Kartogenin mediates cartilage regeneration via stimulating the IL-6/Stat3-dependent proliferation of cartilage stem/progenitor cells

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Research

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Abstract

Background: Declination of endogenous stem cells in cartilage is regarded as the cause of cartilage degeneration. Kartogenin (KGN) is well known to play an important role in chondrogenesis of mesenchymal stem cells (MSC).

Methods: Using MSCs isolated from rat cartilages, we analyzed the changing of transcriptomics after the treatment of KGN *in vitro*. DMM animal models were then applied to identified the effect of MSCs proliferation *in vivo* after KGN capsule injection. Furthermore, we explored the potential mechanisms how KGN mediates cartilage regeneration and proliferation of cartilage progenitor cells.

Results: In this study, we demonstrate that KGN can promote the proliferation of MSC from cartilage, respectively. The percentage of G2-M phase cells in culture reached over 10%, nearly twice as the control group with KGN treatment. Transcriptomic profiling of rat cartilage stem/progenitor cells (CSPC) revealed that the expression of at least 20 cell cycle related genes was significantly changed in response to the KGN treatment. IL-6 and its co-receptor Gp130 gene expression level are much higher than the untreated control. The phosphorylation of the IL-6-downstreamt molecular Stat-3 was enhanced upon the KGN stimulation. The knee joint injury animal model further showed the increased articular cartilage thickness after KGN treatment. Interestingly, the IHC staining also demonstrated the up-regulated level of Stat-3 phosphorylation and enhanced distribution of $CD44^{+}/CD105^{+}$ cells in the KGN-treated cartilage.

Conclusion: Taken together, our data suggest that KGN promotes cartilage regeneration at least partially by stimulating the IL-6/Stat3-dependent proliferation of stem cells resident in the cartilage.

Introduction

Osteoarthritis (OA) is a degenerative multiple joint disease that eventually leads to diminished mobility along with the degeneration progression, affecting millions of populations worldwide. All current therapies for OA are aimed at symptomatic control, which has limited effect on preventing the progression of pathological OA.[1] Drugs such as acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) are designed to control pain and inflammation by blocking the relative inflammatory cytokine pathway. The combined injection of hyaluronic acid and glucocorticoid is used to control local inflammatory mediators. At present, different operation methods such as debridement, autologous osteochondral transplantation and autologous chondrocyte transplantation are applied to promote the repair of articular cartilage.[2] Thus far, disease-modifying treatments for osteoarthritis (DMOADs) still remain lacked.

The main target tissue of OA is articular hyaline cartilage, which is avascular, lack of self-renewal ability and plays a role of bearing articular surface. Normally, articular cartilage consists of more than 90% extracellular matrix (ECM) and less than 10% chondrocytes.[3-5] Chondrocytes in healthy articular cartilage persist and produce ECM, without undergoing terminal differentiation. Though at the early stage, the osteoarthritic chondrocytes may be activated and demonstrate the ability to divide and form cell

clusters,[5, 6] the density and functionality of chondrocytes generally decrease with aging and degeneration. Chondrocytes also become unable to create the complex matrix found in healthy articular cartilage and the response ability of chondrocytes to growth factor stimulation decreased, leading to a decrease in anabolism.[7, 8] In addition, chondrocytes demonstrate increasing senescence and/or apoptosis with aging and OA progression.[6, 9, 10] Severe cartilage damage is caused by many events, such as mechanical changes, biochemical changes of ECM, and biological reactions of chondrocytes, subchondral bone and synovium to inflammation. At the late stage, all the changes at cellular and molecular levels eventually lead to characterized loss of articular cartilage, osteophyte formation, thickening of subchondral bone and subchondral cyst formation.[3]

Tissue resident stem cells/progenitor cells are essential in maintaining homeostasis of all tissue types and are susceptible to disease-causing factors such as aging and inflammatory factors.[11] Cartilage stem/progenitor cells (CSPCs) have been found within human articular cartilage[12-14] and proposed to maintain cartilage homeostasis, including the cell number and their anabolic metabolisms. These CSPCs can respond to injury and migrate to the injured tissue area to achieve minimal cartilage repair, and are characterized as stem/progenitor cells due to their self-renewal ability, multiple differentiation potential and stem cell markers. Some mesenchymal progenitor cell markers, including Notch-1, VCAM (CD166), STRO-1, were expressed in early OA, but less expressed in normal human articular cartilage, suggesting that OA may activate the endogenous CSPCs in the tissue.[15] At the middle to late phases of OA, however, the functionality and number of cartilage progenitors typically decline and may be attributed for the abnormality in pathologies of OA in human and in murine models.[15, 16]

Recently, the small molecule Kartogenin has been proposed to be able to promote the multipotent mesenchymal stem cells (MSC) into chondrocytes by regulating the CBFb-RUNX1 transcriptional program.[17] Since then, the established capability of KGN in mediating cartilage regeneration both *in vitro* and *in vivo* have been documented in a number of studies using different models.[18-21] During the process of mouse limb development, KGN stimulated cartilage nodule formation by up-regulating genes encoding hedgehog and TGFβ.[18] For the purpose of integration with regenerative medicine approaches, KGN has been applied in a number of studies involved in biomaterials and scaffolds for full-thickness cartilage repair.[19] An intra-articular delivery system contained KGN nano- or micro-particles was applied in a surgically-induced OA model in rats, where KGN could induce the chondrogenic differentiation of loaded bone marrow MSC.[20] A pilot study in a rodent model of osteoarthritis using micro-CT as a main evaluation mean showed that the KGN treatment not only reduced the conversion rate of cartilage and bone, but also prevented the change of subchondral bone.[21]

For the action of KGN on the cartilage regeneration, current evidences mainly indicate the differentiation of endogenous MSC within the cartilage by regulating the CBFb-RUNX1 transcriptional program and up-regulating the TGFβ/Smad2/3 pathway. In other experimental sittings, however, KGN may also function in promoting differentiation of MSC towards intervertebral disc cell types or osteogenic lineages.[21, 22] Another study also demonstrated that KGN could increase type-I collagen synthesis in the dermis of BALB/C mice in vivo through activation of the smad4/smad5 signaling.[23, 24] Furthermore, some other

studies have demonstrated that KGN may also stimulate proliferation of several stem cells types originated from none-cartilage tissues.[25] Whether the proliferation of MSC or chondrogenic progenitor cells also plays a significant role in cartilage regeneration remains unknown.

This study originally aimed at further elucidating the role of KGN on promoting chondrogenic differentiation of CSPCs. Unexpectedly, it was repeatedly observed that KGN could stimulate the proliferation of CSPC. The following studies revealed that the IL-6/Stat3 signaling pathway is essential in the KGN action on CSPC proliferation. The study may therefore open a venue in further research to understand and utilize KGN or the IL-6/Stat3-targeting molecules in cartilage regeneration.

Materials And Methods

Isolation and *In Vitro* expansion of CSPCs

Fibronectin (FN, 10 mg/ml) (Gene Operation, 10-50-1101) in PBS was used to coat culture plate overnight at 4 degree for cell transplant. Rats were killed by overdose of chloral hydrate, and separated the joints and intercepted the joint part (keep the upper and lower femur and tibia about 1-2 cm) into the 50 ml Falcon tube containing PBS. The cartilage tissue scissors are about the size of 1-3mm fragments as shown in Figure 1A, transferred to a 15 ml centrifuge tube, adding 3 times volume of the amount of 0.25% trypsin, digest it at 37 degree for 30 min. Following complete medium termination of digestion, adding 300unit/ml type II collagenase (Worthington, 42B13253) dissolved in supernatant for overnight. The supernatant was removed and the complete medium containing 1% gentamicin was suspended and counted. The cell number was adjusted to 1×10^4/ml, and then transplanted into the FN covered culture plate and then separated cells can be observed on the surface of culture dish as shown in left panel of Figure 1B. After 2-3 days, replace fresh culture DF12 medium. On the day 7, cell colonies can be identified as shown in right panel of Figure 1B.

Growth and proliferation assay

After 72 hours of cell culture, cells were counted and collected with cold ethanol solution at 4 degree for overnight. Cells were finally suspended with PBST (containing a TritonX-100 of 0.2% PBS) by adding a 50 μg/ml RNA enzyme (RNase A) at 37 degree for 30-minute incubation. 50 μg/ml PI was added to cell suspension and incubated for 15 mins. After centrifuge above cells at 800 rpm for 5min, and then discard supernatant. Re-centrifugate and remove excess supernatant, afterwards using PBST to suspense cells and transfer to the flow tube before loading to BD FACS Calibur.

Flow cytometry

To characterize the CSPC identity, positive (CD73, CD105 and CD90) and negative (CD45 and CD34) cell-surface markers were detected by flow cytometry. All antibodies are listed as following: CD105-PE (Biolegend, 800504), CD90-FITC (Biolegend, 328108), CD73-APC (Biolegend, 344006), CD34-PE (Biogems, 06421-60-100), CD45-PE (BD, 555483). Harvest primary CSPC cells, prepare a single cell suspension in

Cell Staining Buffer (Cat. No. 420201; BioLegend). One million of cells were pre-incubated with 5μL of Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend Cat. No. 422301) per 100 μL of cell suspension for 5-10 minutes at room temperature. The tubes were centrifuged for 5 minutes at 350 g and supernatant discarded. Appropriately conjugated fluorescent antibodies were added and incubated on ice for 15-20 minutes in the dark. The cells were then washed 2 times with 2 mL of Cell Staining Buffer by centrifugation at 350 g for 5 minutes, and analysed by flow cytometry (Becton, Dickinson and Company, USA). Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry. For G2-M phase detection, cells were stained using DNA-binding dyes include propidium iodide (PI), and cells in G2-M will be approximately twice as bright as cells in G1.

RNA-sequencing and bioinformatics analysis

One million of CSPCs were seeded in T175 bottle and treated with 10 μM KGN for 3 days. Then, total RNA from CSPCs was extracted using TRIzol reagent (Thermo, 15596-026), according to the manufacturer's instructions. RNA-sequencing and bioinformatics analyses were performed as previously described.[11]

IL-6 ELISA assay of rat CSPC in culture

IL-6 secretion in culture supernatants were detected with ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's directions. Briefly, ELISA plates were coated with the capture antibody and incubated overnight at 4°C. Then, washed wells thoroughly and blocked for 1 h. Wells were incubated for 2 hrs with human IL-6 standards and supernatants from CSPC with or without KGN medium, which were diluted 100 times. After extensive wash steps, wells were incubated with detection antibody for 2 hrs at 4°C and then washed several times. Wells were incubated with Avidin-HRP for 30 min, washed thoroughly, incubated with substrate solution for 15 min, and then added stop solution. Finally, A450 values were measured using a microplate reader.

RT-qPCR

5% Agarose gel with identical wells were casted and rat-CSPC were seeded at a density of 1×10^6/10 μL. Cells were cultured in chondrogenic medium with either 10μM KGN or its solvent, DMSO as control. Medium were changed every other day for 28 days before harvested. Total RNA was extracted with TRIzol reagent (Life Technologies) following manufacture's instruction. 500ng RNA was reverse-transcribed to cDNA using PrimeScript^{TM} RT reagent Kit (TaKaRa, Dalian, China). Specific genes were amplified with One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) on CFX96 real-time PCR detection system (BioRad, Hercules, CA, USA). The relative gene expression was normalized to GAPDH transcript level and calculated using the $2-\Delta\Delta Ct$ method.

STAT-3 siRNA knockdown assay

Rat-CSPCs were seeded in 6-well plate at 1×10^5 per well. Serum-free medium was given 2 hours pre-transfection. We designed a STAT-3 siRNA oligonucleotide (5'-AAC AUC UGC CUA GAU CGG CUA dTdT-3';

3'-dTdT GUA GAC GGA UCU AGC CGA U-5') and had it synthesized by GenePharma Limited (Shanghai, China). Lipofectamine™ 3000 Reagent (Invitrogen, Carlsbad, CA, USA) was used as the transfection reagent following manufacturer's instructions with 200–600 nmol siRNA per well. 1 mL per well culture medium with serum was added 3 hours post-transfection. The siRNA transfection were repeated after 72 hours and cells were harvested 5 days after first transfection process. The control siRNA were set as the negative control group.

BrdU administration

The animals were exposed to 1 mg/mL BrdU (Sigma-Aldrich, Steinheim, Germany) for 15 days, which is effective and safe for label-retaining cell studies.[26] Oral BrdU administration was combined with exercise acclimatization, and rats were killed with excessive sodium pentobarbital (Apoteket Produktion & Laboratorier AB (APL), Sweden) at 30, 60 and 90 days after the start point. Two animals, not exposed to BrdU, were killed at day 30 and 90 as negative controls for BrdU technique.

Histology and immunohistochemistry

The knee joint samples were cut vertically at the cartilage defect sites with a thickness of 5 μm and stained with Hematoxylin and Eosin (H&E), Toluidine blue, Safranin O and Fast green.[10] For immunohistochemical (IHC) analysis, the slides were incubated with primary antibodies Phospho-Stat3 (Tyr705) (Mouse mAb #4113, Cell Signaling Technology) and Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (Rabbit mAb #8828, Cell Signaling Technology) at 4 °C overnight. Tissue sections were then incubated with 5% bovine serum albumin (BSA, Sigma) and incubated overnight with mouse-anti Collagen II (Developmental Studies Hybridoma Bank, USA), rabbit-anti Aggrecan (Proteintech, USA), rabbit-anti CD44, and mouse-anti CD105 (Abcam, USA) antibidies. Then, the sections were incubated with Alexa Flour 488/546-conjugated secondary antibody (Invitrogen, USA) for immunofluorescence or HRP-conjugated secondary antibodies (KPL, USA) for immunochemistry. The images were captured with a microscope (Olympus BX51, Japan).

Animals and cartilage regeneration experiments

All animal care and experimental procedures complied with the National Research Council's guide for the care and use of laboratory animals, and all procedures were approved by the Institutional Animal Care and Use Committee and the Ethical Committee for Medical Research of Shenzhen University (Shenzhen, China).

The destabilization of medial meniscus (DMM) model in rats was created according to the previously reported protocol.[26] Briefly, male rats weighted at 100 ± 10 g were anesthetized with 2% isoflurane in air using an anesthesia machine (RWD Life Science Co., Ltd., Shenzhen, China). After opening the capsule of knee joint, the tibial ligament of medial meniscus was cut off to destabilize the joint. Then, the knee joint capsule was closed immediately. Thirty days after surgery, 50μL KGN dissolved in saline at the

concentration of 10 mM was injected into knee capsule every 7 days for seven weeks. Two weeks later, rats were executed followed by knee joint isolation for staining as shown in Figure 5A.

Statistical analysis

All data were expressed as the means ± SD. Differences between groups were evaluated by independent-sample t test or one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test in SPSS 22.0 software. A p-value less than 0.05 was considered to be statistically significant.

Results

Identification of primary CSPC original from rat cartilage tissues

To confirm stem cell characterization of CSPC isolated from cartilage tissue, we checked the stem cell markers including three positive markers CD105/CD90/CD73 and two negative markers CD34/CD45 of these cells. Our results showed that the proportion of CD105/CD90/CD73 positive cells are, respectively, 99.96%, 99.93% and 99.95%; the proportion of CD34/CD45 positive cells are 0.19% and 0.10% suggesting that our CSPC from cartilage tissue are mesenchymal stem cells as shown in Figure 1C.

KGN promotes rat cartilage derived from CSPCs proliferation *In Vitro*

In our study, 10 μM KGN containing media treated CSPC isolated from knee joint of rats aged at 4 weeks led to a significant cell number increase. Cell number counts were performed at day 3, day 5 and day 7 time points with constant KGN concentration. A sustained increase of the total cell number was achieved, as shown in Figure 2A. We applied the flow cytometry to check the percentage of G2-M stage cells corresponding to the increasing cell number. We found the percentage of G2-M stage cells were 8.6%, 9.8% and 15.6% respectively at the end of day3, day5 and day7 as shown in Figure 2B, C. Compared to control groups, both the cell number and the percentage of G2-M of KGN treated group had a significant differences as shown in Figure 2B. At the end of cell culture (day 7), we exanimated not only the percentage of G2-M for both control and KGN treated group but also the percentage of apoptotic cells induced by IL-1β. It was found that the KGN treatment results in the percentage of cell apoptosis is 15.17% which is lower than control treated with IL-1β groups shown in Figure 2D. This means KGN is able to protect cells from the IL-1β cytotoxicity. Taken together, these results shown that the KGN treatment resulted in an increasing of cell growth and a lower percentage of apoptosis.

IL-6 was identified as a key effector of KGN by total RNA sequencing

With the development of NGS, RNA-seq is becoming a powerful tool and has allowed great advances in the characterization and quantification of transcriptomes. With an attempt to reveal the possible mechanism underlying KGN effects on cell proliferation, the comprehensive transcriptomics studies were then carried out between the KGN-treated and the untreated CSPC derived from rat. As shown in Figure 3A, B, 738 genes were identified to be altered for their RNA expression profiling by using 1.5 as a threshold value in duplicate samples. A functional enrichment analysis and a protein-protein interaction

network analysis were performed with bioinformatics, and IL-6 was identified as a key effector following the KGN treatment in vitro, as shown in Figure 3C. The value of log expression multiples of IL-6 between the KGN treatment and control groups reached 13, the highest hit in all genes as shown in Figure 3D. To further verify the elevation of the IL-6 expression by the KGN treatment, qRT-PCR was performed and the results matched the RNA-seq data, i.e. the mRNA level of IL-6 in CSPC was significantly higher than that of control cells (data not shown). IL-6 is one of secreted cytokines by MSC.[27] We first detected the level of IL-6 in the supernatant of KGN treated CSPC using an ELESA kit. The results show that the KGN-treated group contained significantly higher IL-6 level in the supernatant than that of control group, as shown in Figure 4A.

The KGN treatment results in the activation of IL-6/STAT3 pathway

Activation of the TGFβ pathway by KGN has been reported by several groups during introduction of chondrogenic differentiation of MSC.[15, 16] We therefore wondered whether the KGN effects on CSPC proliferation were TGFβ-signaling dependent. To address this question, SB431542, a reported dual inhibitor of activin/Nodal/TGFβ and BMP signaling pathways [28] was added to the experimental system. Interestingly, SB431542 was not able to block the IL-6 excessive secretion (Figure 4A). At the time points of both day 3 and day 5, the KGN treatment resulted in an increasing of IL-6 secretion, which could only be partially inhibited by SB431542. Further, we were able to observe KGN effects on directly phosphating Smad2/3 without the external TGFβ addition, as shown in the column b in Figure 4B. It is interesting that the elevated pSmad2/3 level could be detected by adding KGN even in the presence of the TGFβ inhibitor SB431542, as shown in the column d, meaning that there should other pathways involved in the CSPC regulation by KGN. To investigate this possibility, we detected the Stat3 activation using P-Stat3 antibody and we found that the KGN treatment was able to increase the phosphorylation of Stat3, as shown in Figure 4C. Stat3 is well known to be activated by IL-6 via the IL-6/JAK/STAT3 pathway.[29] Interestingly, we found that KGN could also activate Stat3 (Figure 4E). Phosphorylation of Stat3 was significantly deduced when the IL-6 receptor gp130 was blocked with a gp130 antibody, as compared with either KGN or IL-6 addition alone (Figure 4D, E). Taken all above together, KGN exhibited its role in activating the phosphorylation of Stat3 by increasing the secretion of IL-6.

KGN increases rat cartilage thickness by promoting the stem cells proliferation *In Vivo*

To investigate whether KGN affect CSPCs, we used BrdU labelling[26] to investigate proliferation and label-retaining cells (i.e. slow cycling cells) *in vivo* by ether feeding the rat BrdU containing water or injecting BrdU into the joint space, in combination with immunostaining of the multi-potential stem cell marker nucleostemin. The results showed that articular cartilages were thickened by intra-articular injection of KGN (Figure 5B). The KGN injection group appeared to have in a thicker cartilage layer, implicating a potential pharmaceutical value. Interestingly, the BrdU-labeling experiments showed that slow cycling cells were often assembled as columnar arrangements, like “the string of pearls-like” array. The software ImageJ V1.45 (http://imagej.en.softonic.com/) was applied to quantify the BrdU positive cells, showing a significant increase of the CSPC (the lower right panel in Figure 5B). Nucleostemin, a

nucleolar protein, was first identified in neural stem cells and a number of cancer cell lines.[30] The molecule is mainly expressed in primitive cells and down-regulated before terminal differentiation. It is considered as a stem cell marker of chondrogenic cells. The results here showed that BrdU positive cells were well overlapped with nucleostemin expression (Figure 5C). Furthermore, it was found that the KGN injection could result in the up-regulation of P-Stat3 and P-Smad2/3 positive cells as shown with IHC (Figure 5D), well matching the *in vitro* findings.

KGN shows a good ability for cartilage regeneration *In Vivo* in DMM models

The destabilization of the medial meniscus (DMM) surgical model is a commonly accepted model of OA.[31] Utilizing the model, this study finally tried to assess the therapeutic capacity of the KGN injection. Fifty microliters of KGN at a concentration of 10mM were injected into the knee joints of DMM animal models, twice a week for 8-10 weeks, as schemed in Figure 6A. The DMM surgery could induce obvious degeneration and defects in articular cartilage. The intra-articular injection of KGN mediated a repair of damaged cartilage as shown in Figure 6B. Around the adjacent non-damaged areas, a thicker cartilage layer with more toluidine blue positive cells were observed, presenting an increased number of chondrocytes as shown in left panel of Figure 6B. Both CD44 and CD105 are commonly accepted MSC biomarkers and have been used to identify cartilage-resident stem cells.[12, 13] This inspired us to examine whether cells expressing the two markers could be enhanced with the KGN stimulation. Indeed, around the repairing area within cartilage of the KGN injection group animals, $CD44^{+}CD105^{+}$ double positive cells were found to be clearly more than other groups, even the intact group animals. Therefore, this study further implicated the role of KGN in mediating cartilage repair via enhancing CSPC proliferation (Figure 6C).

Discussion

Well-coordinated sequential proliferation and differentiation of CSPCs are the key cellular process for the cartilage regeneration and defect repair. This study provides evidences indicating that, in addition to the well-documented role in promoting chondrogenic differentiation, KGN also effects on CSPC proliferation as the essential step for cartilage regeneration. Interestingly, we also demonstrate that IL-6, a pro-inflammatory factor well-known in mediating the OA pathology, may actually play a role in stimulating cartilage regeneration mediated with KGN.

A number of previous studies have shown that KGN can promote chondrogenic differentiation of MSC.[18-21] In line with these studies, this study also demonstrated that KGN activated Smad2/3 of in vitro cultured CSPC, implying that the TGFb signaling pathway, one of the most powerful inducers for chondrogenic differentiation, are activated. More importantly, this study demonstrates that KGN could promote CSPC into mitosis and lead to increased percentage of G2-M phase cells and cell counts. Here, we need to clarify the reason why we chose 72h as the time point to carry out RNA-Seq is that 72 hour after KGN treatment is the starting point of the cell proliferation phenotype displayed. Such results seemed in a way controversial because the basic concept would be that a single cell has to exit cell cycle

for differentiation and it would be hard to interpret why a single stimuli could exert effects in both cell proliferation and differentiation. However, it must be noted that MSC from any sources are highly heterogeneous and cells at different cell cycle phases or differentiation stages, and even different functional subgroups co-exist. As a result, the seemingly same or similar types of progenitor cells in vivo may contain different cells and respond differently to the same stimuli. Indeed, KGN has been proposed to exert effects on the proliferation of cells other than the cartilage origin.[25, 32] Interestingly, TGFβ signaling inhibition was not able to inhibit the cell proliferation phenotype, further highlighting the involvement of mechanisms other than the TGFβ signaling pathway. These evidences may imply that the mixed cell subgroups co-exist and differently respond to the KGN treatment. The possible multi-facial functions of KGN on MSC may also be a possible explanation. In this respect, KGN is not alone at all in having effects on both chondrocyte proliferation and differentiation. Several other approaches, such as platelet-rich-plasma, mechanical stimulation, Periostin, and TGFb have been reported to have the positive effects on the both cellular processes and consequently promote cartilage regeneration.[33-35]

Amongst a variety of cellular or soluble factors that contribute to form the local tissue environment are the IL-6 and the family members. IL-6 is generally considered as a pro-inflammatory factor secreted from immune immune cells and senescent tissue cells. IL-6 can bind to the signal transducing subunit of gp130 to induce intracellular signaling. It is highly accepted that persistent activation of the IL-6 signaling is detrimental to tissue integration and might even ultimately result in the development of tumors. In cartilage, IL-6 is mainly thought to be harmful and associated with the catabolic metabolism of chondrocytes and consequently with the OA pathology.[36-38]

On the other hand, a number of studies have demonstrated that IL-6 can also be produced by stem cells, in particular MSC,[39, 40, 27] as a paracrine factor, where their physiological role remains to be clarified. Recent studies indicate that IL-6 is implicated in MSC growth through JAK/STAT3 signaling and that IL-6 contributes to the maintenance of MSC in their undifferentiated state. More importantly, IL-6 has been well demonstrated to be essential for tissue regeneration in several organs, such as hematopoiesis, liver cells, gastrointestinal tract mucosa, and muscle cells. Impaired IL-6 function in some of these organs is associated with reduced regeneration following injury, whereas IL-6 up-regulation during chronic inflammation may in contrast contribute to the dysfunction of the same organs. Apparently, there should be certain regulatory mechanisms to balance the detrimental and anabolic effects of the IL-6 signaling, even for tissues such as liver, where both the effects of IL-6 have been fairly observed. One of the possible mechanisms could be that the cell status or even cell types in these tissues under different pathophysiological conditions responding to the IL-6 stimulation vary. In cartilage, it may be that the mature chondrocytes and stem/progenitor cells respond to IL-6 in different ways, consequently leading to its detrimental or anabolic effects, respectively. The local concentration of inflammatory factors may be another parameter, as shown for many other pro-inflammatory factors, including IL-1a, IL-6 and IFNg, which could also mediate regeneration.[41] In this study, IL-6 production by CSPC following the KGN stimulation was clearly demonstrated and the absolute concentration is not clear but might not be too. Therefore, the anabolic effects may be more protruding in our study but not like most previous studies where mainly the role of IL-6 in mature and degenerative cartilage tissues were involved and the pro-

inflammatory catabolic effects might be dominating. However, this notion remains to be further confirmed by more detailed analysis in different experimental settings, such as the possible role of KGN in IL-6 deficient animal models.

Discovering new roles of KGN on CSPC or MSC and deciphering the underlying signaling mechanisms would be interesting also for several other aspects. As apoptosis and senescence of either differentiated chondrocytes or progenitor cells have been implicated in the OA pathology,[7-10] it might be worth of testing whether KGN could have an effect in delaying or reverse these cellular phenotypes. Considering stem cell therapy for OA and other joint diseases, pre-treatment or chondrogenic priming with KGN or an IL-6/Stat3 analogue, prior to the in vivo transplantation or loading onto a biomaterial scaffold, may be able to enhance the therapeutic efficacy of CSPC or MSC.[41, 42] Furthermore, this study demonstrated that KGN injection could also mediate cartilage regeneration, suggesting the potential of KGN to be developed 8as clinically useful tablets. In line of this and even more importantly, the biosafety and pharmacological kinetics of the systemically and locally administrated KGN may have become the next issue to be addressed in further investigation in order to translate the small chemical to clinical application.

Conclusions

The proliferation of cartilage progenitor cells is crucial for regeneration and repair of cartilage degenerative defects. KGN mediates cartilage regeneration not only by stimulating CSPC differentiation but also by enhancing their proliferation via the IL-6/Stat3 signaling pathway. This study further implicate that KGN may be further translated into a reagent useful both locally and systemically. The involvement of IL-6/Stat3 in cartilage regeneration further implicates an novel insight into the association of cartilage regeneration with inflammation response of the local tissue environmental factors, with particular emphasis on their activating effects on tissue-resident stem/progenitor cells. New therapies for OA may take advantage of the IL-6/Stat3 signaling rather than just inhibiting the inflammatory effects of IL-6, probably with necessary balancing of the IL-6 level and the stage of the OA pathology.

Declarations

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Authors' contributions

XL, TL, TW and JD performed the main *in vitro* experiments. XL, TW, SZ, JL, XC and JD performed the animal *in vivo* experiments. MZ, PF, YZ, YH and YZ assisted on all experiments. TL and GZ designed the project and wrote the manuscript. GZ provided the main funding for the study. All authors approved the manuscript submission.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was also approved by the Ethics Committee of Shenzhen Luohu People's Hospital (ZLNK 03/2014, Shenzhen, China). This article does not contain any studies with patients performed by any of the authors. The rats used in this study were fully qualified with Animal certificate number: SCXK (Yue) 2013-0002 and Animal room license number: SYXK (Yue) 2014-0140.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

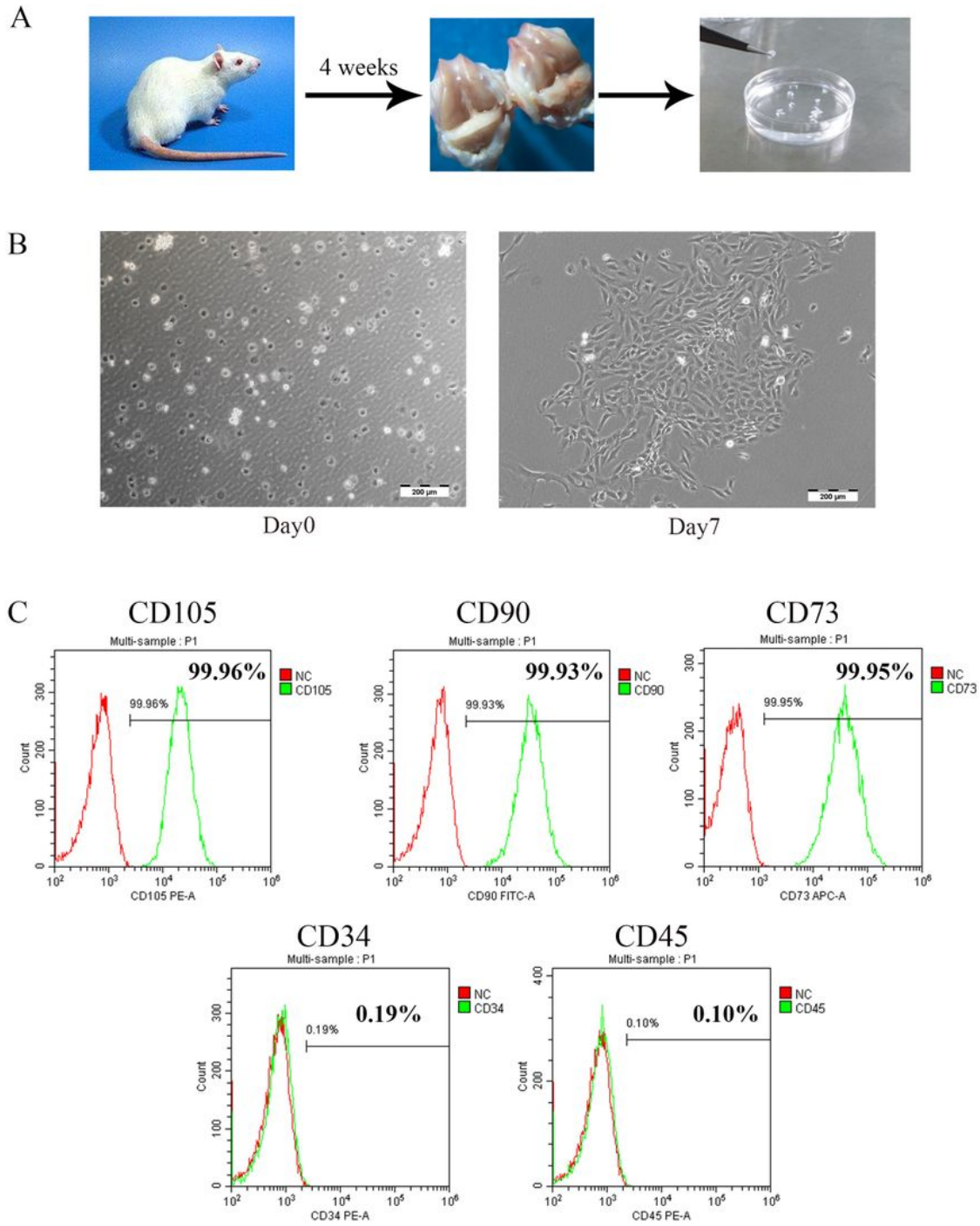

Figure 1

Isolation, purification and identification of primary CSPC original from rat cartilage tissues (a) Isolation and processing of cartilage tissues from 4 week old rat knee joint. All cartilage tissues from different rats were collected and prepared for single cell suspension. (b) Rat primary cartilage stem/progenitor cells (CSPC) grown on fibronectin-coated culture plate original from clones. Scale bar equals 200 μm. (c) Identification of primary CSPC by detection of three positive markers CD105, CD90 and CD73, and two

negative markers CD34 and CD45 using flow cytometry. The results showed that the proportion of CD105/CD90/CD73 positive cells are, respectively, 99.96%, 99.93% and 99.95%; the proportion of CD34/CD45 positive cells are 0.19% and 0.10%, suggesting that our CSPC from cartilage tissue are mesenchymal stem cells.

Figure 2

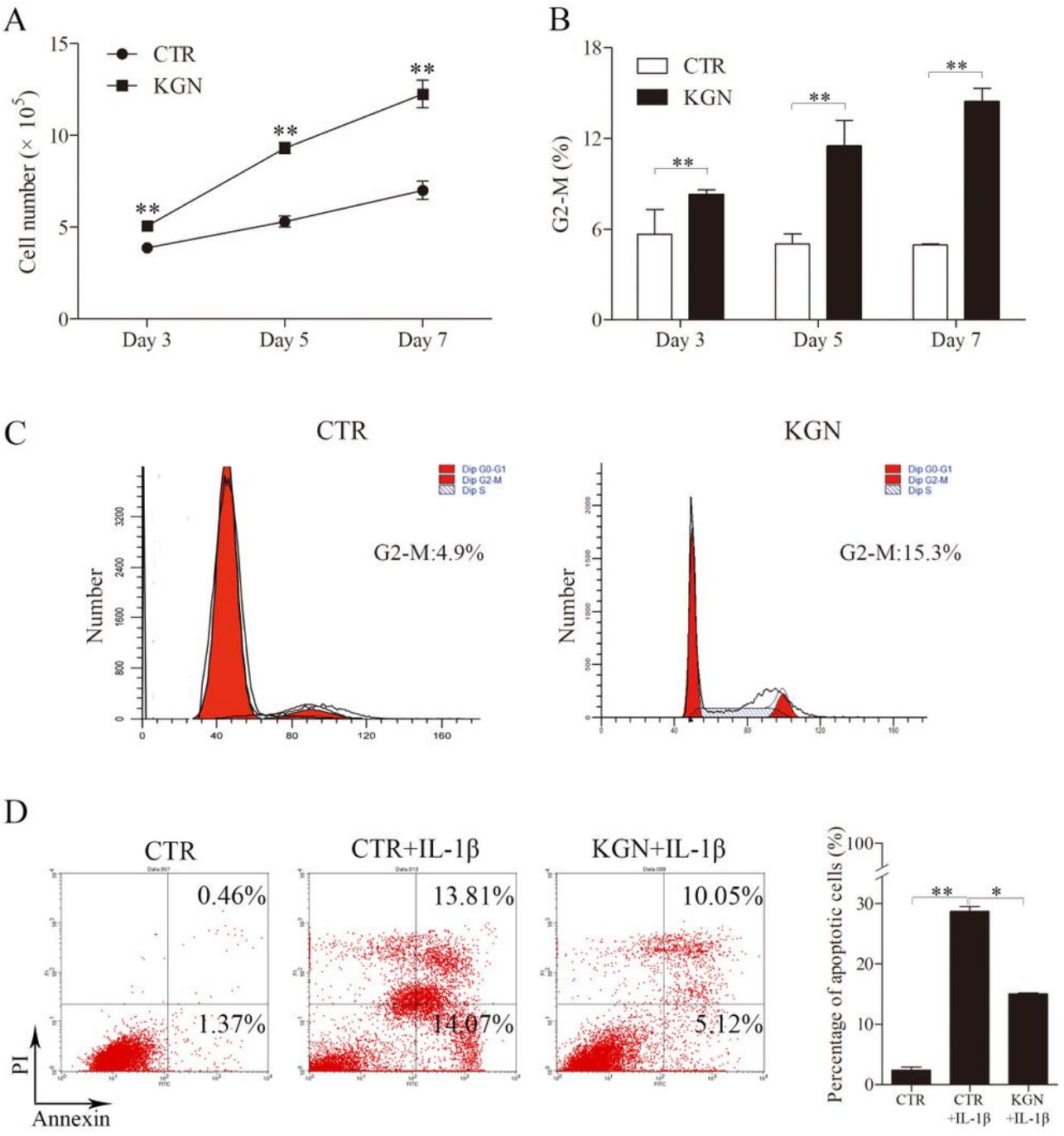

Figure 2

KGN promotes rat CSPC proliferation in vitro (a) Growth curves of CSPC of control (CTR) and KGN-treated group (KGN) 7 days in a row. The cell number at day 3, day 5 and day7 are significant different between ctr and KGN group **P<0.01. (b) Percentage of G2-M stage CSPC of CTR and KGN group. Cell cycle analysis by quantitation of DNA content was applied by flow cytometry. For G2-M phase detection, the percentage of G2-M cells are significant different for all three time points day 3, day 5 and day7. **P<0.01. (c) Flow cytometry detection of G2-M stage CSPC of CTR and KGN group. The result shows that the percentage of G2-M of KGN is 15.3% which is higher than that of control group (4.9%). (d) Detection of apoptotic cells induced by IL-1β of CTR and KGN group respectively. KGN treatment results in the percentage of cell apoptosis decreased from 27.88% to 15.17% . *P<0.05.

Figure 3

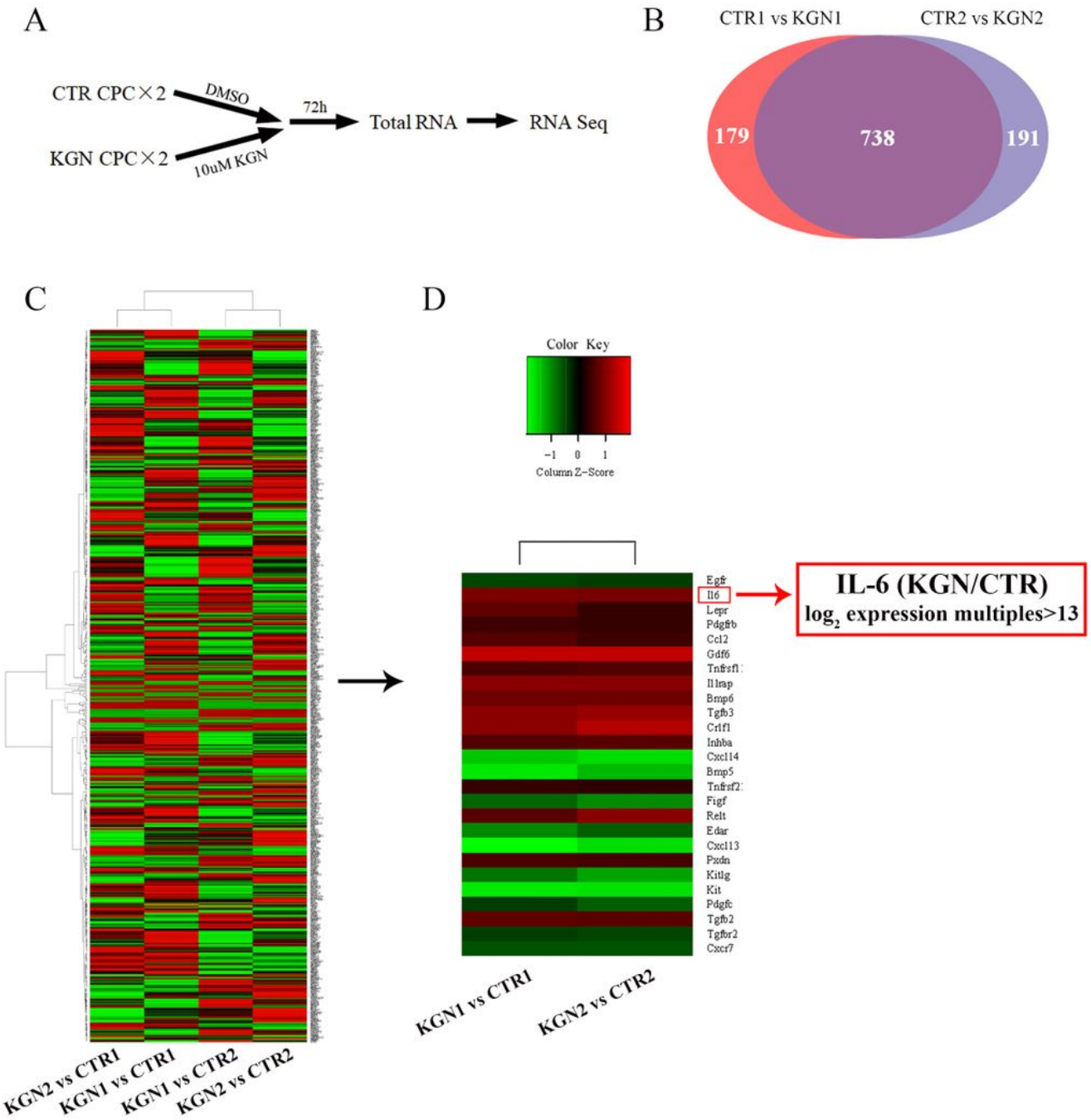

Figure 3

IL-6 was identified as a key effector of KGN by total RNA sequencing (a) CSPC were treated with 10μM KGN or DMSO for total RNA sequencing. (b) Venn diagram of differentially expressed genes between two comparative groups (CTR vs KGN). 738 genes were identified to be altered for their RNA expression profiling by using 1.5 as a threshold value in duplicate samples. (c) Clustering heat map of CTR group and KGN group between duplicate samples. A functional enrichment analysis and a protein-protein

interaction network analysis were performed with bioinformatics. (d) The values of log expression multiples of all hit genes between CTR and KGN group were calculated, and IL-6 was identified as the most differentially expressed gene, which log expression multiple value was more than 13 fold.

Figure 4

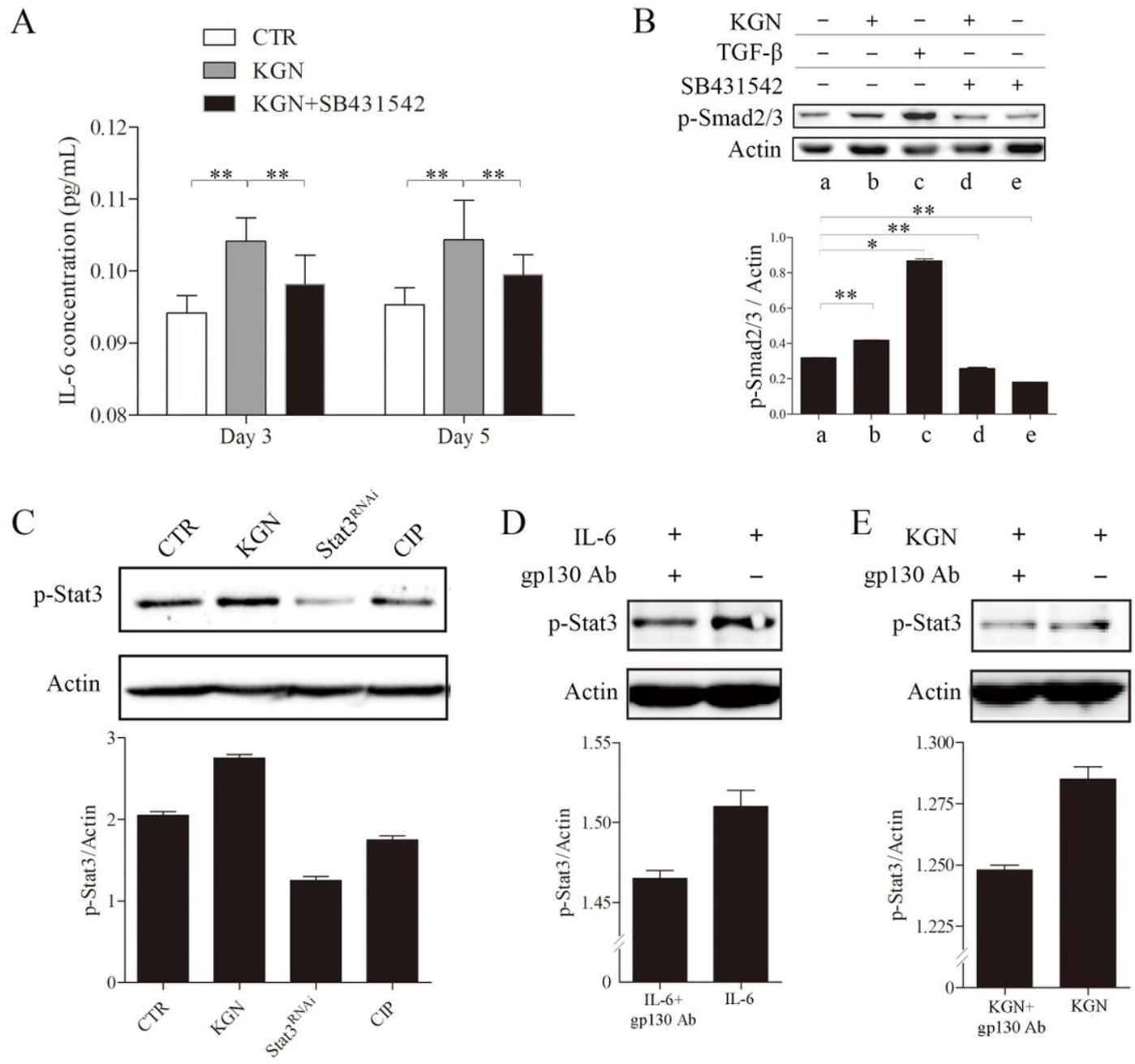

Figure 4

KGN treatment results in activation of IL-6/STAT3 pathway (a) IL-6 concentration of CTR, KGN, and KGN+SB431542 groups. KGN treatment results in the increasement of IL-6 expression of CSPCs, which can be inhibited by TGFβ inhibitor SB431542. **P<0.01. (b) Western blotting analysis of P-Smad2/3 expression levels in CSPC treated with DMSO (a), KGN (lane b), TGF-β (lane c), KGN+SB431542 (lane d)

and SB431542 (lane e). KGN effects on directly phosphating Smad2/3 without the additional TGFβ. **P<0.01. (c) Western blotting analysis of P-Stat3 expression levels in CTR, KGN, Stat3RNAi and CIP treated CSPC. Actin was used as loading control. Stat3 activation using P-Stat3 antibody and we found that the KGN treatment was able to increase the phosphorylation of Stat3 (d) Western blotting analysis of P-Stat3 expression levels in CSPC treated with IL-6+gp130 antibody and IL-6 alone. Actin was used as loading control. Phosphorylation of Stat3 was significantly deduced when the IL-6 receptor gp130 was blocked with a gp130 antibody, as compared with IL-6 treatment. (e) Western blotting analysis of P-Stat3 expression levels in CSPC treated with KGN+gp130 antibody and KGN alone. Actin was used as loading control. Gp130 antibody can partly block the KGN treatment effect on the activation of p-Stat3.

Figure 5

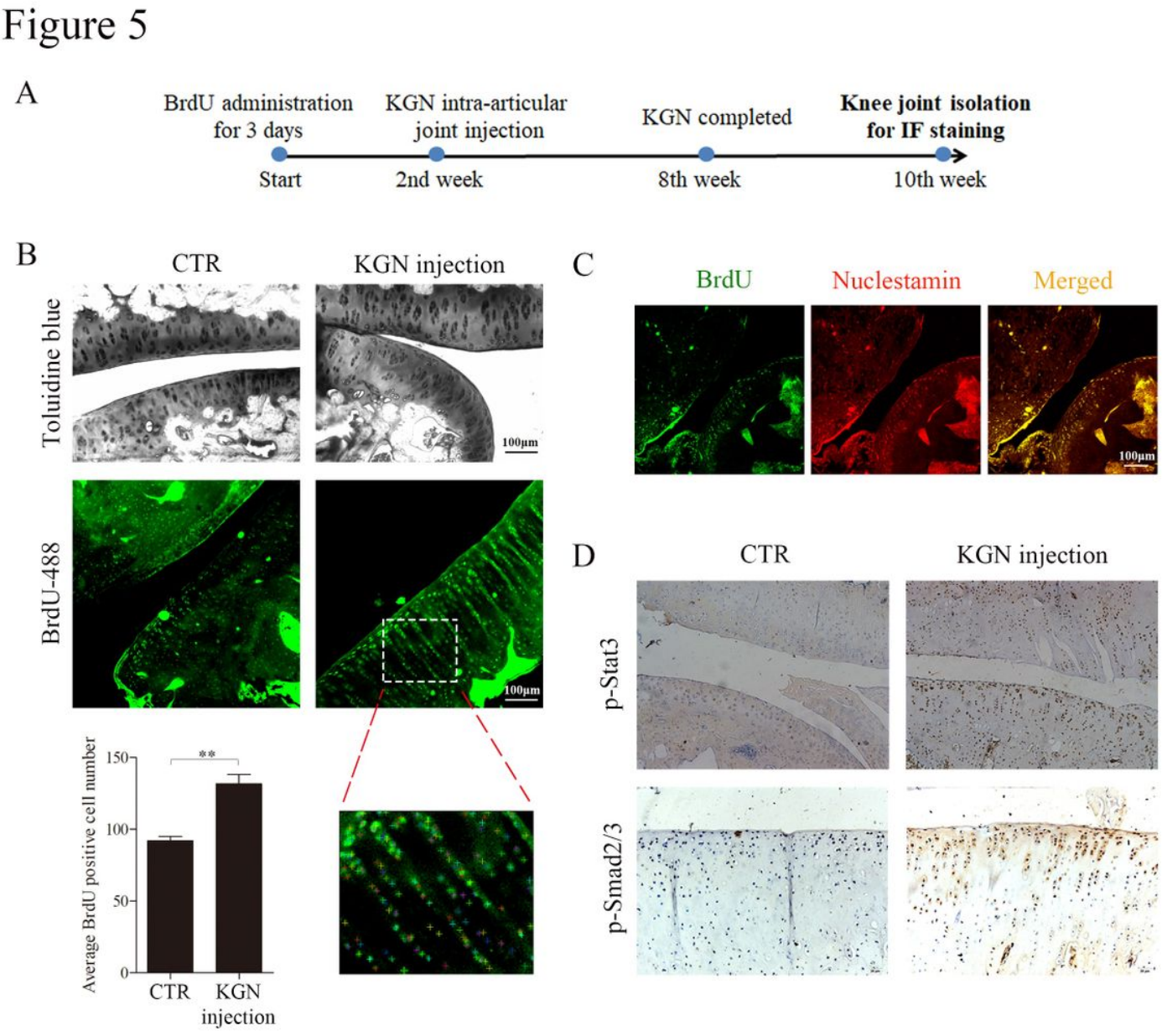

Figure 5

KGN increases rat cartilage thickness by promoting the stem cells proliferation in vivo (a) KGN capsule injection time line of BrdU labeled rat for cartilages regeneration analysis. (b) Toluidine blue staining and BrdU-488 imaging of CTR and KGN injection rat cartilages. The lower left picture was the quantification result of average BrdU positive cell numbers of CTR and KGN injection group. Articular cartilages were thickened by intra-articular injection of KGN. **P<0.01. Scale bar equals 100 μm. (c) Immunofluorescence staining of nucleostemin and BrdU in KGN injection group. Scale bar equals 100 μm. BrdU positive cells were well overlapped with nucleostemin expression which confirm that the labeled cells are CSPCs. (d) IHC staining of P-Stat3 and P-Smad2/3 of CTR and KGN injection group. KGN injection could result in the up-regulation of P-Stat3 and P-Smad2/3 positive cells.

Figure 6

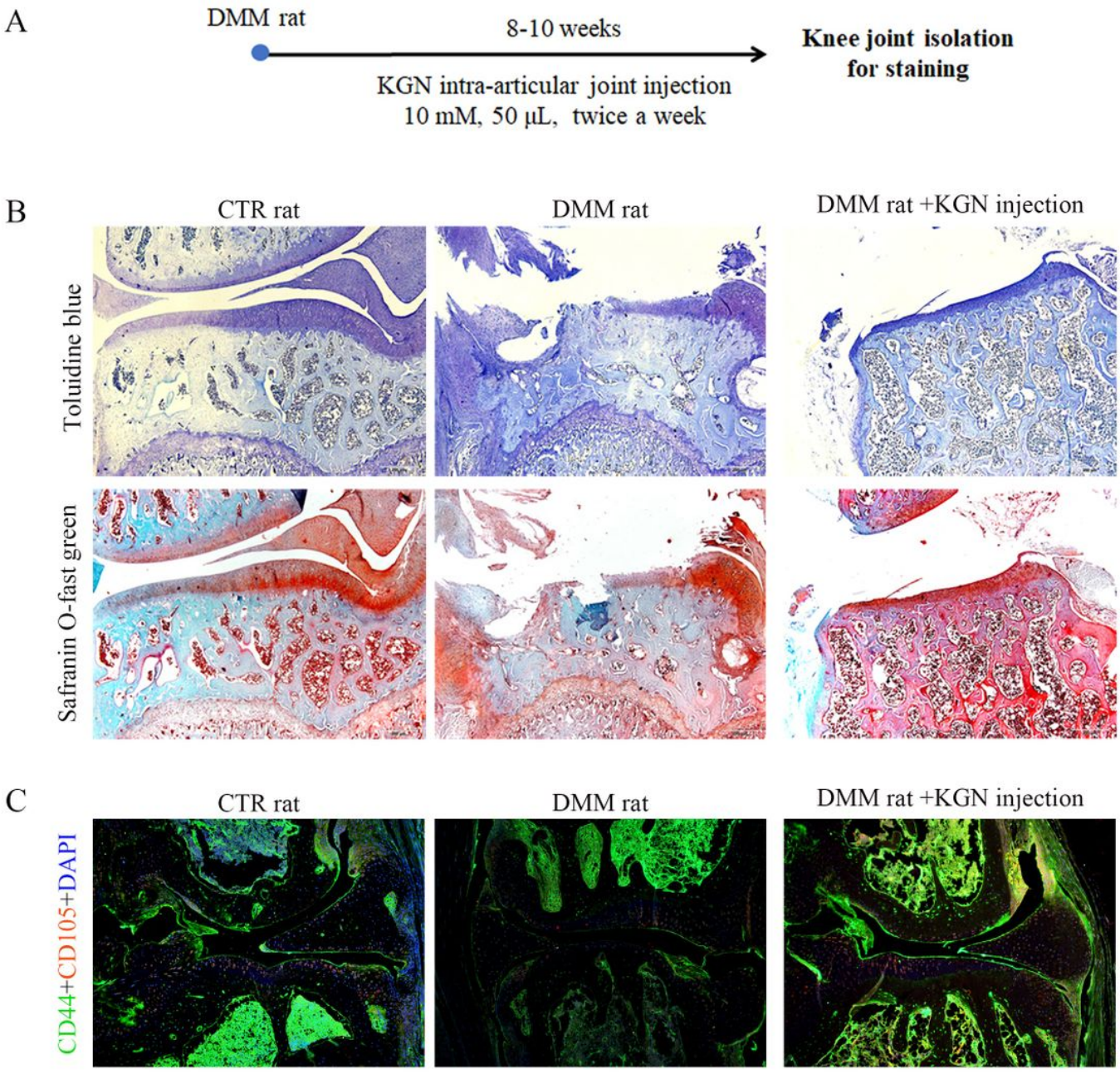

Figure 6

KGN exhibits its effects on cartilage regeneration in vivo in the DMM model (a) Schematic display of time lines for intra-articular injection of KGN into DMM rat. Fifty microliters of KGN at a concentration of 10mM were injected into the knee joints of DMM animal models, twice a week for 8-10 weeks, (b) Toluidine blue and Safranin O and Fast green staining of cartilage tissues isolated from CTR rat (left), DMM rat (middle) and KGN intervened DMM rat (right). The DMM surgery could induce obvious degeneration and defects in articular cartilage. The intra-articular injection of KGN mediated a repair of damaged cartilage. A thicker cartilage layer with more toluidine blue positive cells were observed which means an increased number of chondrocyte. (c) Immunofluorescent double staining for the surface CD44 (green) and CD105 (red) expression in cartilage cells. KGN injection group animals, the number of CD44+CD105+ double positive cells was more than those of other groups, which presented the role of KGN in mediating cartilage repair via enhancing CSPC proliferation.