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The secreted protein WNT5A regulates condylar chondrocyte proliferation, hypertrophy and migration

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ABSTRACT

Objective: Our previous study showed that WNT5A, a member of the noncanonical WNT pathway, is involved in interleukin-1beta induced matrix metalloproteinase expression in temporomandibular joint (TMJ) condylar chondrocytes. The purpose of this study is to further explore the roles of WNT5A in cartilage biology of the TMJ.
Methods: An early TMJ osteoarthritis-like rat model was constructed by a mechanical method (steady mouth-opening). The gene and protein levels of WNT5A during the condylar cartilage changes were measured. Effects of WNT5A on chondrocyte proliferation, hypertrophy and migration were analyzed after WNT5A gain or loss of function *in vitro*. A c-Jun N-terminal kinase (JNK) inhibitor SP600125 was used to evaluate the involvement of JNK pathway in these effects of WNT5A. The expression and transcription activity of cell cycle regulators c-MYC and Cyclin D1 were examined to determine the mechanism behind WNT5A regulation of chondrocyte proliferation.
Results: WNT5A was significantly upregulated in the condylar cartilage of rats in the early TMJ osteoarthritis-like model. Activating WNT5A facilitated condylar chondrocyte proliferation, hypertrophy and migration. Conversely, inhibiting WNT5A activity in chondrocytes decreased their proliferation, hypertrophy and migration. Blockage of the JNK pathway by its inhibitor, SP600125, impaired these effects of WNT5A on chondrocytes. WNT5A regulated both the expression and transcriptional activity of c-MYC and Cyclin D1 in chondrocytes, both of which were upregulated in condylar cartilage of the rat early TMJ osteoarthritis.
Conclusion: WNT5A regulates condylar chondrocyte proliferation, hypertrophy and migration. These findings provide new insights into the role of WNT5A signaling in TMJ cartilage biology and its potential in future therapy for TMJ degenerative diseases.

1. Introduction

Condylar cartilage of the temporomandibular joint (TMJ) is a unique articular cartilage in many aspects, such as its embryonic origin, ontogenetic development, postnatal growth and histological structures (Shen & Darendeliler, 2005; Sriram, Jones, Alatli-Burt, & Darendeliler, 2009). It is thought that the most intriguing biological feature of condylar cartilage, unique from other synovial or epiphyseal cartilage, is its ability to remodel in response to the changes in condylar location, articular function and mechanical loading (Wadhwa & Kapila, 2008). Mechanical loading-induced condylar cartilage remodeling is pivotal, because it has been considered to be closely related to the functional appliance therapies and occlusal changes in orthodontics and prosthodontics, and defects of this process have been linked to degenerative diseases of the TMJ, especially TMJ osteoarthritis (Sobue et al., 2011).

Osteoarthritis, a disease of progressive cartilage degradation, is one of the major pathologic conditions affecting the TMJ (Kuroda et al., 2009; Schminke et al., 2014; Wang, Zhang, Gan, & Zhou, 2015). Despite the involvement of mechanics and numerous identified predisposing factors, the exact pathogenesis of osteoarthritis remains poorly understood. Specifically, the early changes of cartilage occur long before the disease is diagnosed in clinic, rendering them difficult to study in humans (Mastbergen & Lafeber, 2011). During early osteoarthritis, articular chondrocytes display an elevated level of proliferation and try to remodel the composition of the extracellular matrix and maintain cartilage integrity (Goldring, 2012; Sobue et al., 2011). Defining the molecular mechanism underlying these early changes of osteoarthritic cartilage will provide approaches to prevent articular cartilage breakdown and promote cartilage regeneration.

WNT proteins constitute a large family of highly conserved secreted

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signaling molecules that are responsible for a diverse array of functions during development and adult tissue homeostasis. The mammalian genome encodes 19 WNT proteins divided into “canonical” and “non-canonical” classes that activate β-catenin-dependent and independent signaling pathways, respectively (The Wnt Homepage: http://web.stanford.edu/group/nusselab/cgi-bin/wnt/). In the skeletal system, a broad role of WNT signaling in bone and cartilage biology has been reported, from embryonic skeletogenesis to adult homeostasis and diseases. Genetic studies in humans and mice demonstrate a positive correlation between the canonical WNT signaling and bone mass and strength (Baron & Kneissel, 2013). In cartilage, a delicate balance of WNT activity is necessary to maintain its homeostasis. Both repression and constitutive activation of the β-catenin pathway can lead to cartilage breakdown (Nalesso et al., 2016). In the TMJ, high-throughput screening of differential expression genes in condylar cartilage after experimentally-induced TMJ osteoarthritis showed abnormal expression of several WNT members, suggesting a possible involvement of WNT signaling in the condylar cartilage degradation (Meng, Ma, Ma, & Xu, 2005). Despite the wide recognition of WNT signaling in the skeletal biology, its roles in cartilage biology of the TMJ, especially that of noncanonical WNT signaling, remain poorly understood.

WNT5A is a representative member of the noncanonical WNT pathway and is essential for cartilage development by promoting chondrocyte differentiation and inhibiting chondrocyte maturation (Kawakami et al., 1999; Yang, Topol, Lee, & Wu, 2003). In bone, osteoblast-derived WNT5A is important for osteoclastogenesis (Maeda et al., 2012). In limb articular chondrocytes, WNT5A is involved in interleukin-1β regulation of type II collagen expression (Ryu & Chun, 2006). Our previous studies uncovered that WNT5A is important for interleukin-1β mediated matrix metalloprotease (MMPs) expression in TMJ condylar chondrocytes (Ge et al., 2009; Ge et al., 2011). These findings prompted us to further explore the role of WNT5A in TMJ condylar cartilage. In the present study, we provide evidence that links WNT5A with the condylar cartilage remodeling in rat early TMJ osteoarthritis and identify that WNT5A facilitates condylar chondrocyte proliferation, hypertrophy and migration through the c-Jun N-terminal kinase (JNK) signaling pathway.

2. Materials and methods

2.1. Animal model

Early TMJ osteoarthritis-like changes in the rat TMJ were induced as described previously, with slight modifications (Fujisawa et al., 2003; Kawai et al., 2008). Sprague-Dawley male rats at 9 weeks of age were used in the present study. Mechanical overloading was induced in the TMJ by steady mouth-opening for 2 h per day during a period of 5 days. A mouth-opening device was used to keep the maxillary and mandibular incisors 20 mm apart (exerting 2 N of force) (Supplemental Fig. 1A). The magnitude of force exerted by the mouth-opening device was measured with the N5C mechanical test system (Sichuan Dynamometer Plant, China). During the forced mouth-opening, rats were anesthetized with intra-abdominal injections of sodium pentobarbital (SJYF Sciences, China) at a dose of 50 mg/kg body weight. For control animals, no mouth-opening was applied, although the same anesthesia schedule was maintained. The rats did not demonstrate significant weight loss during the mouth-opening procedure (Supplemental Fig. 1B). All procedures in this study were approved by the Institutional Animal Care and Use Committee at Peking University Health Science Center.

2.2. Chondrocyte cultures

Primary TMJ condylar chondrocytes were isolated from the rat condyles as described previously (Ge et al., 2009). Cells were re-suspended in DMEM/F12 media (Gibco) containing 10% fetal bovine serum (FBS) (Hyclone), supplemented with 50 units of penicillin/streptomycin and were plated in 60-mm plates at a density of 1.5×10^6 cells per plate. After primary culture for 5 days, cells were harvested. Secondary cultures were placed in 96-well plates at a density of 2000 cells per well for proliferation analysis.

Human chondrocyte line (SW1353) was purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 containing 10% FBS supplemented with 50 units of penicillin/streptomycin. Prior to treatment for proliferation assay, cells were washed with phosphate buffered saline and then cultured overnight in serum-free medium.

2.3. Histology and immunostaining

After the experimental period, animals were sacrificed with an overdose of anesthesia. Both TMJs were dissected, fixed in 10% buffered formalin, and decalcified with 10% nitric acid for 48 h. Thereafter, samples were embedded in paraffin, and serial sections (5 μm) were cut in the sagittal plane. The sections were stained with hematoxylin & eosin and toluidine blue for histological evaluation.

Immunostaining was performed using goat anti-WNT5A antibody (1:100, AF645, R & D Systems). Immunohistochemistry was carried out using a Polink-2 Plus Polymer HRP Detection System (ZSGB Bio, Beijing, China). Slides were incubated with the WNT5A antibody or normal goat IgG at 4 °C overnight. The localization of WNT5A–positive cells in the condylar cartilage was examined microscopically. Immunofluorescence was examined with a Zeiss LSM 5 EXCITER confocal microscope. Staining specificity was ascertained by substituting the primary antibody with normal goat IgG.

For quantification of WNT5A positive cells in the condylar cartilage, 3 slides were selected from the middle plane of each TMJ. In the posterior region of condylar cartilage, 3 regions were selected at random to count the numbers of WNT5A-positive cells and the total number of cells under the microscopy (40 x objective). The average of the percentage of WNT5A-positive cells from 3 slides was presented for each rat. Four animals were included in each group.

2.4. Western blotting

Western blotting was performed as previously described (Ge et al., 2009). Cells were lysed with the RIPA buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail set I (Calbiochem) and phenylmethanesulfonyl fluoride (PMSF; Sigma). TMJ cartilage tissues were lysed with a Denaturing Lysis Buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2% sodium dodecyl sulfate). Proteins (30 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Goat anti-WNT5A (1:1000 dilution, R & D Systems), rabbit anti-cyclin D1 (1:1000 dilution, Cell Signaling Technology) and rabbit anti-c-Myc (1:1000 dilution, Cell Signaling Technology) antibodies were used to detect the proteins. The protein blots were visualized by either enhanced chemiluminescence detection (ECL) or the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.5. Chondrocyte proliferation assays

Cells in 96-well plates were treated with the indicated concentrations of agents. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8) and BrdU ELISA assays. Each experiment was carried out with 4–6 replicates per treatment and was independently repeated at least three times.

BrdU incorporation assay was performed using the BrdU labelling and detecting kit III (Roche Applied Science). Cells were labelled with 10 μM BrdU in the last 12 h of treatment and then fixed with the Fixodent agent. Incorporated BrdU was detected with monoclonal anti-BrdU-POD antibody, and the bound conjugate was detected with ABST substrate. The absorbance was measured at 450 nm after adding 1 M

H_2SO_4 stop solution.

The CCK-8 assay was used as an alternative way of estimating the change in chondrocyte number. For this analysis, the water-soluble tetrazolium salt (WST-8) (Dojindo Laboratories, Japan) was added in the last 2–4 h of treatment and then the absorbance was measured at 450 nm.

2.6. WNT5A gene overexpression and silence

The mammalian expression vector PGK-WNT5A was transfected into SW1353 cells with LipoFectamine 2000 (Invitrogen). Transfected cells were maintained in complete DMEM/F12 for 48 h and used for further analyses. For *WNT5A* gene silencing, cells were transfected with either 100 NM *WNT5A* SMARTpool siRNA or control siRNA (Dharmacon) for 48 h using Dharma 1 reagent.

2.7. Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and the complementary DNA was synthesized with GoScript reverse transcriptase system (Promega). Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems) and SYBR Green Master Mix (Applied Biosystems). Primer sequences are listed in Supplemental Table 1.

2.8. Luciferase reporter assay

SW1353 cells growing in 96-well plates were transfected with cyclin D1-Luc or c-Myc-Luc structure for 4 h with LipoFectamine 2000. After incubated in complete DMEM/F12 medium for 12 h, cells were treated with 200 ng/ml WNT5A protein or 2 μg/ml anti-WNT5A antibody (R & D) for another 24 h. The activity of the Firefly luciferase was measured and normalized to Renilla luciferase activity using the Dual Luciferase Reporter Assay system (Promega).

2.9. Wound healing and transwell migration assays

For wound healing assay, SW1353 cells were cultured until confluence in 6-well plates and then wounded using a yellow pipette tip. Corresponding reagents were added into the media and migration distance was photographed and measured at zero time and after 12 h.

Transwell migration assay was performed using a Transwell apparatus (Corning). SW1353 cells after siRNA transfection for 48 h were seeded in the upper chamber, and cells that migrated to the bottom surface of the insert were fixed and stained with crystal violet after culture for 12 h.

2.10. Statistical analysis

All data are presented as mean ± SD. Differences between two groups were evaluated by two-tailed Student's *t*-test. One- or two-Way ANOVA followed by Tukey's or Sidak's test for multiple comparisons was performed as indicated. All analyses were performed using Prism 6.0 (Graph Pad). P value < 0.05 was considered significant.

3. Results

3.1. Upregulation of WNT5A in the condylar cartilage of rat early TMJ osteoarthritis

To explore the role of WNT5A in TMJ cartilage biology, an early TMJ osteoarthritis-like model was induced by repetitive, steady mouth-opening in rats. Micro-CT images showed that the mechanical stress was mainly loaded on the posterior region of rat condylar cartilage during the forced mouth-opening (Fig. 1A). Histology showed that normal rat TMJ condylar cartilage can be divided into 4 different cell layers: fibrous, proliferating, mature and hypertrophic cell layers. Immediately after the forced mouth-opening procedure, marked osteoarthritis-like lesions were observed in the condylar cartilage. In the posterior region especially, a decrease in the thickness of the hypertrophic chondrocyte layer, irregular chondrocyte alignment, and reduced proteoglycan staining were observed (Fig. 1A). Gene expression analysis showed decreased expression of *COL1A1*, *COL2A1*, *SOX9*, *ACAN* and *IHH* and increased expression of *COL10A1*, *MMP13* and *RUNX2* in the condylar cartilage (Fig. 1B), which demonstrates chondrocyte dedifferentiation and hypertrophy and supports an osteoarthritis-like change in the TMJ condylar cartilage.

Next, we examined the expression of several WNT ligands that are potentially involved in chondrocyte biology in the rat condylar cartilage after forced mouth-opening. Remarkably, *WNT5A* was the most significantly upregulated gene among these WNT ligands (Fig. 1C). Immunostaining showed an increase of WNT5A positive cells in the proliferating and mature cell layers after the mouth-opening procedure, with notably intense staining in the mature cell layer. The percentage of WNT5A positive cells in the posterior area of rat TMJ condylar cartilage also increased (Fig. 1D–F). Western blotting confirmed the upregulation of WNT5A in the rat condylar cartilage (Fig. 1G). Together, these results demonstrate an increased expression of WNT5A in condylar cartilage of the early TMJ osteoarthritis.

3.2. Expression of WNT5A in normal TMJ condylar cartilage

Next, we sought to study the potential roles of WNT5A in TMJ cartilage by using *ex vivo* cultured rat primary condylar chondrocytes and human chondrocyte line SW1353 cells. The expression of WNT5A in normal condylar cartilage tissue and chondrocytes was examined by immunostaining and Western blotting. WNT5A was mainly expressed in the proliferating and mature cell layers of normal rat condylar cartilage at 10 weeks of age (Fig. 1D). The expression of WNT5A was further confirmed in *ex vivo* cultured primary condylar chondrocytes from 5- and 10-week-old SD rats and in human SW1353 cells (Fig. 2A and B). Secreted WNT5A protein in the media of these cells was also detected by Western blotting (Fig. 2C).

3.3. WNT5A regulates condylar chondrocyte proliferation

Elevated chondrocyte proliferation is a feature of early osteoarthritis, by which articular cartilage attempts self-repair to block the structural breakdown (Dijkgraaf, de Bont, Boering, & Liem, 1995; Goldring, 2012; Haskin, Milam, & Cameron, 1995; Wadhwa, Embree, Kilts, Young, & Ameye, 2005). Increased condylar chondrocyte proliferation has also been displayed in the mouth-opening induced TMJ osteoarthritis-like changes (Fujisawa et al., 2003; Sobue et al., 2011). WNT5A purified protein significantly promoted the proliferation of *in vitro* cultured primary rat condylar chondrocytes determined by BrdU incorporation and WST-8 assays (Fig. 3A). Meanwhile, overexpression of the *WNT5A* gene in the human SW1353 chondrocyte line also elevated the cell proliferation (Fig. 3B). Conversely, siRNA knockdown of *WNT5A* in primary rat condylar chondrocytes significantly inhibited cell proliferation (Fig. 3C). Interestingly, introduction of purified WNT5A protein into the medium of *WNT5A*-knockdown condylar chondrocytes rescued the reduced cell proliferation (Fig. 3D), suggesting that decreased cell proliferation due to *WNT5A* siRNA results from the impaired WNT5A secretion rather than other indirect effects of silencing the intrinsic *WNT5A* gene.

3.4. Involvement of c-MYC and cyclin D1 in regulation of chondrocyte proliferation by WNT5A

Previous reports showed that WNT5A activates the JNK signaling pathway in chondrocytes (Ge et al., 2009; Ryu & Chun, 2006), and JNK signaling can regulate cell cycle genes c-MYC and Cyclin D1 (Gururajan

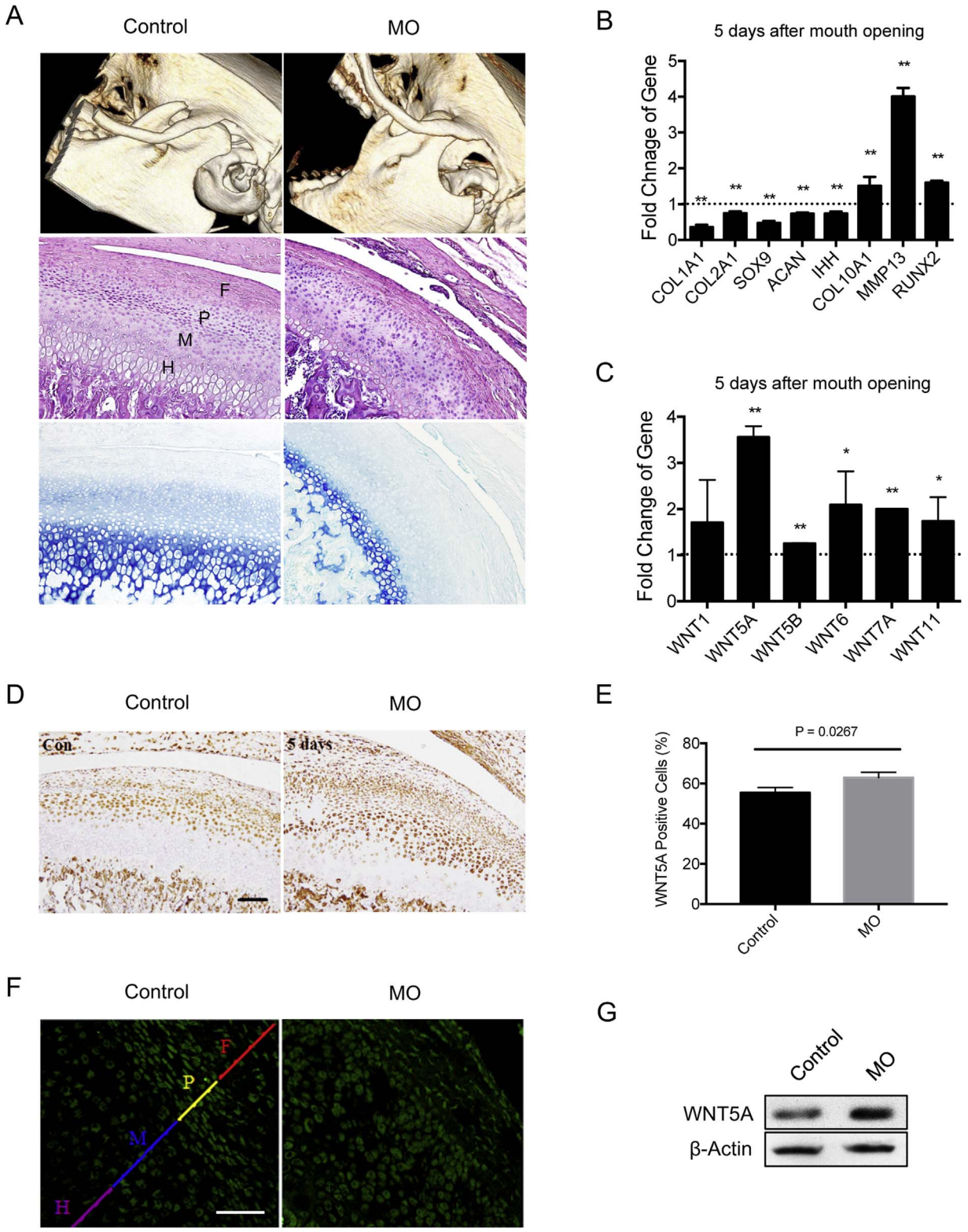

Fig. 1. Upregulation of WNT5A in condylar cartilage of the rat early TMJ osteoarthritis. (A) Micro-CT, H & E and toluidine blue stain of TMJ condylar cartilage from control and experimental animals after mouth-opening (MO) for 5 days. The normal condylar cartilage (Control) is divided into 4 different cell layers: fibrous (F), proliferating (P), mature (M) and hypertrophic (H). Original magnification, 200×. Images are representative of 8 animals in each group. (B) Real-time PCR determining fold changes of chondrocyte-related genes in the TMJ condylar cartilage after 5 days of mouth-opening. n = 5 animals for each group. (C) Real-time PCR determining fold changes of WNT genes in condylar cartilage of the early TMJ osteoarthritis induced by steady mouth-opening. n = 5 animals for each group. (D and E) Immunohistochemical staining of WNT5A and quantification of WNT5A positive cells in the rat TMJ condylar cartilage after the mouth-opening procedure. Scale bar, 50 μm. (F) Immunofluorescence staining of WNT5A in the rat condylar cartilage. Scale bar, 50 μm. (G) Western blotting confirming the upregulation of WNT5A in TMJ condylar cartilage after 5 days of mouth-opening. Protein of condylar cartilage was pooled from 5 rats in each group. This experiment was repeated twice. All data are mean ± SD. *, $P < 0.05$; **, $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

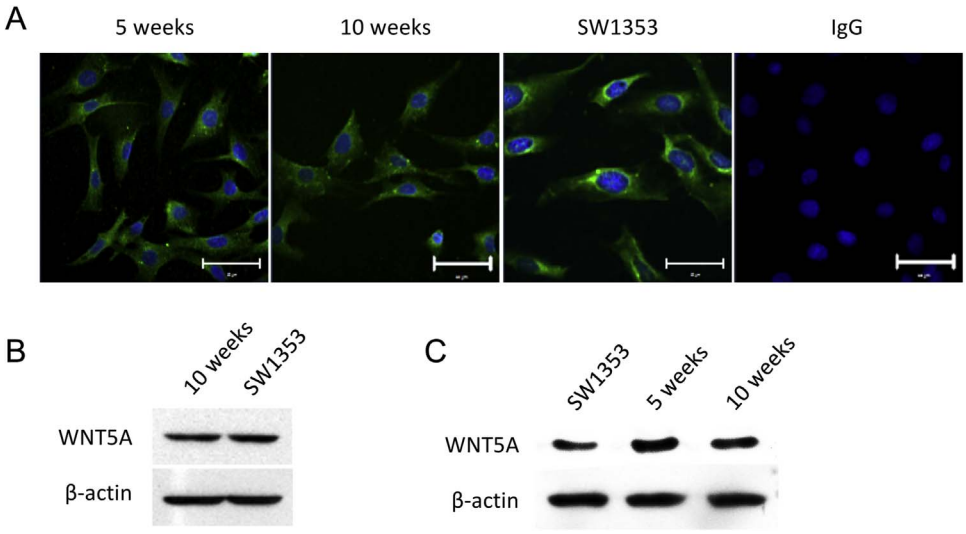

Fig. 2. Expression of WNT5A in TMJ condylar chondrocytes. (A) Immunofluorescence staining of WNT5A in primary condylar chondrocytes from 5-week or 10-week old rats and in SW1353 cells. Scale bar, 50 μm. (B) Western blot determining WNT5A expression in primary condylar chondrocytes from 10-week-old rats and in SW1353 cells. (C) Western blot determining secreted WNT5A in the culture media of human SW1353 cells and primary condylar chondrocytes of 5-week or 10-week old rats.

et al., 2005; Schwabe et al., 2003). To dissect the mechanism of WNT5A regulation of chondrocyte proliferation, expression levels of c-MYC and Cyclin D1 were examined after activation or inhibition of WNT5A signaling in SW1353 cells. Remarkably, overexpression or knockdown of *WNT5A* dramatically upregulated or downregulated the expression of c-MYC and Cyclin D1, as determined by western blotting (Fig. 4A). Similarly, in rat primary condylar chondrocytes, WNT5A purified protein increased the expression of c-MYC and Cyclin D1, and a specific JNK inhibitor, SP600125, inhibited the upregulation of c-MYC and Cyclin D1 by WNT5A (Fig. 4B), suggesting an involvement of JNK pathway in regulation of the cell cycling genes by WNT5A. Meanwhile, the JNK inhibitor SP600125 can also block WNT5A-induced condylar chondrocyte proliferation in a dose-dependent manner (Supplemental Fig. 2).

The effect of WNT5A on the promoter activity of c-MYC and Cyclin D1 was evaluated by luciferase reporter assay. Activating or blocking WNT5A signaling by the purified WNT5A protein or a specific anti-WNT5A antibody efficiently increased or decreased the promoter activity of *c-MYC* and *Cyclin D1* (Fig. 4C and D). Consistent with these *in vitro* findings, an upregulation of *c-MYC* and *Cyclin D1* expression was observed in the condylar cartilage of rat early TMJ osteoarthritis after 5-day mouth-opening (Fig. 4E). Together, these results suggest an involvement of cell cycle genes c-MYC and Cyclin D1 in the regulation of chondrocyte proliferation by WNT5A.

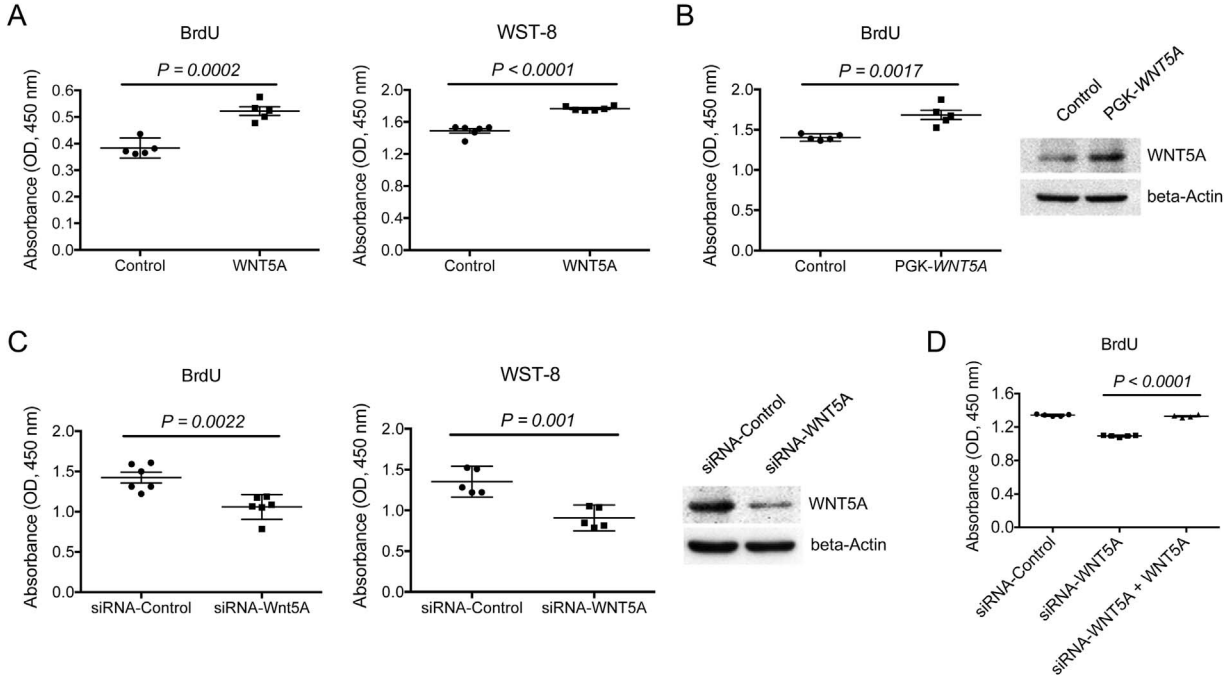

Fig. 3. WNT5A regulates condylar chondrocyte proliferation. (A) BrdU and WST-8 assays examining the proliferation of primary rat condylar chondrocytes after treatment with purified WNT5A protein or control for 24 h. (B) Proliferation analysis of SW1353 cells by BrdU incorporation after transfecting a mammalian WNT5A expression vector (PGK-WNT5A) or an empty vector (Control) for 48 h. The overexpression of WNT5A is confirmed by Western blot (right panel). (C) BrdU and WST-8 assays determining the proliferation of primary rat TMJ condylar chondrocytes after transfecting siRNA-Control or siRNA-WNT5A for 48 h. Knockdown of WNT5A was confirmed by Western blot (right panel). (D) Proliferation analysis of primary rat condylar chondrocytes by BrdU incorporation 24 h after introducing WNT5A protein (200 ng/ml) into the media following transfection with siRNA-WNT5A for 36 h. One-way ANOVA followed by Tukey's test for multiple comparisons was performed. All data are mean ± SD of 4–6 replicates per group. The results are representative of three independent experiments.

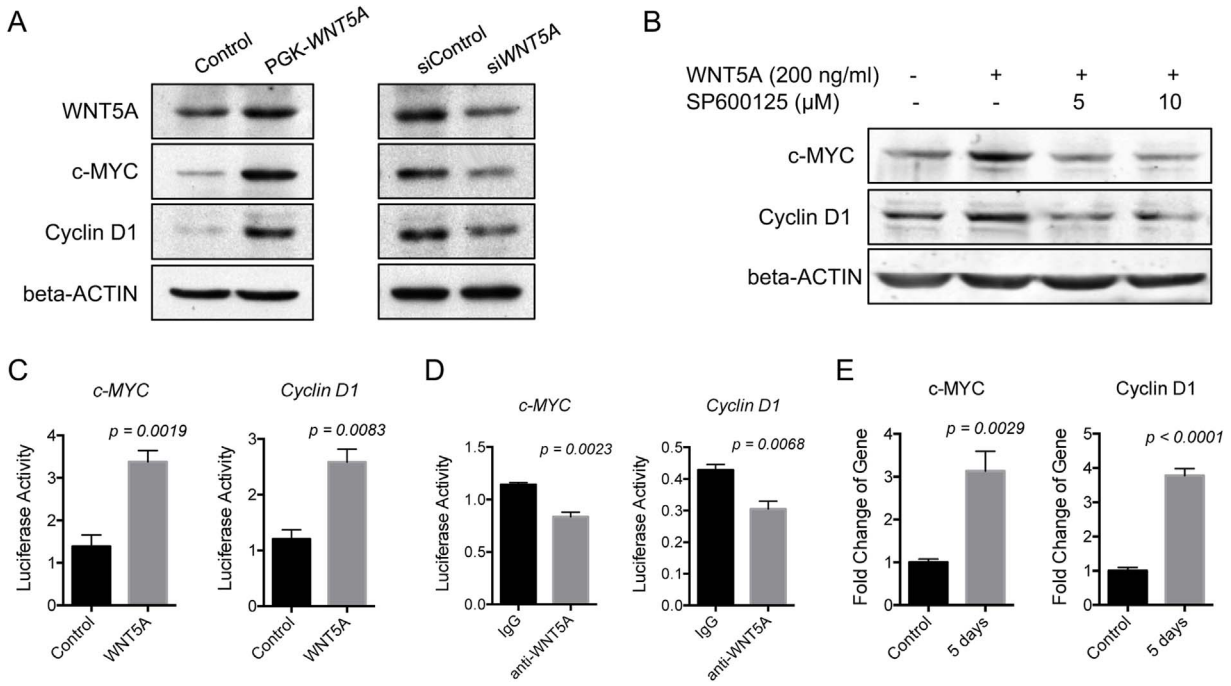

Fig. 4. Involvement of c-MYC and Cyclin D1 in regulation of chondrocyte proliferation by WNT5A. (A) Western blots examining the expression of c-MYC and Cyclin D1 in SW1353 cells transfected with PGK-WNT5A (left panel) or WNT5A siRNA (siWNT5A, right panel). Cells were collected 48 h post-transfection. (B) Western blots showing the effect of the JNK inhibitor SP600125 on WNT5A-meidated upregulation of c-MYC and Cyclin D1 in primary rat TMJ condylar chondrocytes. (C and D) Transcription activity of c-MYC and Cyclin D1 in SW1353 cells transfected with c-MYC-Luc and Cyclin D1-Luc constructs and then treated with WNT5A protein (C) or WNT5A blocking antibody (D) for 36 h. (E) Real-time PCR analyzing the expression of *c-MYC* and *Cyclin D1* in the rat TMJ condylar cartilage after steady mouth-opening for 5 days. All data are mean ± SD of 3–5 replicates per group. Results shown are representative of three independent experiments.

3.5. WNT5A promotes condylar chondrocyte hypertrophy

Chondrocyte hypertrophy, characterized by decreased expression of chondrocyte-elated genes like COL2A1, ACAN, IHH and increased expression of hypertrophic markers, including COL10A1 and MMP13, occurs in both early and late osteoarthritis (van der Kraan & van den Berg, 2012). Real-time PCR analysis displayed a significant decrease of *COL2A1*, *ACAN* and *IHH* expression, and increase of *MMP13* expression in rat primary condylar chondrocytes treated with WNT5A purified protein for 24 h (Fig. 5A). Knocking down *WNT5A* by siRNA inhibited the expression of *COL2A1* and *IHH*, while *ACAN* and *MMP13* were not affected (Fig. 5B). These results suggest that elevated WNT5A is enough to cause condylar chondrocyte hypertrophy by inhibiting COL2A1, ACAN and IHH and promoting MMP13 expression. In the physiological conditions, WNT5A is only necessary for COL2A1 and IHH expression, but not for the expression of ACAN and MMP13. There were no changes of other fibrochondrocyte related genes including *COL1A1*, *SOX9*, *COL10A1* and *RUNX2* after activating or inhibiting WNT5A signaling in rat condylar chondrocytes. Involvement of the JNK pathway in the regulation of chondrocyte genes by WNT5A has been reported in previous studies (Ge et al., 2009; Ryu & Chun, 2006), suggesting that the JNK pathway also mediates the regulation of condylar chondrocyte

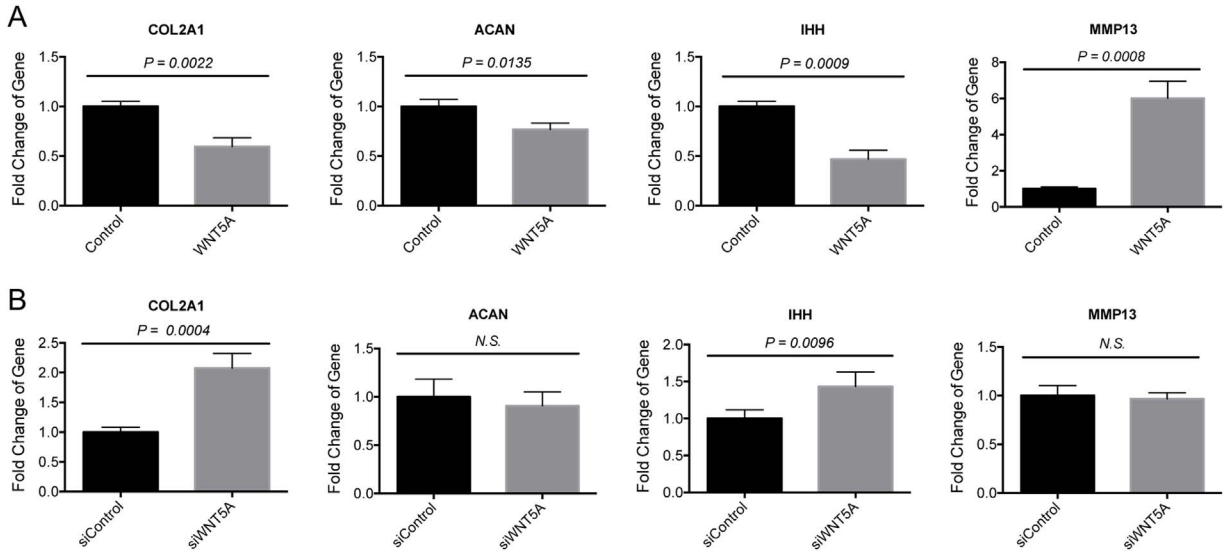

Fig. 5. WNT5A promotes condylar chondrocyte hypertrophy. (A) Real-time PCR examining the expression of *COL2A1*, *ACAN*, *IHH* and *MMP13* in rat primary condylar chondrocytes treated with WNT5A protein for 24 h. (B) Expression of *COL2A1*, *ACAN*, *IHH* and *MMP13* genes in rat primary condylar chondrocytes 48 h after transfecting with siRNA-Control (control) or siRNA-WNT5A (siWNT5A). All data are mean ± SD of 3 independent experiments. *N.S.*, not significant.

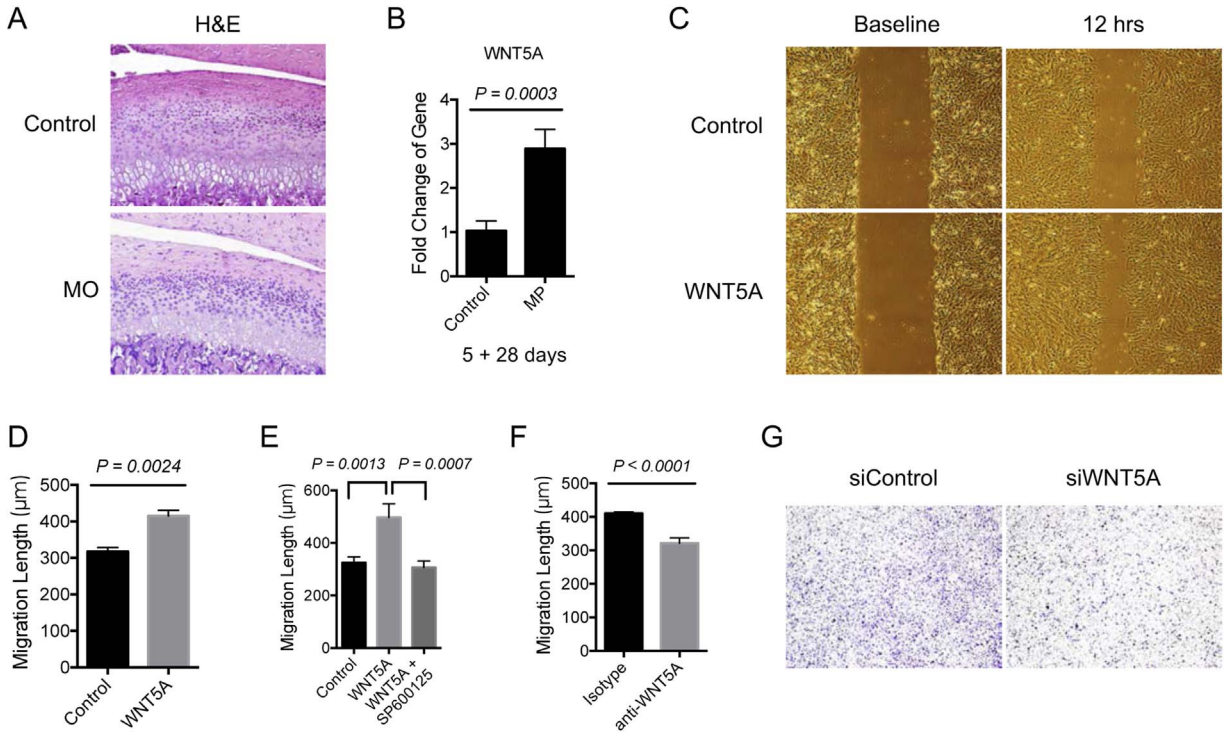

Fig. 6. WNT5A regulates chondrocyte migration. (A) Histology (H & E stain) of TMJ condylar cartilage from control and experimental animals after a 28-day recovery period after 5-day mouth-opening (MO) (5 + 28 days). Original magnification: 200×. Images are representative of 6 animals per group. (B) Real-time PCR showing upregulation of *WNT5A* in TMJ condylar cartilage at the time 5 + 28 days. (C and D) Wound healing assay showing increased cell migration after treatment with WNT5A protein for 12 h. (E) The JNK specific inhibitor SP600125 (5 μM) inhibits WNT5A-dependent cell migration in SW1353 cells. One-way ANOVA followed by Tukey's test for multiple comparisons was performed. (F and G) Wound healing (F) and transwell migration (G) assays demonstrating decreased cell migration after knockdown of WNT5A by siRNA in SW1353 cells. All data are mean ± SD of 4–5 replicates per group. Results shown are representative of three independent experiments.

hypertrophy by WNT5A.

3.6. *WNT5A facilitates chondrocyte migration*

The long-term impact of the early TMJ osteoarthritic changes after repetitive mouth-opening in rats remains understudied. Interestingly, 4 weeks later, without mouth-opening, histology showed an adaptive remodeling in the rat TMJ condylar cartilage, including an increased thickness of the hypertrophic cell layer and return of the four-layer alignment of chondrocytes (Fig. 6A). Expression of *WNT5A* was still elevated at this time (Fig. 6B). In addition, the cell cycle genes *c-MYC* and *Cyclin D1* remained upregulated in the rat condylar cartilage (Supplemental Fig. 3A), suggesting that increased cell proliferation during this period may contribute to condylar cartilage repair. Notably, except for increased chondrocyte proliferation and hypertrophy, over-activated WNT5A signaling may also regulate chondrocyte migration to remodel the normal cell alignment in TMJ condylar cartilage.

The wound healing assay using SW1353 cells demonstrated that WNT5A significantly promoted chondrocyte migration after treatment with WNT5A purified protein for 12 h (Fig. 6C and D). This effect was inhibited by the JNK specific inhibitor SP600125 (Fig. 6E), suggesting that JNK signaling mediates the effect of WNT5A on chondrocyte migration. Meanwhile, inhibition of WNT5A by blocking antibody or siRNA in SW1353 cells blocked chondrocyte migration by wound healing and transwell migration assays (Fig. 6F and G). The influence of WNT5A on chondrocyte migration was further verified in primary TMJ condylar chondrocytes by the wound healing assay (Supplemental Fig. 3B). These results indicate a role for WNT5A in regulating chondrocyte migration, which may facilitate condylar cartilage repair in early TMJ osteoarthritis.

4. Discussion

Here we report a novel role of WNT5A in TMJ condylar chondrocytes. Our results show that WNT5A is significantly upregulated during the condylar cartilage changes of rat early TMJ osteoarthritis induced by mouth-opening. Increased WNT5A activity can promote chondrocyte proliferation, hypertrophy and migration through the JNK signaling pathway. Thus, WNT5A may facilitate condylar cartilage repair through these effects.

The TMJ osteoarthritis-like model by steady mouth-opening is noninvasive and finite, and the external mechanical loading is quantifiable (Fujisawa et al., 2003; Kawai et al., 2008; Tanaka et al., 2005). Importantly, this model mimics the early changes of condylar cartilage and represents a subset of TMJ conditions resulting from increased loading by altered occlusion or musculoskeletal function in the craniofacial region. However, it is not clear whether these early osteoarthritic changes are enough to cause advanced osteoarthritis characterized by cartilage deterioration. In the present study, our results show that four weeks after the mouth-opening procedure, the cartilage are mostly repaired, suggesting that this model represents a very early, reversible change of condylar cartilage. This model may help to further characterize the mechanism of condylar cartilage remodeling and lead to new approaches for cartilage repair and regeneration.

The effect of WNT5A on cell proliferation is complex, depending on different cell types. Inactivation of WNT5A results in reduced proliferation rate of the progenitor cells in the developing limb (Yamaguchi, Bradley, McMahon, & Jones, 1999) but leads to an increased level of cell proliferation during distal lung morphogenesis (Li et al., 2005; Li, Xiao, Hormi, Borok, & Minoo, 2002) and mammary tissue (Roarty & Serra, 2007). It appears to exert opposite roles in the regulation of cell proliferation in different regions of the developing palate (He et al., 2008). In addition, the role of WNT5A in cancer cell proliferation has been extensively researched and is still controversial

(Pukrop & Binder, 2008). While some reports demonstrate WNT5A as a tumor suppressor through inhibiting cell proliferation (Kremenevskaja et al., 2005; Liang et al., 2003; Ying et al., 2008), other studies have showed that WNT5A induces cell proliferation as an oncogenic gene (Huang et al., 2005; Masckauchan et al., 2006). WNT5A has also been shown to promote self-renewal of stem cells (Yeh, Zhang, & Nagano, 2011) and promote angiogenesis through inducing endothelial cell proliferation (Masckauchan et al., 2006). In this study, our results provide the first evidence that WNT5A is indispensable to maintenance of normal condylar chondrocyte proliferation, as silencing WNT5A by siRNA inhibits proliferation. Meanwhile, elevated WNT5A level can facilitate chondrocyte proliferation, which is crucial for condylar cartilage repair and regeneration.

Notably, in the present study, a high concentration of SP600125 (10 μM) demonstrates a stronger effect in inhibiting condylar chondrocyte proliferation (Supplemental Fig. 2) than blocking WNT5A-induced c-MYC and Cyclin D1 expression (Fig. 4B). A possible reason is that other molecules, like alternative cyclin genes, cyclin-dependent kinases (CDKs) and CDK inhibitors, may be involved in WNT5A regulation of chondrocyte proliferation. In addition, the high concentration of SP600125 might inhibit the baseline proliferation of chondrocytes *via* a mechanism independent of c-MYC and Cyclin D1 (Du et al., 2004), and thus results in a more potent inhibition of cell proliferation than that on WNT5A-induced c-MYC and Cyclin D1 expression. Our results in this study demonstrate that SP600125 at 5 μM is enough to block WNT5A-induced condylar chondrocyte proliferation and the expression of c-MYC and Cyclin D1, providing critical evidence for the implication of JNK pathway in the pro-proliferative activity of WNT5A.

Gene expression data in the present study show that WNT5A promotes condylar chondrocyte hypertrophy (Fig. 5). This phenotype coincides with that of osteoarthritic chondrocytes (van der Kraan & van den Berg, 2012). The inhibition of COL2A1 by WNT5A in condylar chondrocytes is consistent with previous reports in limb articular chondrocytes (Ryu & Chun, 2006). Importantly, our results suggest that chondrocyte hypertrophy in the mature articular cartilage is not unique to pathological conditions like osteoarthritis, but also may contribute to cartilage repair by increasing the thickness of the hypertrophic cell layer in the TMJ condylar cartilage. It should be highlighted that the role of WNT5A in osteoarthritic cartilage deterioration remains unclear. A recent study reported that WNT5A is upregulated in osteoarthritic cartilage of both human and mouse knees (Nalesso et al., 2013). Thus, it is possible that elevated WNT5A boosts cartilage degradation by regulating chondrocyte hypertrophy and expression of MMPs (Ge et al., 2009; Ryu & Chun, 2006) after sustaining biomechanical or biochemical factors that initiate the process of articular cartilage degradation. Future studies should carefully examine the expression and function of WNT5A in condylar cartilage of advanced TMJ osteoarthritis.

The migration of articular chondrocytes is receiving more attention, especially in the setting of cartilage repair (Morales, 2007; Onuora, 2015; Schubert, Kaufmann, Wenke, Grassel, & Bosserhoff, 2009). Human osteoarthritic cartilage explants or chondrocytes, as well as numerous animal models for articular cartilage injury, have been utilized to characterize chondrocyte migration (Hopper, Henson et al., 2015; Hopper, Wardale et al., 2015). However, chondrocyte migration in physiologic conditions remains controversial, as the hyaline cartilage in limb joints contains condensed extracellular matrix and seems difficult to penetrate (Akkiraju, 2015). TMJ condylar cartilage is a fibrocartilage and contains rich cells that align into different layers. Importantly, the condylar cartilage of the TMJ demonstrates robust remodeling activity in response to functional changes of orofacial muscles and dental occlusion. In addition, the fibrocartilage of TMJ condyles is softer than the limb hyaline cartilage (Tanaka, Detamore, & Mercuri, 2008) and thus might be more conducive to chondrocyte migration under physiological and pathologic conditions. Indeed, repair of rat condylar cartilage in early TMJ osteoarthritis in the present study strongly suggests that chondrocyte migration may be involved in this process, by which the disorganized chondrocytes return to the normal array. The TMJ condylar cartilage may therefore represent a valuable tissue model for *in vivo* chondrocyte migration.

In conclusion, our results show a critical role for WNT5A in TMJ chondrocyte biology by regulating cell proliferation, hypertrophy and migration. Future studies should carefully characterize the roles of WNT5A in the pathological conditions of TMJ, like osteoarthritis, and modifying WNT5A activity as a potential therapy for TMJ cartilage diseases warrants exploration.

Conflict of interest

The authors declare that no conflict of interest exists.

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Ethical approval

Animal procedures in this study were approved by the Institutional Animal Care and Use Committee at Peking University Health Science Center. Reference number: LA2010-003.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2017.06.019.

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