-PCR and real-time fluorescent quantitative PCR were then undertaken using PrimeScript RT reagent kit and SYBR Green PCR kit (Takara, Japan), respectively.

Western Blot

Samples of human OA cartilage tissue, normal cartilage tissue and chondrocyte line were collected, from which cell and tissue proteins were extracted using cell lysate and tissue extraction kit. After quantitative analysis using BCA method, protein buffer was added and boiled in boiling water for 10 min. Same

amount of protein (40 μg) was separated using 10% SDS - PAGE gel. The first antibody including Rock2 (ab-71598) was used to incubate overnight based on manufacturer's instructions, and second antibody of corresponding species was then used to incubate. Membrane was washed using 1 x TBST, and specific antibody interaction was then observed using ECL luminescent reagent. Expression of ROCK2 protein was finally observed using chemiluminescence gel imaging analyzer.

CCK-8 assays

CCK-8 method was used to detect and analyze cell activity in treatment and control groups. 100μL chondrocytes suspension was dispensed in 96 well plate and preincubated for 24 hours (37°C, 5% CO_2). Cells were then treated with inhibitor and plates were then incubated for 24, 48, and 72 h. 10μl CCK8 solution was then added and incubated for 2 h. Absorbance of each well was determined at 450 nm using Bio-Rad (USA) and mean absorbance of each well was then computed.

Edu Assay

Chondrocytes in logarithmic growth phase in cell culture bottle were digested with 0.25% trypsin and centrifuged at 1000 rpm for 5 min to prepare single cell suspensions. Small amount of cell suspension was taken to cell counting plate, and cell concentration was computed after counting under microscope. Cells were seeded on 96 well plates, and 1× 104 cells (1 cell per well) were cultured in 200μl complete medium for 24 h, following which medium was changed. After corresponding treatment, based on Edu reagent instructions, each well containing 100μL Edu medium was incubated at 37°C for 2 h. 200μL of 4% paraformaldehyde was fixed for 30 min, and 100μL glycine was then added, followed by 100 μL Apollo. 100μL 0.5% Triton-X was added, washed twice using PBS, and 100μL Hoechst 33342 was decolorized at room temperature, shaken for 30 min, and washed twice with PBS. Cell proliferation was then observed using fluorescence microscope.

Flow Cytometry (FCM) Assay

Chondrocytes ($1x10^5$) in treatment and control groups were digested with 0.25% white trypsin. After centrifugation at 500-1000 R/min for 5 min, culture medium was removed, medium was precooled, washed with PBS, centrifuged twice, and then centrifuged with 100μl. Cells were resuspended using BD Biosciences (USA) in apoptosis detection kit. 5μL annexin, v-pe and PI were incubated at room temperature for 15 min in dark, and 400mg/L were added. Flow cytometry was used to evaluate proportion of apoptosis in treatment and control groups.

Alkaline Phosphatase (ALP)Assay

Chondrocytes at 5 × density of 104/well were inoculated in lower chamber of 24 well Transwell plate. Chondrocytes in each group were treated and added to 200μl DMEM medium. ALP staining was undertaken after 7 days using alkaline phosphatase staining kit.

Alizarin Red S (ARS) Assay

After 21 days, cells were washed with PBS twice, fixed with 4% formaldehyde for 10 min, and stained with alizarin red for 30 min. Staining solution was removed, cells were washed, photographed and analyzed.

Statistical Analyses

All data were analyzed using SPPS 26.0 and Graphpad prism 8 softwares. Student's t-test was used to analyze differences between the two study groups. Differences between the two groups were compared by one-way ANOVA. ($P<0.05$ was considered statistically significant.

Results

Rock2 was over-expressed in OA cartilages

To explore expression of Rock2 in OA cartilage, the current study analyzed 15 OA artistic samples and 15 normal artistic samples by qRT-PCR and Western blot. Findings showed that expression levels of Rock2 protein and mRNA in OA artistic samples were significantly increased ($p< 0.05$, Fig 1A-B).

Inhibition of Rock2 expression significantly reduced proliferation of chondrocytes

To further clarify role of Rock2 in osteoarthritis, the current study first used Rock2 interference fragment (siRNA) and Rock2 inhibitor to downregulate expression of Rock2 in chondrocytes (Fig 2A-B, $p<0.05$. Findings of CCK8 and EdU assays showed that proliferation significantly decreased in knockdown of Rock2 (Fig 2C-D). However, rate of apoptosis was significantly increased (Fig 2E, $p< 0.05$). Furthermore, the current study established that cell cycle was blocked in G1 phase after down regulating expression of Rock2 in chondrocytes (Fig2F, $p< 0.05$).

Inhibition of Rock2 expression significantly inhibited differentiation of chondrocytes

Effect of Rock2 expression on chondrocyte differentiation was further clarified in the current study and findings are shown in Fig 3A and B. Findings showed that differentiation was significantly related to decrease in si-Rock2 cells, and expression of Runx2 and osterix protein also decreased.

Rock2 activated Wnt/β-catenin signaling pathway in OA chondrocytes

Findings of the current study indicated that Rock2 plays important roles in OA cartilages, although mechanism is unclear. Previous studies aver that Wnt/ β-catenin signaling pathway plays important roles in progress of osteoarthritis[16]. The current study first used siRNA and Y27632 to reduce expression of Rock2 in chondrocytes, where expression of β- catenin,Gsk-3 and TCF4 was significantly inhibited (Fig 4A). Furthermore, the current study upregulated expression of Rock2 in chondrocytes and then inhibited β-catenin signaling pathway using xav939. Western blot was used to detect expression of β-catenin, TCF4, and GSK-3β proteins. (Fig 4b). Findings of CCK8 and EdU assays showed better inhibition effect, indicating that β-catenin signaling pathway attenuates effect of Rock2 on chondrocyte proliferation (Fig 4C and D). Similarly, findings of flow cytometry showed that cells were inhibited, an indication that β-

catenin signaling pathway reduces effect of Rock2 on chondrocyte apoptosis and G1 phase tissue. The current study also examined effect β- catenin signaling pathway on cartilage differentiation. β-catenin signaling pathway weakens effect of Rock2 on chondrocyte differentiation. These findings indicated that Rock2 activates β-catenin signaling pathway, further affecting proliferation, apoptosis and differentiation of chondrocytes.

Discussion

Osteoarthritis (OA) is a common degenerative joint disease, whose incidence rate increases with age. Main OA symptoms include joint pain, joint stiffness and reduced movement[17]. Statistics indicate that about 240 million people are affected by OA worldwide, accounting for about 10% of men and 18% of women. Occurrence of OA leads to gradual decline of patient's function, which adversely affects quality of life of patients[18, 19]. OA is characterized by articular cartilage destruction, subchondral bone lesions and associated synovitis. OA is mainly caused by degeneration of articular cartilage. Maintenance of chondrocytes is an important factor protecting whole cartilage[20, 21]. Epidemiology of OA is very complex. Previous studies reported that some risk factors related to occurrence and development of OA include aging, obesity, inflammation, trauma, genetics, biology and biomechanics[22]. Current treatment of osteoarthritis complements palliative pain control, physical therapy or prosthetic joint replacement. Effective drug therapy target for OA has not yet been developed. Therefore, there is urgent need elucidate effective molecular targets to provide more theoretical basis for targeted therapy of osteoarthritis.

Ras homologous gene family member A (RhoA) is a small GTPase protein in Rho family. In human, RhoA is encoded by *RhoA* gene located on chromosome 3. It comprises effector domain, four exons, hypervariable region and CaX box motif. RhoA protein is expressed in all tissues including normal human tissues, embryonic tissues and stem cells[23]. RhoA is mainly located in plasma membrane and cytoplasm, and plays important roles in several cellular processes, including cell growth, transformation and cytoskeleton regulation, mainly formation of actin stress fibers and actin contractility. Rho associated protein kinase (rock) is downstream effector of RhoA, which exists in two isotypes; Rock1 and Rock2[24, 25]. Rho/Rock signaling pathway is an important signal transduction system, which is closely related to cell growth and differentiation. Previous studies established that RhoA/Rock signaling is involved in initiation and progression of OA through response to abnormal mechanical stimuli[26]. Change of RhoA/Rock in articular chondrocytes is considerably a new marker for OA development. RhoA/Rock pathway plays important roles in OA development. Inhibition of RhoA/Rock pathway through selective inhibitors benefits treatment of OA. Some successful *in vivo* experiments have demonstrated potential value of RhoA/Rock pathway inhibition in treatment of OA. Therefore, there is need to further explore biological characteristics of RhoA/Rock signaling pathway, develop strategies to target RhoA/Rock in treatment of OA, and establish new therapies for OA in future. Findings of the current study indicated that Rock2 mRNA and protein were significantly overexpressed in OA cartilage compared with expression in normal cartilage. Rock2 is often overexpressed in autoimmune diseases and in OA. Findings of the current study showed that proliferation of chondrocytes was significantly inhibited after decrease in expression of Rock2. However, apoptosis was significantly increased. Findings also showed

that cells were arrested in G1 phase. The current study findings also confirmed that down-regulation of Rock2 expression in chondrocytes reduced their differentiation ability. Moreover, findings of the current study established that Rock2 knockdown inhibited propagation and induced apoptosis of OA cells, which can be utilized in development of effective anti-inflammatory methods in autoimmune diseases before thorough investigation on downstream molecular activities is undertaken.

Previous studies have shown that Wnt/ β-catenin signaling pathway plays key roles in development of embryonic cartilage, development of postnatal cartilage, and growth of osteoblasts and osteoclasts[27]. Findings of the current study showed that down-regulation of Rock2 expression, significantly inhibited β-catenin, Tcf4 and GSK-3β expression. Furthermore, the current study undertook inhibition of Wnt expression in Rock2 overexpressing β-catenin. These findings indicated that overexpression of catenin signaling pathway inhibited chondrocyte proliferation, apoptosis and differentiation. The current study confirmed that Rock2 activates Wnt by activating Wnt/β-catenin signaling pathway, thereby promoting growth and differentiation of chondrocytes.

Conclusions

The current study established overexpression of Rock2 in OA cartilage and chondrocytes. Furthermore, knockdown of Rock2 inhibited proliferation and differentiation while promoting apoptosis of OA chondrocytes, indicating that siRock2 protects cartilage tissues. To the best of our knowledge, this is the first study that established that Rock2 regulates Wnt/β- catenin signaling pathway in OA, which provides novel insights to OA treatment.

Declarations

Acknowledgements

Not applicable

Authors' contributions

All authors made substantial contribution to conception and design, acquisition of the data, or analysis and interpretation of the data; take part in drafting the article or revising it critically for important intellectual content; gave final approval of the revision to be published; and agree to be accountable for all aspect of the work. The author(s) read and approved the final manuscript.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The present study was approved by the ethical review committee of Huashan Hospital of Fudan university. Written informed consent was obtained from all enrolled patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

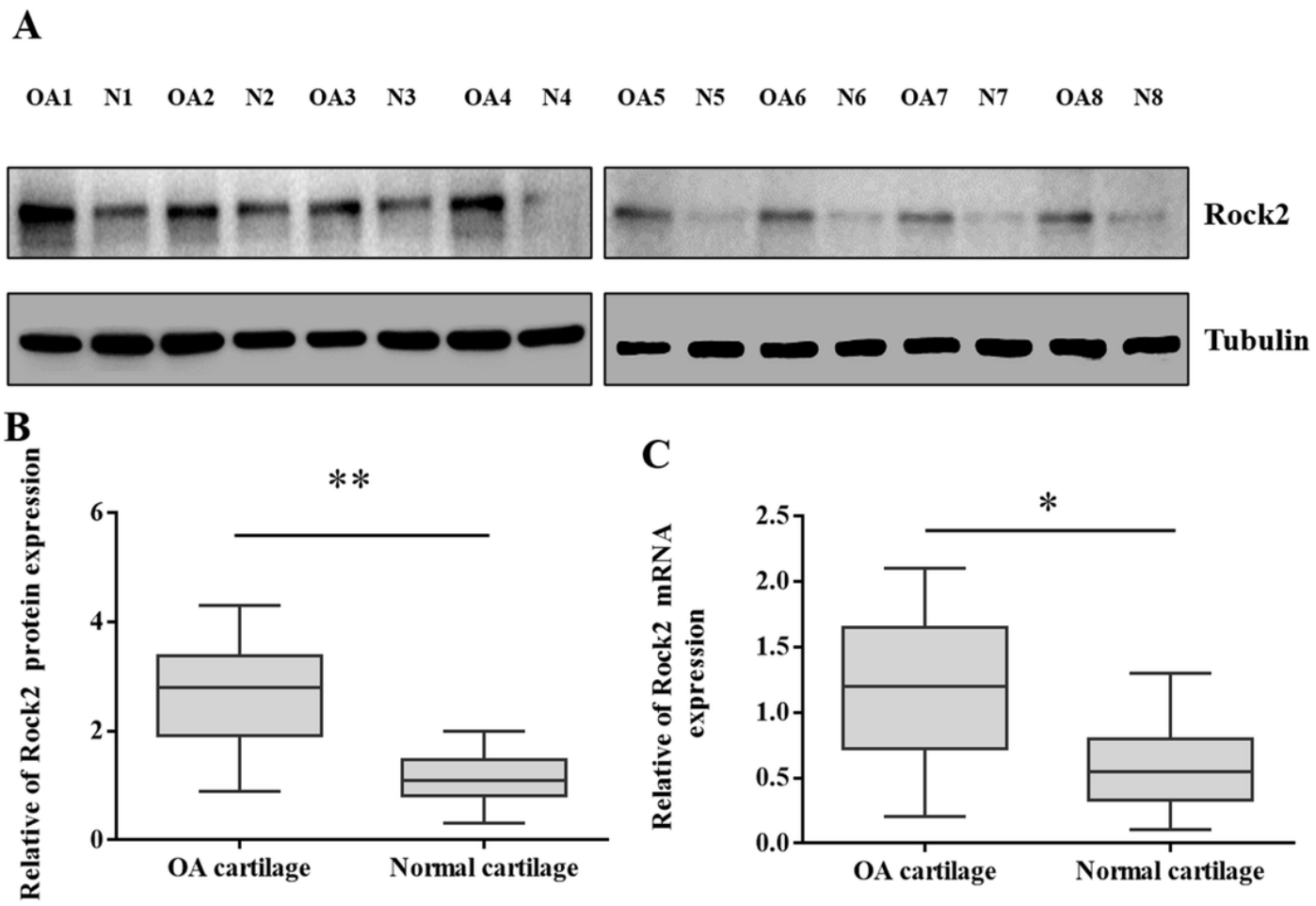

Figure 1

Rock2 was over-expressed in OA cartilages A -B: Western blot result showed that protein expression of Rock2 was increased in OA cartilages compared with in normal cartilages(**p<0.01); C, qRT-PCR result showed that mRNA expression of Rock2 was increased in OA cartilages compared with normal cartilages(*p<0.05).

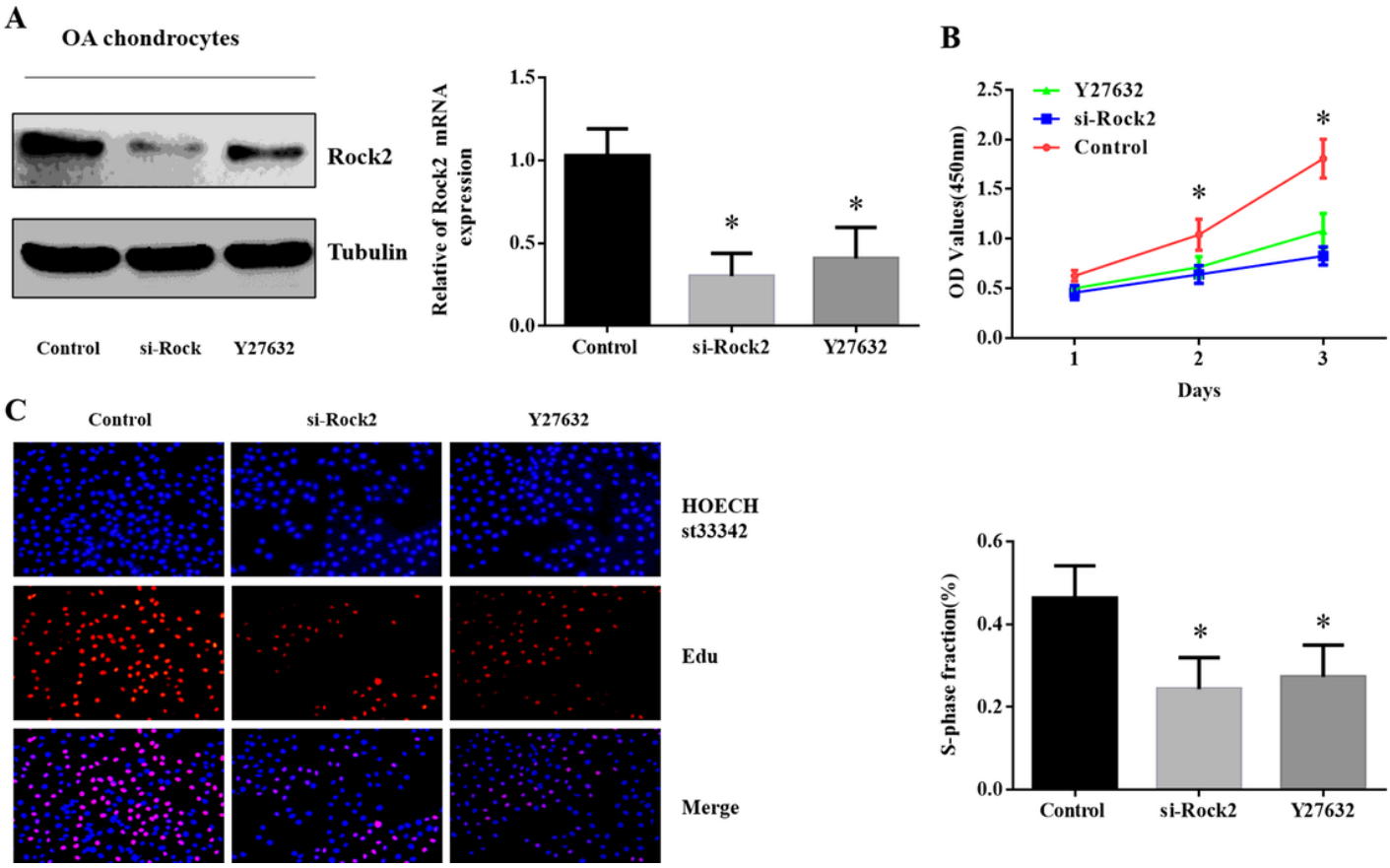

Figure 2

Inhibition of Rock2 expression significantly reduced proliferation of chondrocytes AB Western blot and qRT-PCR results showed that Rock2 was successfully down-regulated in chondrocytes. B and C: Findings of CCK8 and Edu showed that after down-regulating expression of Rock2 in chondrocytes, its proliferation ability was significantly weakened (*p<0.05).

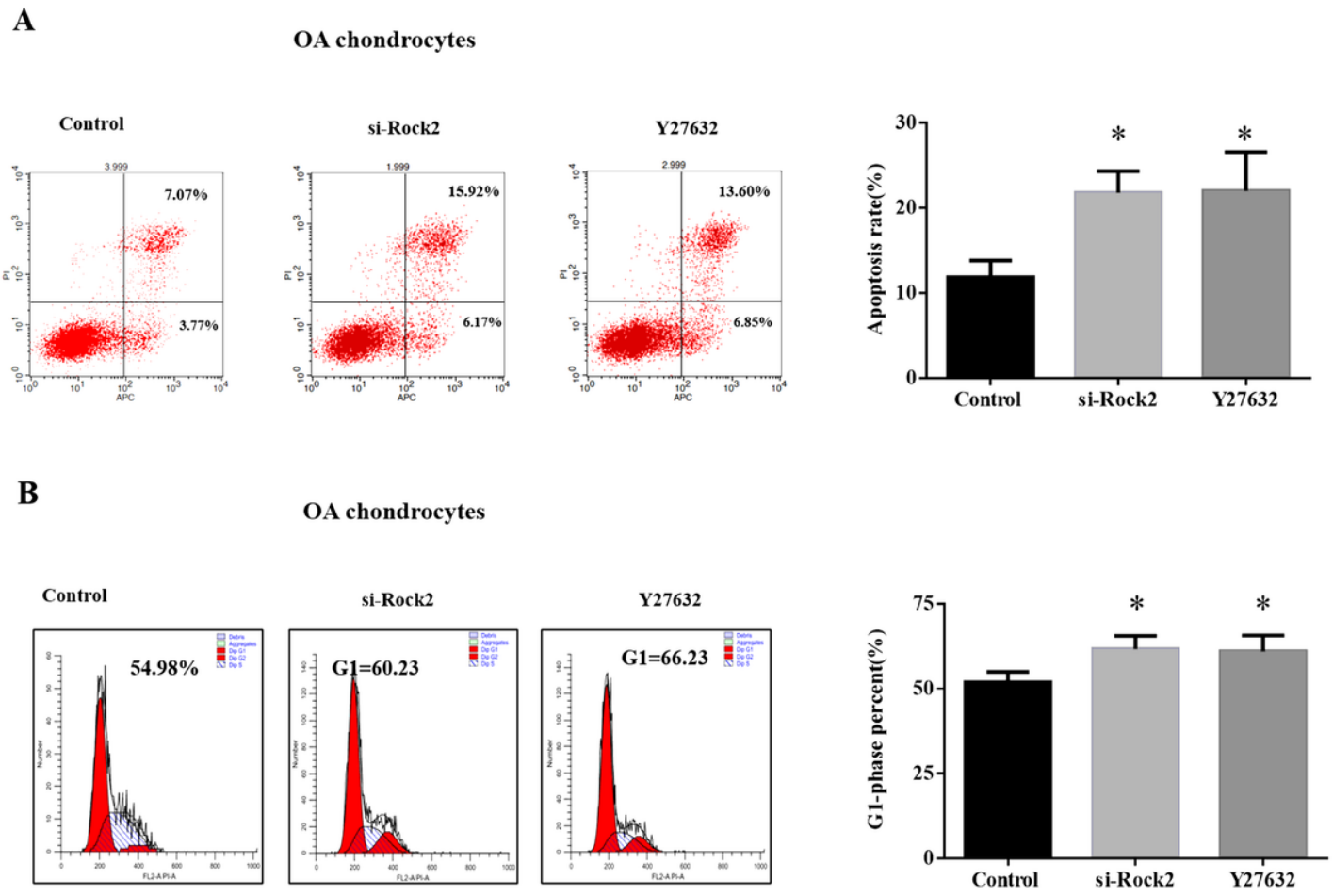

Figure 3

Inhibition of Rock2 expression promoted the rate of apoptosis of chondrocytes A -B: Flow cytometry assay results showed that after down-regulating expression of Rock2 in chondrocytes, apoptotic ratio was significantly increased, and cell cycle was blocked in G1 phase (*p<0.05).

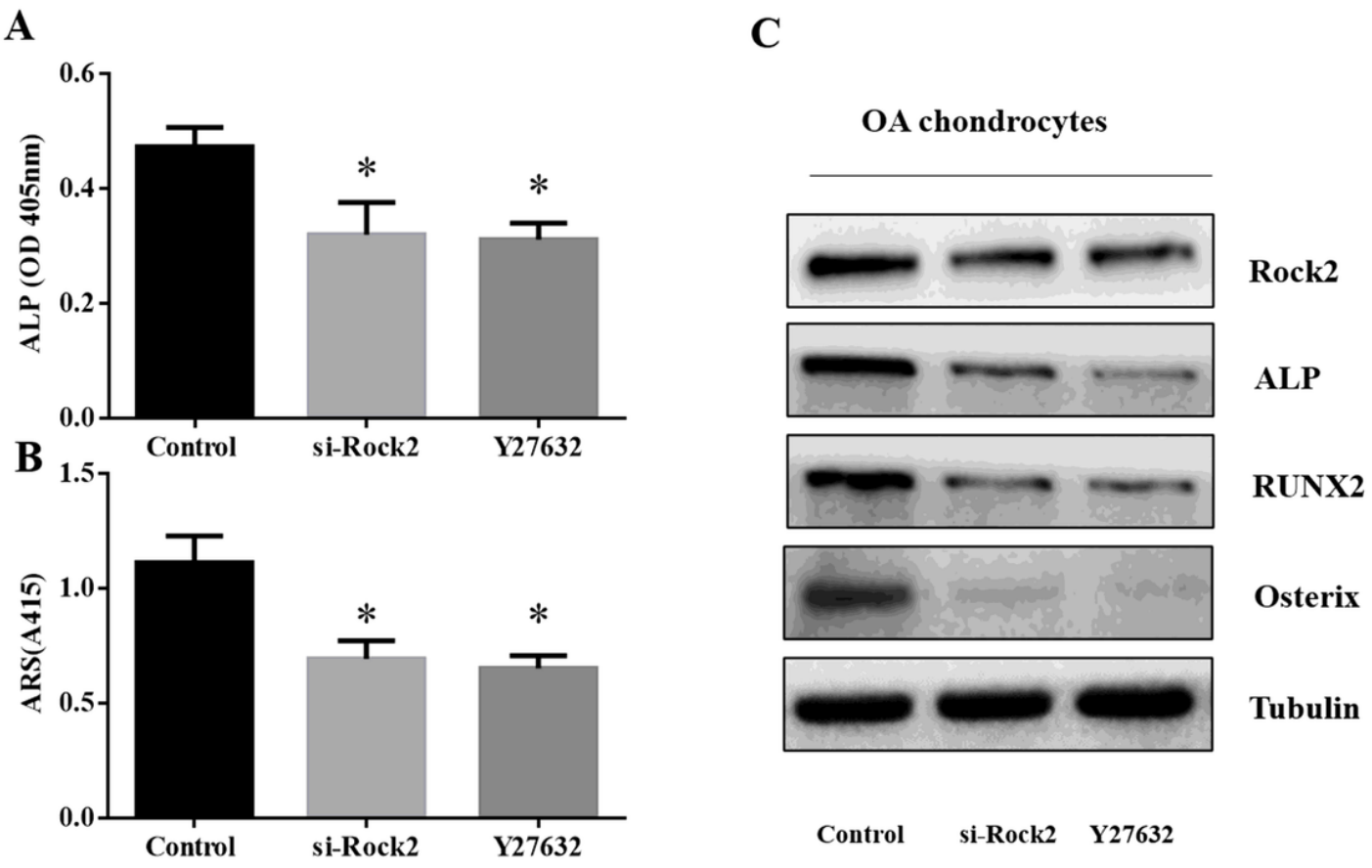

Figure 4

Inhibition of Rock2 expression significantly inhibited differentiation of chondrocytes A: Quantitative analysis of alkaline phosphatase in different groups, B: Quantitative analysis of alizarin red staining in different groups C: Western blot analysis of osteogenic proteins (Runx2, ALP and Osterix).

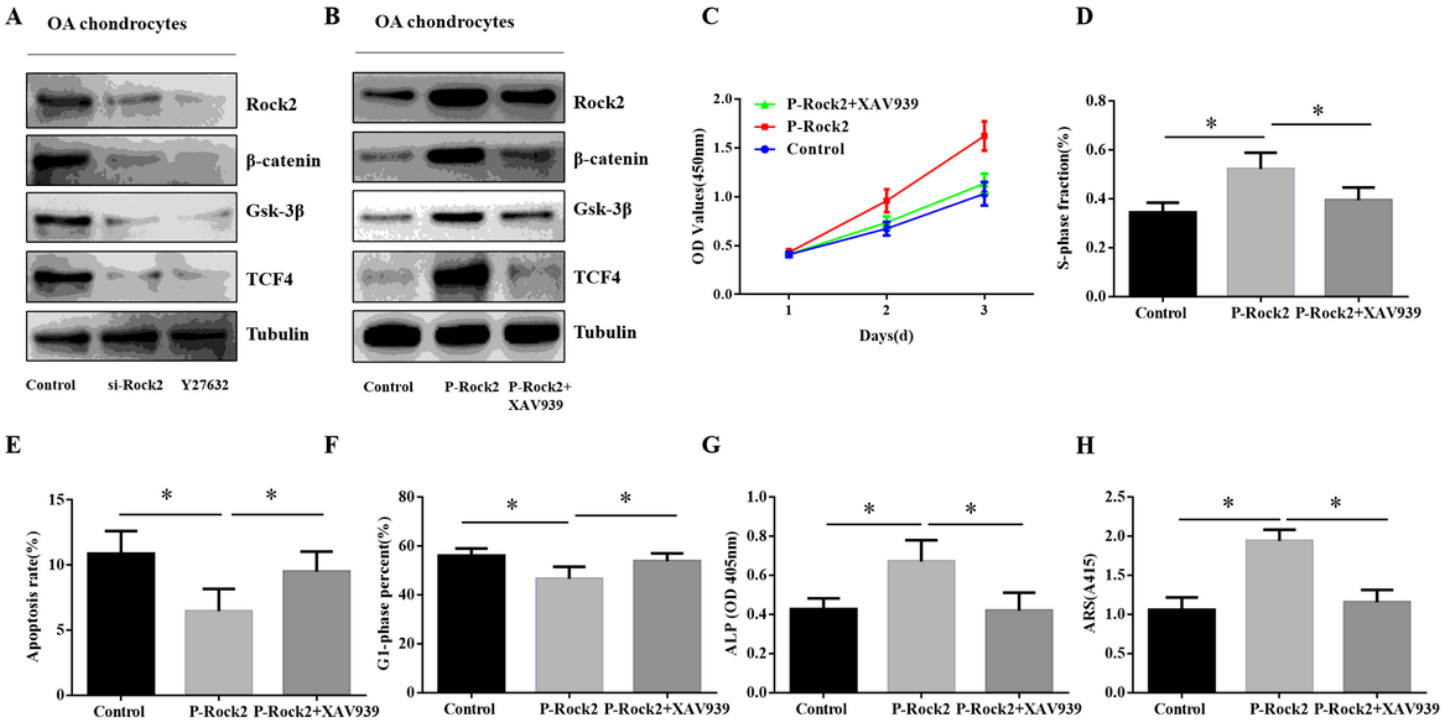

Figure 5

Rock2 activated Wnt/β-catenin signaling pathway in OA chondrocytes A: After down-regulating expression of Rock2 in chondrocytes, Western blot detects expression of Wnt/β-catenin signaling pathway proteins (β-catenin, TCF4, GSK-3β); B: up-regulates chondrocytes and inhibits Wnt/β-catenin signaling pathway, Western blot was used to detect expression of β-catenin, TCF4, and GSK-3β proteins. C: up-regulates chondrocytes and inhibits Wnt/β-catenin signaling pathway. CCK8 findings showed that up-regulation and inhibition of Wnt/β-catenin signaling pathway in chondrocytes attenuates up-regulation of promotion of proliferation by Rock2. D: Findings of Edu indicated that up-regulation and inhibition of Wnt/β-catenin signaling pathway in chondrocytes attenuates up-regulation of promotion of proliferation by Rock2. E: Flow cytometry assay findings showed that inhibition of Wnt/β-catenin signaling pathway in chondrocytes attenuates up-regulation of promotion of apoptosis by Rock2. F: Flow cytometry showed that cells were inhibited and β- catenin signaling pathway reduces the effect of Rock2 on chondrocyte apoptosis and G1 phase tissue. G: and H: Cartilage differentiation. β- Catenin signaling pathway weakens effect of Rock2 on chondrocyte.