Wnt/PCP-YAP-BIRC2 axis maintains cartilage stem/progenitor cell homeostasis in osteoarthritis

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Research Article

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Abstract

Background

The cartilage stem/progenitor cells (CSPCs) play a critical role in maintaining cartilage homeostasis. However, the regulation of CSPC function in the microenvironment of Osteoarthritis (OA) is not fully understood.

Methods

The joint uid of 10 OA patients were collected and analyzed for WNT signaling. CSPCs derived from these patients were isolated, identified, and evaluated for cellular functions. The effect of non-canonical WNT signaling and Yes-associated protein (YAP) expression on CSPCs was investigated in vitro. The OA rat model was established by Hulth's method. Lentivirus-mediated YAP (Lv-YAP) or lentivirus-mediated YAP RNAi (Lv-YAP-RNAi) was injected intra-articularly to modulate YAP expression in rat joint. In addition, allogeneic CSPCs overexpressing or silencing YAP were transplanted by intra-articularly injection. We also evaluated the functions of CSPC and the OA-related cartilage phenotype in the rat model. Finally, the transcriptome of OA-CSPCs overexpressing YAP was examined to explore the downstream targets of YAP in CSPC.

Results

OA-CSPCs exhibited impaired function in the microenvironment dominated by Wnt/β-catenin signaling and activated function in Wnt/PCP signaling. Wnt5A/B improved the proliferative activity and migration ability of OA-CSPCs. In OA-CSPCs, Wnt/PCP signaling upregulated YAP, which maintained the homeostasis and functions of CSPCs. Upregulation of YAP in the joint delayed OA progression and improved the cartilage regeneration capacity of CSPCs. Using transcriptomic analysis, we found that YAP regulates CSPC function by upregulating Baculoviral IAP repeat-containing 2 (BIRC2). Furthermore, knockdown of BIRC2 significantly impaired the cellular functions of CSPCs and largely blocked the regulation of YAP on CSPCs.

Conclusion

In this study, dysregulation of the Wnt/PCP-YAP-BIRC2 signaling axis contributes to OA-CSPC dysfunction, impairs cartilage's intrinsic reparative ability and accelerates OA progression. This provides a potential therapeutic target for OA.

Background
Osteoarthritis (OA) is a disease characterized by cartilage degeneration affecting the whole joint. Nowadays, articular cartilage is no longer considered a kind of tissue lacking intrinsic reparative ability but is in a dynamic balance between micro-damage and regeneration\[1, 2\]. The regenerative capacity of cartilage primarily originates from cartilage stem/progenitor cells (CSPCs)\[3\]. In 2003, a cell population with multi-differentiation potential was first isolated from the superficial zone of bovine articular cartilage\[4\]. Alsalameh et al. isolated a cell with similar properties to bone marrow mesenchymal stem cells in human normal and OA cartilage. Subsequently, these cells were identified as CSPCs, which mainly reside in the superficial zone of cartilage\[5\]. The proportion of CSPCs in normal cartilage is about 4% and increases to more than 8% under the stimulation of injury and degeneration\[6\], where they play a critical role in maintaining cartilage homeostasis\[7\]. In response to injury, CSPCs are activated and migrate to the cartilage injury area for tissue regeneration through proliferation, differentiation, and production of new extracellular matrix. Furthermore, CSPCs release various functional molecules, such as lubricin, that regulate surrounding cells and create a beneficial microenvironment for tissue regeneration. However, the role of CSPC in the pathomechanism of OA is not fully understood\[8, 9\]. Some studies have observed the aggregation of CSPCs in the cartilage fissures in early OA\[10–12\]. It was also found that CSPCs derived from some OA patients exhibited a premature senescence phenotype\[13\], which is similar to those residual stem-cell failures observed in other age-related diseases\[14\]. Together, these suggest an association between the dysfunction of CSPCs and the development of OA.

Wnt signaling is a highly conserved pathway involved in organogenesis and stem cell maintenance\[15–17\]. Notably, it is a key regulator of stem cell function. Generally, Wnt signaling is divided into the dependent and independent β-catenin canonical pathways and independent noncanonical pathway. The balance between these two signals maintains joint homeostasis\[18\]. In OA, this balance is dysregulated, leading to the overactivation of the canonical Wnt signaling, whereas the noncanonical signaling is inhibited \[19–22\]. Consequently, this dysregulation may impair the function of stem cells residing in the microenvironment. For example, it has been found that the overactivation of canonical Wnt signaling leads to the failure of hematopoietic stem cells [23]. Therefore, the dysregulation of Wnt signaling can potentially impair the function of CSPCs and promote OA progression. In this study, we explored this issue and investigated the critical role of the Wnt/PCP-YAP-BIRC2 signaling axis in maintaining the homeostasis of CSPCs and OA cartilage.

**Material And Methods**

**Human cartilage and synovial fluid sample collection**

Human OA cartilage tissue and synovial fluid (SF) from total knee arthroplasty (n=10, female, average ages 76.2 yrs.). The SF samples were centrifuged at 3,000 x g for 10 min at 4 °C, and the supernatant was collected. The BCA Protein Assay Kit (P0012S, Beyotime, China) was used to measure the total protein concentrations. The protein level of β-catenin, c-Jun N-terminal kinase (JNK) and p-JNK, Calmodulin dependent kinase II (CAMK II) and p- CAMK II, Protein kinase C (PKC) and p-PKC in SF were detected by Western blotting.
CSPC isolation and identification

Cartilage tissue was washed, cut into 1-2 mm² fragments, and digested by 0.2% collagenase type II (Sigma, USA) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, USA) for 4 hours. The isolated cells were seeded into six-well plates (Corning, NY) coated with 10 µg/ml of fibronectin (Solarbio, China) in DMEM/F-12 medium with penicillin (100 U/ml), streptomycin (100 µg/ml, Gibco, USA) and 10% fetal bovine serum (FBS, Gibco, USA) for 20 minutes. The nonadherent cells were removed. Adherent cells were collected for further identification. Multipotential differentiation assays and flow cytometry were used to identify CSPC. Cells in Passage 3 were plated in 6 well plates (2×10⁴/well) and cultured for 14 days in OriCell osteogenic or adipogenic culture medium (Cyagen, USA). Then cells were stained with alizarin red S or an oil red solution. 3×10⁵ cells were put into a 15ml conical tube, centrifugated into pellets, and cultured for 21 days in OriCell chondrogenic culture medium (Cyagen, USA) and stained with Alcian blue. Antibodies against CD44, CD90, CD31, and CD45 (Thermofisher, USA) were used for flow cytometry (BD FACSCalibur, USA). 1×10⁵ cells were suspended in 500 µl PBS containing 20 µg/ml antibody before analyses.

5-ethyl-2'-deoxyuridine (EdU) incorporation assay

Proliferating cells were determined by the EdU Imaging Kits (APExBIO, USA). Cells were incubated with 10 µM EdU for 2 hours before EdU staining, and cell nuclei were stained with Hoechst 33342 at 5 µg/mL for 30 minutes. The cells were counted in five random fields per well. The percentage of EdU-positive cells was calculated using Image-Pro Plus version 6.0 for Windows (Media Cybernetics, USA). For detecting proliferating cells in the rat OA model, 100 µl 10 µM EdU was injected intra-articularly on day 38. Four days later, rats were sacrificed, and the joints were harvested. Paraffin sections were evaluated using the EdU Kit and stained with Hoechst 33342. Each paraffin section was counted in five random fields.

Transwell migration assay

Cell migration assays were performed using 24-well transwell plates with polycarbonate membranes with an 8 µm pore size (Corning, USA). CSPC in DMEM/F12 was added to the upper chamber at a density of 2.5×10⁵ (200 µl/well), and the lower chamber was filled with 10% FBS in the culture medium. Cells that did not penetrate the membrane were removed. Cells were stained with 0.1% crystal violet and counted in six random fields per well.

Lentivirus infection and small interfering RNA (siRNA) transfection

Lv-YAP, Lv-YAP-RNAi, and Lv-Vector were synthesized and packaged by Genechem (Shanghai, China). The lentivirus infected rat OA CSPCs following the manufacturer's protocol. Si-YAP, si-Birc2, and nonspecific control siRNA (si-Ctrl) were synthesized by JTSBio (Wuhan, China). The siRNAs were transfected into rats OA-CSPC using jetPRIME transfection reagent (Polyplus, France) following the manufacturer's protocol. The siRNAs sequences are shown in Table.1.
Table 1. RNA oligo sequences for transfection

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<th>Name</th>
<th>RNA oligo sequences (5' to 3')</th>
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<td>si-YAP1-2</td>
<td>CCGGGAUGACUCAGGAAUUTT AAUUCUCUGAGUCAUCCCGGTT</td>
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<td>si-Yap1-2</td>
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<td>si-Birc2-3</td>
<td>UCAUUAUCUGAUCUCGUUCAUCCA GAUGAGAUUCAGAAUAUGAAG</td>
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The co-culture of OA-CSPC with OA-chondrocyte

A co-culture system used six-well transwell plates (Corning, USA). 2.5×10^5 CSPC, CSPC(YAP+), or CSPC(YAP-) were seeded in the upper compartments, and 2.5 × 10^5 OA-chondrocyte were cultured in the lower compartments in DMEM/F12 with 10% FBS. Co-cultures were maintained for 7 days before evaluation.

Animal experiments

Hulth's surgery was performed on 8-week-old male rats. For YAP regulation, 20μl lentivirus expressing YAP (Lv-YAP) or YAP-RNAi (Lv-YAP-RNAi) was injected intra-articularly 7 days after surgery. For CSPC
transplantation, 100μl PBS containing 5×10^6 allogeneic CSPCs was injected intra-articularly 7 days after surgery. CSPCs were infected or transfected with Lv-YAP or si-YAP 3 days before transplantation. The rats were sacrificed for evaluation at weeks 2, 4, or 6.

**Histological evaluation**

The joint is fixed with 4% Paraformaldehyde and decalcified for 1 month before being dehydrated and embedded with paraffin. Paraffin sections were stained for histological evaluation using safranin O-fast green, anti-CD44 (15675-1-AP, Proteintech, China), anti-CD90 (66766-1-lg, Proteintech, China), anti-YAP (AF6328, Affinity, China), anti-Collagen II (Col II,GB11021, Servebio, China), anti-Collagen I (Col I,bs-0578R, Bioss, China) and anti-Collagen X (Col X,DF13214, Affinity, China). Secondary antibodies were detected using a fluorescent secondary antibody (Proteintech, China) or Rabbit streptavidin-biotin detection system kit (ZSGB-Bio, China) according to the manufacturer's protocol. Joint pathology was quantified using the Osteoarthritis Research Society International (OARSI) scoring system.

**RNA-sequencing and functional annotation**

5×10^6 cells were infected with or without Lv-YAP for 72 h and then subjected to further RNA preparation, library construction, sequencing, and analysis. Genes with an adjusted P < 0.05 and an absolute value of |log2(fold change)| > 1 found by the “limma” R package were assigned as differentially expressed genes (DEGs). Gene Ontology (GO) term enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and annotation were conducted with “clusterProfiler” and “org.Hs.eg.db” R packages. Expression heatmaps were visualized with “pheatmap” R package.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

RT-qPCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) according to the manufacturer's protocol. An initial denaturation step was carried out at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. Gene transcriptional levels were normalized to those of β-actin and calculated using the log2–ΔΔCt method. The primer sequences are shown in Table.2.

**Table.2.** Gene-primer sequences for RT-qPCR
<table>
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<th>Forward primer(5’ to 3’)</th>
<th>Reverse primer(5’ to 3’)</th>
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<td>MMP3</td>
<td>CTCACAGACTGACTCGGTT</td>
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<td>ACCAGAAGCTTTGCTCTG</td>
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<td>BMP4</td>
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<td>TCF4</td>
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<td>CCCTGAGCCGAGATGAATCC</td>
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<td>RYK</td>
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<td>FZD7</td>
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<td>RUNX2</td>
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<td>CCCGTTACAGCCTCCTCTAC</td>
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</tr>
<tr>
<td>Birc2</td>
<td>GGGACAGTCCGGAAGGC</td>
<td>TCTTGAGGGCTTAAATCGCAG</td>
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</table>
Western bolting

Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in blocking buffer (5% skimmed milk) for 1 hour. The membranes were incubated overnight at 4°C with primary antibodies against β-catenin (8480S,CST, USA), JNK and p-JNK (BS3630 and BS4763, Bioworld, China), CAMK II and p-CAMK II (BS5510 and BS5009, Bioworld, China), PKC and p-PKC (AP0290, Bioworld, China), Lubricin (bs-11175R, Bios, China), YAP (AF6328, Affinity, China), fibroblast activation protein alpha(FAP,AF5344, Affinity, China), vitamin D receptor(VDR AF6159, Affinity, China), telomerase reverse transcriptase(TERT,DF7129, Affinity, China), matrix metallopeptidase 3 (MMP3,AF0217, Affinity, China), Focal adhesion kinase (FAK,BS6899, Bioworld, China), Col I (bs-0578R, Bios, China), Col II (GB11021, Servicebio, China), Col X (DF13214, Affinity, China), Birc2 (DF6167, Affinity, China), snail family transcriptional repressor 2 (Snai2,AF4002, Affinity, China), zinc finger E-box binding homeobox 2(Zeb2,AF5278, Affinity, China), cyclin D2(Ccnd2,AF5410, Affinity, China), and glyceraldehyde-3-phosphate dehydrogenase(GAPDH). The following day, the membranes were incubated with fluorophore-conjugated secondary antibody or HRP-conjugated secondary antibody at room temperature for 1 hour and developed in electrochemiluminescence (ECL) Western blot detection reagents (Biosharp, China). The band was analyzed using LI-COR Odyssey infrared imaging system (LI-COR Corp., NE) or UVP Chem studio PLUS 815 (Analytik Jena, Germany).

Statistical analysis

Values in the text and figures are expressed as the mean ± SD unless otherwise noted. Statistical significance was determined by Student’s t-test or analysis of variance (ANOVA) using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Spearman correlation analysis was applied to evaluate correlations among continuous variables. R version 4.0.3 was used for omics data analyses. Differentially expressed genes (DEGs) between two subclusters were calculated with the R package “limma” (log2 FC>1 and adjusted P < 0.05). P values in GO and KEGG enrichment analyses were adjusted using the “Benjamini & Hochberg” method. Differences with p < 0.05 were considered statistically significant.

Results

Wnt signaling has a critical impact on OA-CSPCs

To investigate the correlation between Wnt signaling and CSPC function in the OA microenvironment, the Wnt signaling in SF of 10 OA patients was evaluated, and the CSPCs from these patients were isolated and identified. These OA patients exhibited differential Wnt signaling patterns. A large proportion of patients (P1, P2, P5, P6, P9, and P10) revealed activation of canonical Wnt/β-catenin signaling, while others (P3, P4, P7, and P8; red arrows) exhibited a predominance of noncanonical Wnt/PCP signaling (Fig.1a; supplementary Fig.1a). The isolated cells were negative for endothelial cell markers CD31 and
CD45 and positive for mesenchymal stem cell markers CD45 and CD90 (Fig. 1b). Cells were successfully induced to undergo chondrogenic, osteogenic, and adipogenic differentiation (Fig. 1c). We evaluated the functions of CSPCs, including proliferative activity (EdU assay), migration ability (transwell migration assay), and lubricin production. We found a tendency that CSPCs exhibited impaired function in a microenvironment dominated by Wnt/β-catenin signaling (P1, P2, P5, P6, P9, and P10). In contrast, CSPCs demonstrated activated functions in a Wnt/PCP microenvironment (P3, P4, P7, and P8) (Fig. 1d, e). Spearman's analysis confirmed this correlation between Wnt signaling and CSPC function (Fig. 1f). Next, we treated OA-CSPCs with ligands for Wnt/PCP signaling (Wnt5A and 5B) and found that Wnt5A/B significantly improved the proliferative activity and migration ability of OA-CSPCs (Fig. 2a, b).

**YAP maintains the function of CSPCs**

To investigate the downstream activity by which Wnt/PCP signaling regulates CSPC function, we examined genes associated with the regulation of stem cell function. The mRNA expression of YAP, FAP, TERT, VDR, and MMP3 were increased in CSPCs after Wnt5A/B treatment (Fig. 3a), but only the protein level of YAP was found to be increased (Fig. 3b, c). In addition, we also observed the upregulation of YAP in OA CSPCs derived from patients with activated Wnt/PCP signaling (P3, P4) (Fig. 3d).

Next, we explored the role of YAP in OA-CSPCs. In the co-culture of OA-CSPC and OA-chondrocytes, CSPCs overexpressing YAP (+Lv-YAP) exhibited increased expression of lubricin and FAK. Meanwhile, OA-chondrocytes co-cultured with CSPCs exhibited an alleviated OA phenotype, including elevated Col II and decreased Col I and Col X. Notably, this anti-degenerative paracrine effect of CSPCs was significantly enhanced by overexpression of YAP. In contrast, YAP silencing impaired this effect of CSPCs on OA-chondrocytes (Fig. 4a; supplementary Fig. 1b). Additionally, YAP activated the proliferative activity (EdU assay) and migration ability (transwell migration assay). YAP also attenuated the cellular senescence (SA-β-Gal staining) of OA-CSPCs, while silencing YAP aggravated the dysfunction of OA-CSPCs (Fig. 4b, c, and d; supplementary Fig. 1c).

**YAP contributes to CSPCs for cartilage regeneration**

Hulth's surgical method induced cartilage injury in rats. 2 weeks after injury, YAP expression was upregulated and progressively enhanced in cells in the superficial zone of cartilage. Surface markers revealed that most of these YAP+ cells were CSPCs (CD44+CD90+) (Fig. 5a). By isolating CSPCs from injured cartilage, we detected upregulation of YAP and its representative target genes in CSPCs (Fig. 5c, d). Concomitant with YAP expression, CSPCs began to proliferate and increase in number after cartilage injury (Fig. 5a, b).

To investigate the contribution of YAP to cartilage homeostasis, we injected Lv-YAP or Lv-YAP-RNAi intra-articularly into OA rats models. Lv-YAP activated the proliferative activity (EdU positive, from 2.9% to 9%) and increased the number of CSPCs (CD44+CD90+, from 6% to 11%) in cartilage. In contrast, Lv-YAP-RNAi had the opposite effect (Fig. 6a; supplementary Fig. 1d). Lv-YAP significantly alleviated the OA phenotype of cartilage, including increasing Col II production, inhibiting Col I and X expression, and improving OARSI
score in Safranin O staining, while inhibition of YAP exacerbated OA progression (Fig.6b and supplementary Fig.1e).

To explore the role of YAP in CSPCs for cartilage regeneration, we injected CSPCs overexpressing YAP (+Lv-YAP), and YAP silenced CSPCs (+si-YAP) into the OA rat model. Intra-articularly, CSPC transplantation improved the OA phenotype of cartilage. To a large extent, this effect positively correlated with the expression of YAP in CSPC. Overexpressing YAP in CSPCs markedly reinforced its anti-degenerative effect on cartilage, while knocking down YAP impaired this effect (Fig.6c).

Transcriptomic analysis

RNA sequencing was used to investigate the transcriptome of OA-CSPCs overexpressing YAP. Compared to control CSPCs, a total of 4,723 DEGs, including 2,534 upregulated and 2,189 downregulated, were identified in CSPCs transfected with Lv-YAP. KEGG enrichment exhibited that these DEGs were primarily enriched in pathways associated with cell cycle, cellular senescence, autophagy, apoptosis, hippo signaling, and TNF signaling (Fig.7a). The detailed DEGs enriched in these pathways are shown in Figure.7b.

YAP regulates CSPC function by upregulating Birc2

Next, we examined the direct target genes of YAP -TEAD complexes in the transcriptome [24]. We identified four YAP target genes, including Birc2, Snai2, Zeb2, and Ccnd2, upregulated in the Lv-YAP-induced DEGs described above (Fig.7c). Notably, Western blotting showed that the protein level of Birc2 elevated markedly after Lv-YAP treatment (Fig.7d). We further examined the role played by Birc2 in regulating CSPC function. We identified that knockdown of Birc2 significantly impaired the cellular functions of CSPCs, including lubricin production, proliferative activity, and migration ability. More importantly, we found that Birc2 knockdown largely blocked the regulation of YAP on the cellular functions of CSPCs (Fig.8).

Discussion

Due to its structural characteristics of lacking blood vessels, articular cartilage has long been thought to lack the ability to repair. However, current studies suggest that cartilage has an intrinsic reparative ability associated with cartilage stem/progenitor cell (CSPC) [1–3]. Therefore, the role of CSPCs in OA still needs to be further investigated to better understand these physiological properties. One limiting aspect to these kinds of studies is that there are no widely recognized specific surface markers for CSPCs. Usually, CSPCs are identified by markers of mesenchymal stem cells. In several studies, CD44, CD71, CD90 and CD105 have been used to identify CSPCs[4, 6, 25]. This study used CD44 and CD90 as surface markers to identify CSPC (Fig. 1b, c). Notably, Seol et al. showed that 7–14 days after blunt mechanical impact injury to healthy cartilage explants, a class of migrating cell population appeared at the injury site around the ECM of the explants and showed characteristics of cartilage progenitor cells[8]. This suggests that CSPCs are involved in the repair of cartilage injury. A series of studies also confirmed that CSPCs play an
important role in cartilage repair. In healthy cartilage, CSPCs are generally found in the superficial zone of cartilage[5]. When damage occurs in cartilage, CSPCs respond to the biological stimuli, migrate to the cartilage damage to proliferate, and secrete collagen II, chitosan, and lubricin for joint resurfacing[8]. Lubricin also exerts a protective effect by inhibiting synovial cell hyperproliferation and suppressing chondrocyte senescence[26–28]. In this study, we found that CSPC activation enhanced gradually in the superficial zone of cartilage along with the progression of cartilage injury (Fig. 5a). Additionally, the percentage of CSPC in normal articular cartilage was about 4% and increased to about 8% in OA cartilage[6]. This change suggests that CSPCs are involved in the pathological process of OA. In early OA, aggregated CSPCs were found in the fissures of articular cartilage[10–12], implying that CSPCs exert a protective effect on articular cartilage by participating in the remodeling of the ECM. In late OA, due to the destruction of the ECM, CSPCs migrate between cartilage tissue, subchondral bone, and bone tissue[9], suggesting that it may play a role in information exchange. In this study, we co-cultured OA-CSPC with OA-chondrocytes and observed that after 7 days of co-culture, OA-chondrocytes showed an increase in Col II and a decrease in OA-related phenotypes such as Col I and Col X (Fig. 4a). In the OA rat model, we delayed the osteoarthritis process and OA-related phenotypes in cartilage by intra-articular injection of normal CSPCs (Fig. 6d). These findings suggest the involvement of CSPCs in the maintenance of cartilage homeostasis.

Although CSPCs are critical for maintaining cartilage health, little is known about regulating their function. Fellows CR et al. have shown that the presence of a premature failure phenotype of CSPC in OA joints[13] is similar to the stem cell failure in other age-related diseases[14]. In the present study, we found varying degrees of CSPC dysfunction in the cartilage of OA patients, including diminished proliferative, migratory, and paracrine capacities (Fig. 1d,e). We also found that the severity of their dysfunction was closely related to the dysregulation of the Wnt signaling in the microenvironment. Wnt signaling is highly conserved during evolution and regulates many fundamental developmental processes, including organogenesis and the maintenance and differentiation of progenitor and stem cell populations[15–17]. The canonical Wnt signaling functions through β-Catenin/TCF transcriptional activity (i.e., Wnt/β-Catenin pathway), whereas the noncanonical Wnt pathway, independent of β-Catenin, includes the PCP pathway and the calcium pathway. G Nalesso et al. have shown that fine-tuning the balance between canonical and noncanonical Wnt signaling in the joint contributes to the maintenance of tissue homeostasis[18]. However, activation of the canonical Wnt signaling and inhibition of the noncanonical Wnt signaling had been observed in OA joints[19–22]. In particular, excessive activation of the canonical Wnt signaling pathway led to chondrocyte hypertrophy, matrix mineralization, and increased expression of matrix metalloproteinase 13 (MMP-13), accelerating the OA process[19, 21, 22, 29, 30].Canonical Wnt signaling pathway inhibitors are currently considered promising disease-modifying OA drugs (DMOADs)[31]. The Wnt signaling is also essential for maintaining stem cell function, and its dysregulation can lead to stem cell dysfunction[15, 16, 32]. In hematopoietic stem cells, persistent activation of the canonical Wnt signaling pathway leads to failure of their hematopoietic function, inhibition of erythroid differentiation, inhibition of lymphatic system development, and inhibition of regenerative stem cell activity[23]. However, no studies point to a relationship between dysregulation of the Wnt signaling and CSPC dysfunction in
OA. In this study, we found Wnt/β-catenin pathway activation in most OA patients' joints (Fig. 1a), which is consistent with the dysfunctional situation of CSPC. In contrast, activation of the noncanonical Wnt/PCP pathway in OA CSPCs results in a certain degree of improvement in its function, including the enhanced proliferative and migratory capacity (Fig. 2a, b). This suggests that dysregulation of the Wnt signaling in the OA microenvironment may contribute to CSPC dysfunction and thus accelerate the progression of OA.

We screened the genes related to stem cell function under the activation of Wnt/PCP signaling. We observed that the transcriptional co-activators YAP was activated and translocated to the nucleus in CSPCs (Fig. 3c). It has also been noted that YAP is reduced in CSPSc, findings consistent with the inhibition of noncanonical Wnt signaling. YAP is a key effector downstream of the Wnt/PCP pathway[33], essential for regenerating multiple organs. Its ectopic activation in adult mice drives the overgrowth of specific organs, promotes dedifferentiation of mature cell types, and triggers the expansion of stem and progenitor cell pools[24, 33–35]. YAP is essential for maintaining stem cell functions in different species, including 1. YAP is necessary for organ growth and development; 2. YAP promotes stem cell reprogramming and maintenance of stemness; 3. YAP promotes stem cell repair functions; 4. YAP delays stem cell aging. However, the effect of YAP on CSPC function is still unclear. In this study, we observed differential YAP expression in the CSPCs of different OA patients. Dysregulation of Wnt signaling is likely to promote dysfunction of CSPCs in OA by affecting the expression of YAP. To further validate the effect of YAP on CSPC function, we regulated YAP expression within OA CSPCs. We found that along with increased YAP expression, the dysfunction was alleviated, and proliferation capacity, migration capacity and anti-senescence capacity were enhanced. In addition, overexpression of YAP in OA CSPCs improved its anti-degenerative paracrine effect to OA-chondrocytes. In contrast, knockdown of YAP further impaired the function of OA-CSPCs. These findings suggest that YAP is essential for the maintenance of CSPCs (Fig. 4). Furthermore, Fu et al. have shown that the expression of YAP in the superficial zone of cartilage gradually decreases with OA progression, and that upregulation of YAP expression in the OA model alleviates OA progression and delays cellular senescence in local bone joints[35]. In this study, we confirmed this finding and demonstrated that upregulation of YAP in the OA model maintains cartilage homeostasis by improving CSPC functions. By using transcriptomic analysis, we revealed that BIRC2 is a critical downstream target of YAP in CSPC (Fig. 7d, e). Previous studies showed that YAP induces BIRC2 in mammalian cells[36]. Some studies showed that BIRC2 is physically linked to YAP in mice (chromosome 9qa1) and humans (chromosome 11q22)[37, 38]. BIRC2 is a member of the anti-apoptotic protein family, which inhibits apoptosis and promotes proliferation by binding to the tumor necrosis factor receptor-associated factors TRAF1 and TRAF2. It belongs to the same family as BIRC5, which has recently been recognized as a new biological marker of CSPCs [39]. In support of this, Paulina Gil-Kulik et al. showed that Mesenchymal Stem Cells of the Umbilical Cord Wharton's Jelly (WJSC) in younger women giving birth naturally had higher BIRC2 expression and were hypothesized to have better stem cell function[40].

In this study, we propose for the first time that dysregulation of the Wnt signaling pathway in OA joints leads to dysfunction of CSPCs, and we found that the YAP-BIRC2 axis is critical for the maintenance of
CSPCs. Additionally, overexpression of YAP expression in the CSPC improves CSPC function and delays OA progression. This suggests a new therapeutic target for OA treatment. But, the CSPCs and OA chondrocytes were cultured in pattern of monolayer in this study may impair the cell phenotype and affect the outcomes [41]. And the mechanism by which BIRC2 regulates CSPCs function has not been elucidate. This pathway’s precise and targeted regulation in CSPC remains to be further investigated.

**Conclusion**

In this study, we found that the dysregulation of the Wnt/PCP-YAP-BIRC2 signaling axis contributes to OA-CSPC dysfunction, impairs cartilage's intrinsic reparative ability, and accelerates OA progression. These findings provide a potential therapeutic target for the treatment of OA.

**Abbreviations**

- CSPCs: Cartilage progenitor/stem cells;
- YAP: Yes-associated protein;
- BIRC2: Baculoviral IAP repeat-containing 2
- OA: Osteoarthritis;
- JNK: c-Jun N-terminal kinase,
- CAMK II: Calmodulin dependent kinase II
- PKC: Protein kinase C
- SF: synovial fluid
- DMEM/F-12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
- FBS: fetal bovine serum
- Lentivirus: Lv
- siRNA: small interfering RNA
- EdU: 5-ethynyl-2'-deoxyuridine
- Col II: Collagen II
- Col I: Collagen I
- Col X: Collagen X

DAPI: 4,6-diamidina-2-phenylin;

FAP: fibroblast activation protein alpha

VDR: vitamin D receptor

TERT: telomerase reverse transcriptase

MMP3: matrix metallopeptidase 3

FAK: Focal adhesion kinase

Snai2: snail family transcriptional repressor 2

Zeb2: zinc finger E-box binding homeobox 2

Ccnd2: cyclin D2

GAPDH: glyceraldehyde-3-phosphate dehydrogenase;

ANOVA: analysis of variance

DEG: differentially expressed genes.

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

RT-qPCR: Real-time quantitative polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF: polyvinylidene fluoride

ECL: electrochemiluminescence

WJSC: Umbilical Cord Wharton's Jelly

DMOADs: disease-modifying OA drugs

Declarations

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**Availability of data and materials**

All data generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

**Authors’ contributions**

LZ conceived and performed most of the experiments. XW wrote the manuscript. GX and JH(u) collected and analyzed the data. ZW and CL raised the animals. XC provided experimental advice and supervised the study; SW provided the funding support. All authors read and approved the final manuscript.

**Ethics approval**

This study protocol was approved by the institutional review board (IRB) of the Third Xiangya Hospital, Central South University (No: 2020-S221), and a signed written consent form was obtained from all study subjects. All experiments involving human tissues and animals were performed per the IRB’s guidelines. Each sample was processed only after receiving a signed informed consent form.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


Figures
Figure 1

Wnt signaling in the OA microenvironment is associated with CSPC dysfunction. **a** protein level of β-catenin, p-JNK, and total JNK in the synovial fluid of OA patients. **b** The chondrogenesis, osteogenesis, and adipogenesis of CSPCs. **c** Expression of the surface markers CD44, CD90, CD31, and CD45 as determined by flow cytometry. **d** The EdU staining (Red) and transwell migration assays of CSPCs from OA patients. Nuclei were stained by Hoechst 33342 (blue). **e** Expression of lubricin in the CSPC of OA
Spearman’s analysis was used to analyze the correlation of functions of OA-CSPC with the Wnt/β-catenin pathway and Wnt/PCP pathway. Bars = 100 μm.

Figure 2

Wnt5A/B regulates the function of OA-CSPCs. The CSPCs derived from OA patients were assessed by EdU proliferation assay (a) and transwell migration assay (b) after Wnt5A/B treatment. The cells were counted in five random fields per well. Bars = 100 μm. ***p < 0.001.
Figure 3

Wnt5A/B upregulates YAP in OA-CSPCs.  

a The mRNA expression of the genes associated with stem cell function in OA-CSPCs.  

b The protein levels of YAP, FAP, VDR, TERT, and MMP3 in OA-CSPCs.  

c Immunofluorescence staining of YAP (red) in OA-CSPCs. Nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI, blue), Bars=100 μm.  

d The protein levels of YAP in OA-CSPCs derived from OA patients.
Figure 4

YAP maintains the function of CSPCs by regulating protein levels of FAK, Lubricin, and YAP in OA-CSPCs (left) and Col II, Col I, and Col X in OA-chondrocytes (right) after co-culture for 7 days. OA-CSPCs overexpressing or silencing YAP were evaluated by EdU proliferation assay (b), transwell migration assay (c), and SA-β-Gal staining (d). The cells were counted in five random fields per well. Bars = 100 μm. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 5

Cartilage injury-induced YAP expression in CSPCs. **a** Immunohistochemistry staining of rat cartilage for CD44 (green), CD90 (red), DAPI (blue), and YAP (brown). Bars=100μm. **b** The percentage of CD44+/CD90+ cells in the superficial zone of rat cartilage. **c, d** Expression of YAP (c) and downstream target genes (d) in CSPCs derived from rat cartilage injury model. **p < 0.01; ***p < 0.001.
YAP contributes to CSPCs for cartilage regeneration. a, b. After Lv-YAP-RNAi or Lv-YAP intra-articular injection, cartilage was assessed by EdU proliferation staining (red, left), CD44 (red, right), and CD90 (green) immunofluorescence staining (a). YAP, Col II, Col I, Col X immunohistochemistry staining and safranin O staining (b). c. After intra-articular injection of CSPCs overexpressing or silencing YAP,
cartilage was evaluated by Col II, Col I, Col X immunohistochemistry, and safranin O staining. The severity of cartilage degeneration was evaluated by OARSI scoring. Bars = 100 μm. *p < 0.05; ***p < 0.001.

Figure 7

Transcriptomic analysis. a KEGG and GO enrichment analysis of DEGs. b The detailed DEGs that enriched in cell cycle, cellular senescence, autophagy, apoptosis, Hippo signaling pathway, and TNF
signaling pathways. c The direct downstream target genes of YAP. d The protein level of Birc2, Snai2, Zenb2, and Ccnd2 in CSPCs.

Figure 8

YAP regulates rat OA-CSPC function by upregulating Birc2. a. The expression of Lubricin, YAP, and Birc2 in CSPCs. The protein levels were normalized to GAPDH. b. CSPCs were evaluated by EdU proliferation.
assay and transwell migration assay. The cells were counted in five random fields per well. Bars = 100 μm. *p < 0.05; ***p < 0.001, ns: no significance.

Supplementary Files

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