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Original Article

Taladegib controls early chondrocyte hypertrophy via inhibiting smoothened/Gli1 pathway

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Abstract: Chondrocyte hypertrophy is a common phenomenon in osteoarthritis (OA). Indian hedgehog (Ihh) is secreted by pre-hypertrophic chondrocytes, which regulates the hypertrophy and mineralization of chondrocytes during cartilage osteogenesis. Smoothened (Smo) is a connecting protein among the Ihh signaling pathway that triggers glioma-associated oncogene homologue 1 (Gli1) to active the hypertrophic process. In this study, we aimed to examine a new inhibitor of Smo in the prevention of chondrocyte hypertrophy during OA. We collected human joint cartilage from the OA patients undergoing knee arthroplasty. Chondrocytes in different OA degrees were isolated and divided them into mild and severe groups. Alginate beads (ABs) was used to establish a chondrocyte hypertrophy model. The expression of type X collagen, MMP-13, Runx-2, type II collagen, SOX-9, and aggrecan were determined using immunofluorescence, Western blot, real-time polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay was performed to assess the viability of chondrocytes. ABs treatment accelerated the process of chondrocyte hypertrophy and upregulated the expression of type X collagen, Matrix metalloproteinase-13 (MMP-13), Runx-2 but decreased type II collagen, SOX-9, and aggrecan both in protein and mRNA levels, which abolished by the present of Taladegib with the activation of Smo and Gli1. However, in the severe OA chondrocytes, Taladegib lost the ability to reverse hypertrophic chondrocytes to a healthy state and made no sense to the expression of type X collagen and Gli1. Our results reveal Taladegib as a novel drug in controlling chondrocyte hypertrophy depending on Smo blocking, which plays a vital role in the homeostasis of cartilage and the development of OA. Besides, we found that Taladegib only works in the previous stage of chondrocytes hypertrophy but not in the later of the process.

Keywords: Taladegib, chondrocyte hypertrophy, Indian hedgehog, smoothened, Gli1, cartilage

Introduction

Osteoarthritis (OA) is one of the universal joint diseases in the clinic, characterized by degradation and destruction of articular cartilage and causes changes in joint morphology, which leads to chronic joint pain and even disability [1]. Several studies have shown that articular cartilage cells will undergo abnormal differentiation during the degradation of cartilage in OA, and this process is similar to the distinction of chondrocytes during osteogenesis of cartilage [2]. With the development of OA, normal chondrocytes undergo pre-hypertrophy, hypertrophy, terminal differentiation, and mineralization. Specific gene expression significantly changes during each stage of differentiation: healthy chondrocytes mainly secret type II collagen and aggrecan; pre-hypertrophic chondrocytes primarily secrete Indian hedgehog (Ihh); hypertrophic chondrocytes specifically secrete type X collagen, matrix metalloproteinase-13 (MMP-13) and proteoglycanase (ADAMTs), among which MMP-13 and ADAMTs can further decompose cartilage matrix and accelerate cartilage calcification and promote OA development [3].

As an essential secretory protein, Ihh acts in chondrocyte differentiation, proliferation, and maturation, especially during endochondral ossification [4]. Patched-1 (Ptch1) and Smoothened (Smo) are two specific membrane proteins secreted on the surface of Ihh target cells in the Ihh signaling pathway [5]. Smo is in a state of inhibition with the absence of Ihh, and Smo activates under the condition of Ihh binding with Ptch-1 Activated Smo following acti-

vates transcription factor glioma-associated oncogene homologue 1 (Gli1), regulates transcription of its target gene and then regulates Cell proliferation and differentiation [6]. There are three types of Gli family in mammals: Gli1, Gli2, and Gil3, of which Gli1 is mainly responsible for the activation of the Ihh signaling pathway [7]. Ihh plays an intricate role in cartilage osteogenesis; the abnormal differentiation of chondrocytes of OA is similar to the differentiation process of chondrocytes in the process of cartilage osteogenesis [8]. In recent years, attention has been paid to the role of Ihh in OA. Beaupre et al. [9] found the activation of the downstream in Ihh signaling led to thinning of cartilage and decreased proteoglycan (PG) content, while inhibition of Ihh signaling led to increased cartilage thickness and PG. Mak et al. [10] suppressed Ihh signaling by removing Smo, and chondrocyte hypertrophy was delayed.

Small-molecule inhibitors of the Ihh pathway receptor Smo have been put into use in treating patients [6]. In this present project, we aimed to explore a new Smo specific inhibitor [11], and whether it could control chondrocyte hypertrophy to slow down the progress of OA. We elucidate several managerial abilities of Taladegib, including suppression of Gli1, overexpression of SOX-9, type II collagen and aggrecan expression, and inhibition of Runx-2, MMP-13, and type X collagen expression. Our findings reveal a possible usage of Taladegib protein for chondroprotective therapy.

Patients and methods

Patient tissue samples collection

This retrospective project was approved by the Ethics Committee of the Yantaishan Hospital. A total of 8 patients (4 males, 4 females; every age: 47 years, from 39 to 67 years) undergoing total knee replacement surgeries with OA from February 2018 to March 2019 took part in the study. Informed consent from all the patients or the relatives were obtained before the operations. We divided the knee cartilage tissues into two groups: 1) Mild OA group (with even, shiny and light pinks surface), 2) Severe OA group (with uneven, abrasive, and red surface). We conserved the tissues in a sterile culture medium immediately after gaining from patients.

NP cells isolation and cell culture

Cartilage was removed from the articular surface without fibers and ligaments. We cut the cartilage into small pieces and washed them using the sterile phosphate-buffered saline solution (PBS). Fragments were digested with collagenase XI (Sigma Aldrich, St. Louis, MO, USA) and Penicillin/Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA), shaking overnight at 37°C. The digested solution was centrifuged to get globules and diluted them in a new culture medium (contains DMEM, with 10% fetal bovine serum (FBS), and Penicillin/Streptomycin) (Gibco, Rockville, MD, USA). The passage 1 chondrocytes were used in the following experiment. 1.2% wt/vol alginate (dissolved in PBS, pH 7.4) was used to culture chondrocytes to establish hypertrophic model [12].

Western blot analysis

Total protein was isolated from cartilage tissues or chondrocytes using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), following added in the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Protein membranes were blocked with nonfat 5% milk and then incubated with primary antibodies against Smo (ab236465, Abcam, Cambridge, MA, USA), Gli1 (ab49314, Abcam, Cambridge, MA, USA), MMP-13 (ab39012, Abcam, Cambridge, MA, USA), Runx-2 (ab192256, Abcam, Cambridge, MA, USA), aggrecan (ab3778, Abcam, Cambridge, MA, USA), SOX-9 (ab185966, Abcam, Cambridge, MA, USA), and β-Actin (as loading control, Abcam, Cambridge, MA, USA) at 4°C overnight. Membranes were then incubated with secondary antibody for 1 h at 37°C. Finally, Ehemiluminescent electrochemiluminescence (ECL) substrate (Beyotime, Shanghai, China) was used to expose the bands.

Immunofluorescence

Chondrocytes seeded on coverslips were fixed with 4% paraformaldehyde for 15 min. 0.5% Triton-X was used to permeabilize the membrane of chondrocytes for 15 min at 37°C.

Table 1. Primer sequences of the genes for RT-PCR

Gene name	Forward (5'>3')	Reverse (5'>3')
Smo	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
Gli1	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
Collagen X	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
Runx-2	GGTTCCTGCCCCAGGATGTTG	GGAACATCTCGAAGCGCTCA
SOX-9	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

Then, the chondrocytes were blocked with 5% bovine serum albumin (BSA) for 1 h at 37°C. Chondrocytes were following incubated with primary antibodies against collagen X (ab58632, Abcam, Cambridge, MA, USA), collagen II (ab34712, Abcam, Cambridge, MA, USA), Smo (ab236465, Abcam, Cambridge, MA, USA), Gli1 (ab49314, Abcam, Cambridge, MA, USA), at 4°C overnight. Finally, incubate the coverslips with Alexa Fluor 488/568 conjugated secondary antibody and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA) for 1 h in the dark at 37°C.

Real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cartilage tissues or chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was following reverse-transcribed into complementary deoxyribose nucleic acid (cDNA) with a Primescript RT reagent kit (Roche, Basel, Switzerland). RT-PCR was performed using SYBR Green (TOYOBO, Osaka, Japan) with a thermal profile containing beginning with 95°C for 5 minutes, followed by 94°C for 30 seconds of 40 cycles, 60°C for 30 seconds, and end with 72°C for an extension. The expression of the target molecules, including type X collagen, MMP-13, Runx-2, type II collagen, SOX-9, aggrecan, Smo, and Gli1were achieved by normalization to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta\Delta Ct}$ method. The primers sequences used for RT-PCR are shown in **Table 1**.

Cell viability assay

Cell counting kit-8 (CCK-8) test was performed to determine the viability of chondrocytes treated with Taladegib. A density of 1×10^4 cells per well was prepared in a 96-well plate. After treatments, chondrocytes were incubated with CCK-8 reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions. Cell viability was shown as the intensity at 450 nM relative to non-treated value.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) Version 22.0 (IBM, Armonk, NY, USA). Differences between two groups were analyzed by using the Student's t-test. A comparison between multiple groups was made using one-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $P<0.05$ was considered a significant difference between groups.

Results

Smo and Gli1 levels in different degree of OA tissues

Human OA tissues were collected from the patients, and the little serious cartilage degenerated the healthier it looked. Growth regulation of chondrocyte is disordered in OA, with differentiating from maturation to hypertrophy and then to ossification. As shown in **Figure 1A**, cartilages were grouped into mild and severe subgroups according to the exteriors. We first texted the hypertrophic related gene Smo and Gli1 in OA cartilage of different degrees. The result suggested that the protein levels of Smo and its downstream gene Gli1 were increased in the severely degenerated cartilage compared to the mild one (**Figure 1B**, **1C**). The mRNA expression was paralleled with the protein (**Figure 1D**). These results indicate that Smo and Gli1 are thought to be crucial for the differentiation of chondrocytes in the development of OA.

Taladegib inhibits Smo and Gli1 expression of chondrocytes in vitro

To evaluate whether Taladegib can control the progress of chondrocytes hypertrophy, we iso-

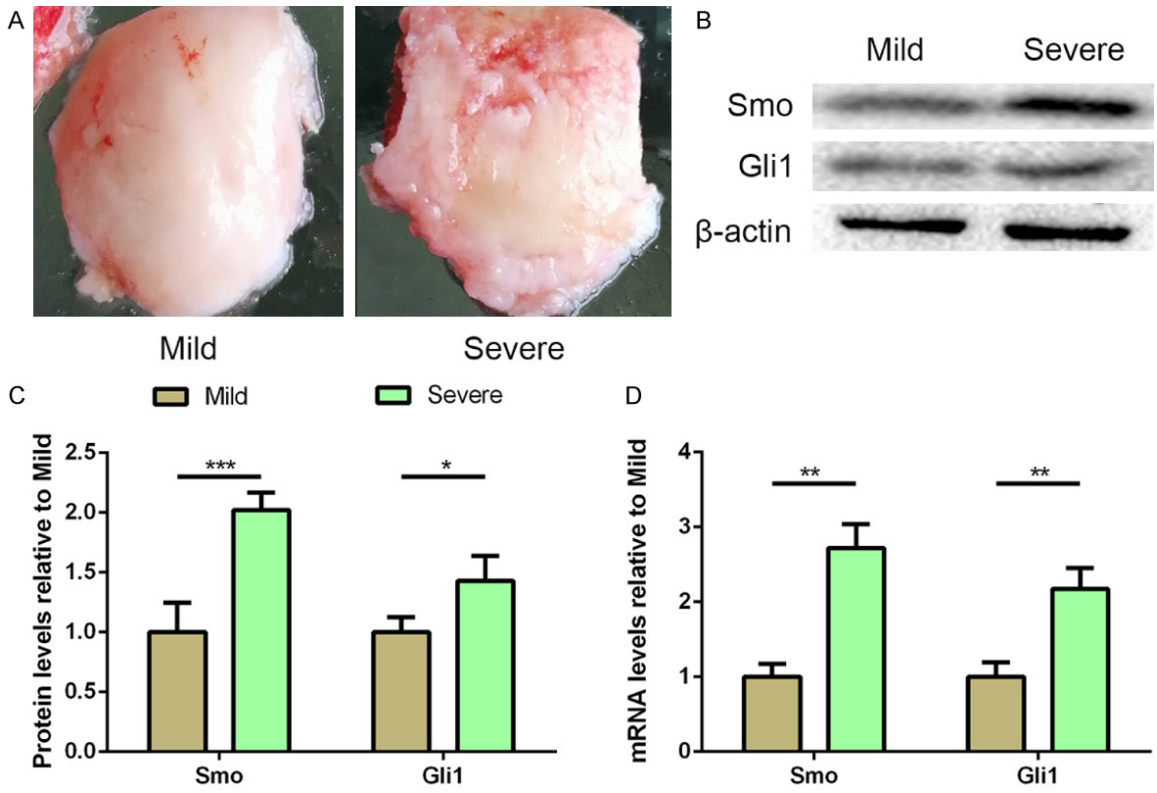

Figure 1. Smo and Gli1 levels in different degrees of OA cartilage collected from the patients. (A) Cartilage tissues were divided into two groups according to the degenerated degree. (B, C) Protein and mRNA were extracted from the cartilage tissue. The protein levels of Smo and Gli1 were determined by Western blot (B) and quantification analysis (C). (D) mRNA levels of Smo and Gli1 were determined by RT-PCR (D). The values are mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with Mild group.

lated chondrocytes in mild degeneration as control and treated with alginate beads (ABs) to induce chondrocyte hypertrophy [13]. To test an optimized method to manage chondrocytes with Taladegib, we used CCK-8 assay to test cell viability under ranged time points and drug concentration. Firstly, we cultured chondrocytes with a ranged concentration of Taladegib from 1 nM to 5 nM for 1 h. As shown in **Figure 2A**, it made no significant difference in cell viability until the concentration up to 4 nM compared to 1 nM, so 3 nM was chosen as the optimized concentration for the following experiment. To explore the optimized stimuli time, we used 3 nM Taladegib to stimulate chondrocytes ranged from 1 h to 12, and the data shown no visible difference (**Figure 2B**). In addition to this, we also performed RT-PCR to analyze Smo and Gli1 mRNA levels under Taladegib treatment (**Figure 2C**, **2D**). The results indicated that the mRNA expression of Smo showed a downtrend till 6 h later and stayed stably in the longer term, which suggested an efficient, optimized treated time of Taladegib was 6 h at least. Summarize above, we stimulated chondrocytes with Taladegib in a concentration of 3 nM for 12 h and collected for the following experiments.

Taladegib controls chondrocyte hypertrophy in vitro

To investigate the effects of Taladegib in the chondrocyte hypertrophy, we firstly surveyed the cell shape of each group. As shown in **Figure 3A**, the cells changed to a hypertrophic and branched shape after Abs treatment compared to the controls, and Taladegib somehow prevented these morphological changes. We next explored the function of Taladegib by analyzing the expression of genes involved in specific hypertrophic chondrocyte, including type X collagen, MMP-13, Runx-2, and chondrocytes makers such as type II collagen, SOX-9, and aggrecan. As compared with controls, the IF results suggested a significant upregulation of type X collagen and decreased type II collagen genes involved in hypertrophic induction. In-

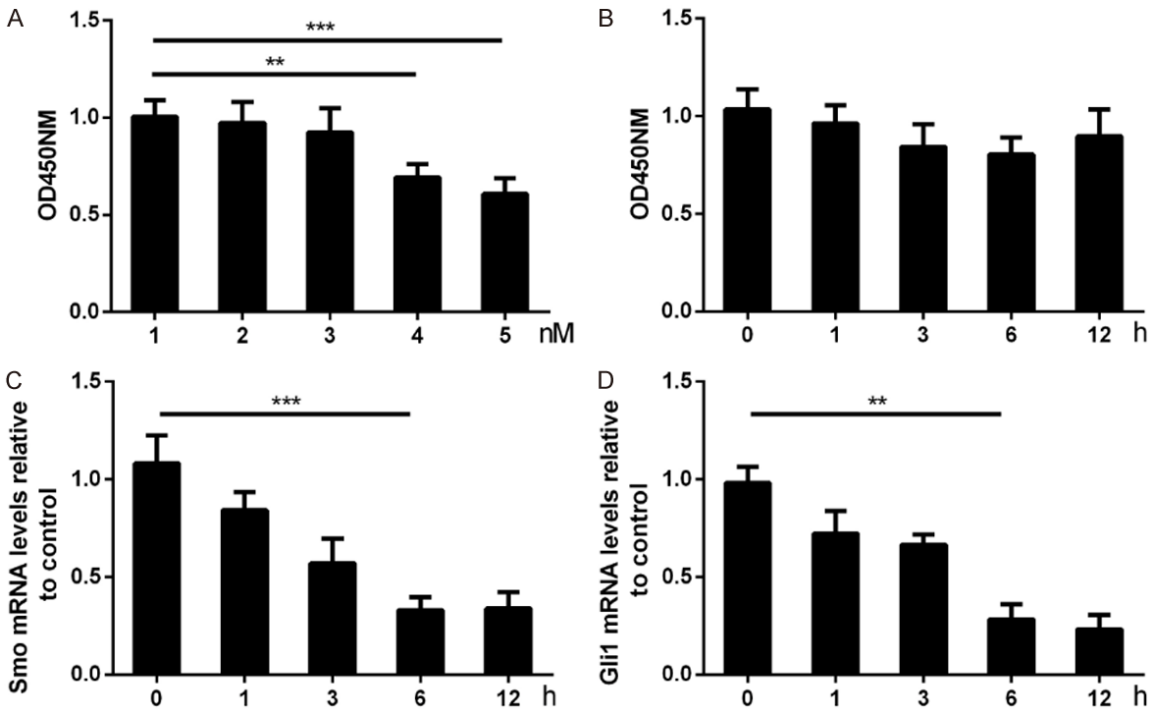

Figure 2. Taladegib inhibits Smo and Gli1 expression of chondrocytes. Chondrocytes in mild OA conditions were pretreated with Taladegib in ranged concentration and time-points. (A, B) CCK8 assay was performed to detect the cell viability under drug treatments. (C, D) The mRNA expression levels of Smo (C) and Gli1 (D) were determined by RT-PCR. The values are mean ± SD of three independent experiments. **P<0.01, ***P<0.001.

terestingly, Taladegib antagonized the effect of Abs treatment with inhibiting type X collagen expression (**Figure 3A**, **3B**). Apart from these, the data of WB showed the levels of MMP-13 and Runx-2 were increased under ABs treatment with a reduction of SOX-9 and aggrecan expression. However, Taladegib obviously prevented these hypertrophic gene expressions and kept chondrocytes stay healthy (**Figure 3C**, **3D**). More detailed analysis of the mRNA expression revealed that chondrocytes in the Abs treatment expressed more hypertrophic gene and less chondrogenic phenotype compared to the control, but successfully affected by Taladegib. Collect above, Taladegib virtually delayed the development of chondrocytes hypertrophy by suppressing type X collagen and Runx-2 expression and kept the chondrogenic phenotype by inhibiting MMP-13 expression and maintaining type II collagen, SOX-9, and aggrecan (**Figure 3E**).

Taladegib controls chondrocyte hypertrophy via Smo/Gli1 pathway

Next, we investigated the effect of Taladegib in the Smo and Gli1 expression by IF and RT-PCR. We co-stained Smo and Gli1 using IF, and the result indicated that the expression of Smo increased after ABs treatment and finally suppressed by Taladegib. Aa a crucial hypertrophic gene, Gli1 was activated in the presence of ABs. However, the Gli1 level was also reduced by Taladegib associated with the suppression of Smo (**Figure 4A**, **4B**). Taladegib also suppressed Smo and Gli1 mRNA levels in the condition of ABs cultural condition (**Figure 4C**). These data indicate that Smo-suppressed chondrocytes were sufficient to suppress Gli1 activity by the pharmacologic effect of Taladegib, suggesting the abrogation of chondrocyte hypertrophy underlies Taladegib is related to Smo/Gli1 pathway inhibition.

Taladegib is deficient in the reversion of hypertrophic chondrocyte

Our results above revealed that Taladegib could prevent the process of chondrocytes hypertrophy; in addition to this, whether it also could reverse the hypertrophic chondrocytes reminded unknown. We immediately following isolated the much hypertrophic chondrocytes (HT) from the severely degenerated cartilage as control.

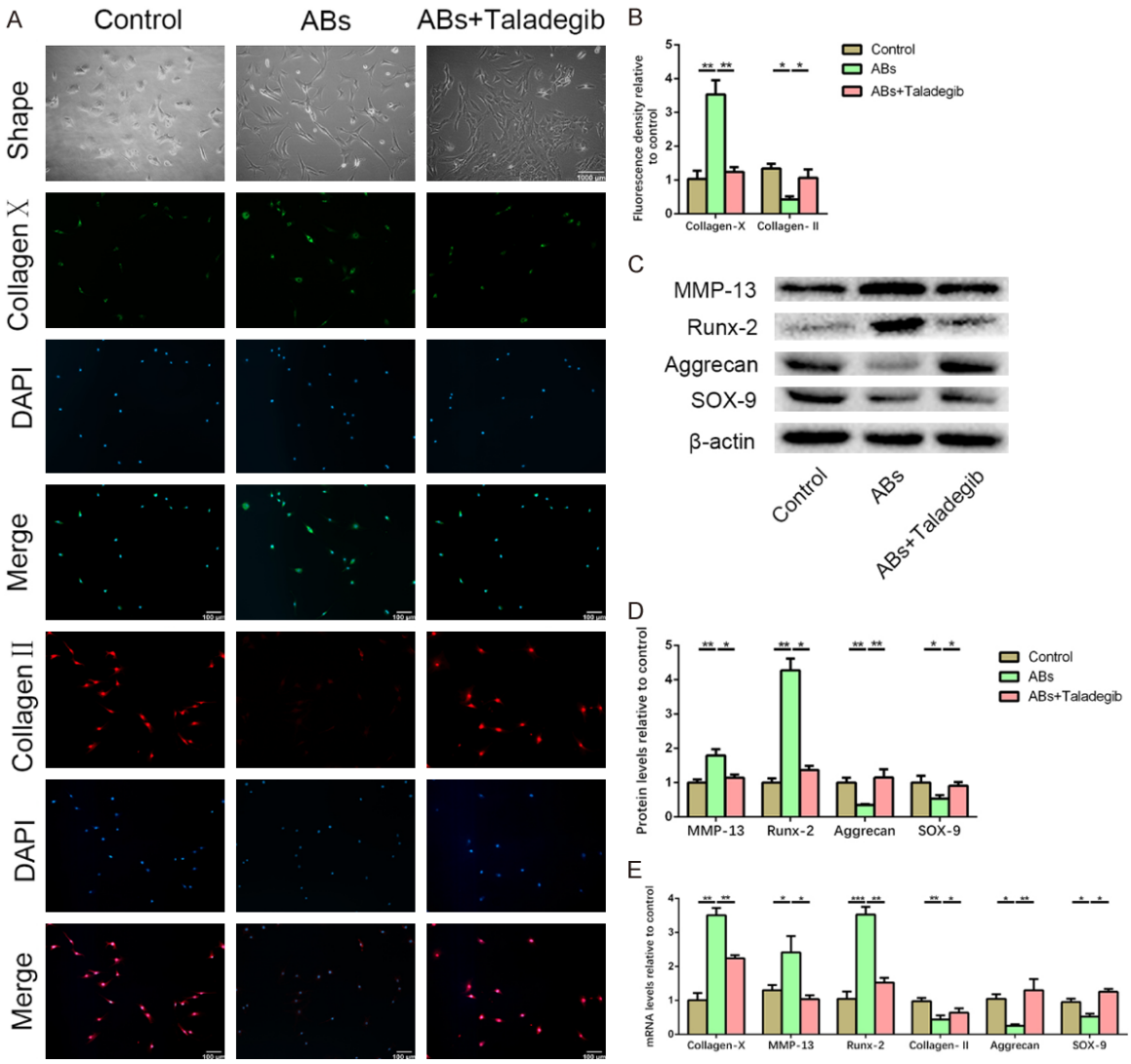

Figure 3. Taladegib controls chondrocyte hypertrophy. Chondrocytes in mild OA condition were pretreated with ABs to the hypertrophic formation, following cultured with or without Taladegib (3 nM) for 12 h. (A) Cell shape was visible under a white light microscope. (A, B) The protein expression level of collagen X and II were determined by immunofluorescence (A) and quantification analysis (B). (C, D) The protein expression level of MMP-13, Runx-2, aggrecan, and SOX-9 were determined by Western blot (C) and quantification analysis (D). (E) The mRNA expression levels of collagen X, collagen II, MMP-13, Runx-2, aggrecan, and SOX-9 were assayed by RT-PCR. The values are mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

However, Taladegib seemingly made no visible difference to the shape of the hypertrophic cells compared to the HT (**Figure 5A**). Type X collagen level was significantly suppressed within the appearance of Taladegib in the hypertrophic process of chondrocytes, as previously described. But we did not detect a significant difference in type X collagen level after Taladegib treatment compared to the HT. Similarly, Gli1 changed inconspicuously even though the existence of Taladegib (**Figure 5A**, **5B**). We further explored the expression of type X collagen and Gli1 mRNA levels, and the trend was similar to IF. These result suggested that Taladegib could prevent the development of chondrocytes in mild hypertrophic state and getting worse, but could not reverse the chondrocytes in severe hypertrophic to a better state (**Figure 5C**).

Discussion

Ihh is a secreted protein that plays a vital role in controlling chondrocyte hypertrophy in the developing skeletal system [14]. Ptch1 and Smo are two receptors on the cell membrane of

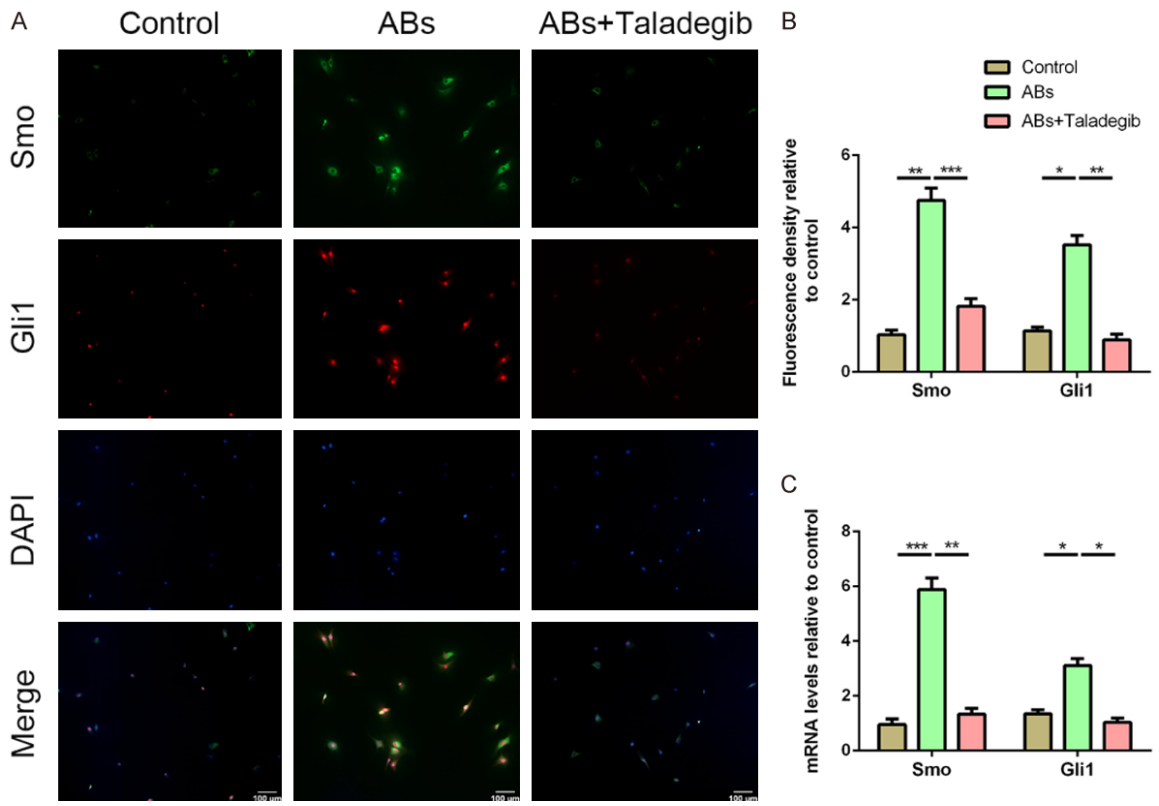

Figure 4. Taladegib controls chondrocyte hypertrophy *via* Smo/Gli1 pathway. Chondrocytes in mild OA conditions were treated as mentioned above. (A, B) The protein expression level of Smo and Gli1 were determined by immunofluorescence (A) and quantification analysis (B). (C) The mRNA expression levels of Smo and Gli1 were assayed by RT-PCR. The values are mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

the Ihh signaling pathway, of which Ptch1 negatively regulates the signal path; Smo is one of the necessary receptors for signal path activation. The downstream transcription factor Gli1 binds to Runx-2 [15] to achieve transcription and expression of type X collagen, thereby causing hypertrophy and ossification of chondrocytes [16]. In this study, we first texted the Smo and Gli1 levels in the cartilage of different OA degrees and found the more serious cartilage degenerated, the more Smo and Gli1 accumulated.

As a mesenchymal source of highly specific cells, chondrocytes are the most basic unit of cartilage, with the function of the production, maintenance, and degradation of extracellular matrix (ECM). Type II collagen and aggrecan are the main components of the ECM that recognized as important markers for chondrocytes [17]. Changes in its content can affect the structure and function of the ECM, which in turn leads to related diseases. MMP-13 and type X collagen, are the hallmarks of hypertrophy in the differentiation of chondrocytes into hypertrophy and osteogenesis. MMPs are a series of proteases that destroy the ECM, which can lead to the destruction of the cartilage matrix and lead to the disorder of joint structure and function [18, 19]. Among them, the substrate of MMP-13 is the most extensive, and the digestion of collagen in articular cartilage is also the strongest [20]. Because of type X collagen cannot form a fibrous scaffold structure as type II, IX, and XI collagen, it weakens the strength of the bone matrix and prepares for calcification [21]. The confirmation of type X collagen can be used as a chondrocyte phenotype in OA, which eventually leads to thinning of articular cartilage and hardening and thickening of the articular cartilage bone plate. The Runx-2 expression is down-regulated in the proliferating chondrocytes during the aggregation phase of mesenchymal stem cells and up-regulated during chondrocyte hypertrophy [22]. In this case, under the appearance of ABs, the chondrocytes turned out a hypertrophic phenotype, including changed shape, overexpressed type X

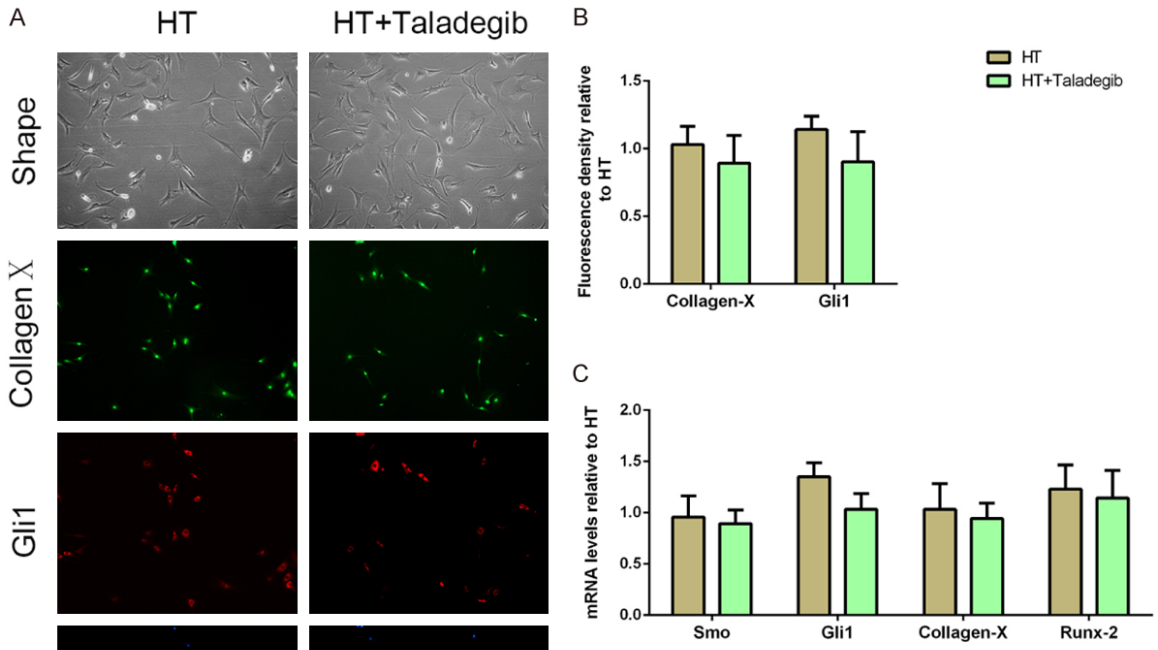

Figure 5. Taladegib is deficient in the reversion of hypertrophic chondrocyte. Chondrocytes in severe OA conditions were cultured with or without Taladegib (3 nM) for 12 h. (A) Cell shape was visible under a white light microscope. (A, B) The protein expression level of collagen X and Gli1 were determined by immunofluorescence (A) and quantification analysis (B). (C) The mRNA expression levels of collagen X and Gli1 were assayed by RT-PCR (C). The values are mean ± SD of three independent experiments.

collagen, MMP-13 and Runx-2, and a decrease in type II collagen, aggrecan, and SOX-9. Taladegib is a specific molecule antagonist of Smo that has been shown to inhibit signaling mediated of the Ihh pathway [23]. However, the negative effect of ABs was efficiently inhibited by Taladegib through the suppression of Smo and Gli1. Therefore, the Smo is a target for delay the chondrocyte hypertrophy in the process of OA.

However, for chondrocytes that have been hypertrophied, whether Taladegib can reverse its hypertrophic phenotype becomes another concern for us. The chondrocytes in severe OA conditions supplied us with a natural source of hypertrophic chondrocytes. The results indicated it was not apparent for the Taladegib to bring hypertrophic chondrocytes to the undifferentiated state. So we hypothesized that Taladegib prevents the new accumulation of Smo and its downstream gene Gli1, but bot clears the existent Smo and Gli1, suggesting the Taladegib may be only functional at the beginning of chondrocytes hypertrophy.

Conclusions

In conclusion, the present study elucidated that targeting of Smo in Ihh signaling pathway by Taladegib control the early chondrocytes hypertrophy *via* inhibition of type X collagen, MMP-13, and Runx-2 expression. This concept provides a new option of developing therapeutic strategies for the control of the early chondrocytes hypertrophy in OA.

Disclosure of conflict of interest

None.

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