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Original Article

JD-312 – A novel small molecule that facilitates cartilage repair and alleviates osteoarthritis progression

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ABSTRACT

Background: The chondrogenic differentiation of mesenchymal stem cells (MSCs) to enhance cartilage repair and regeneration is a promising strategy to alleviate osteoarthritis (OA) progression.
Method: The potency of JD-312 in inducing chondrogenic differentiation of MSCs was assessed and verified. The efficacy of JD-312-treated MSCs was evaluated using a Sprague–Dawley rat DMM model. Additionally, the capacity of JD-312 to successfully recruit bone marrow-derived mesenchymal stem cells (BMSCs) for the treatment of OA in *vitro* was confirmed via intra-articular injection. The repair status of the articular cartilage was analyzed in *vivo* through histological examination.
Result: In this study, we identify JD-312 as a novel non-toxic small molecule that can promote chondrogenic differentiation in human umbilical cord-derived MSCs (hUCMSCs) and human bone marrow MSCS (hBMSCs) *in vitro*. We also show that transient differentiation of MSCs with JD-312 prior to *in vivo* administration remarkably improves the regeneration of cartilage and promotes *Col2a1* and *Acan* expression in rat models of DMM, in comparison to kartogenin (KGN) pre-treatment or MSCs alone. Furthermore, direct intra-articular injection of JD-312 in murine model of OA showed reduced loss of articular cartilage and improved pain parameters. Lastly, we identified that the effects of JD-312 are at least in part mediated via upregulation of genes associated with the focal adhesion, PI3K-Akt signaling and the ECM-receptor interaction pathways, and specifically cartilage oligomeric matrix protein (COMP) may play a vital role.
Conclusion: Our study demonstrated that JD-312 showed encouraging repair effects for OA *in vivo*.
The translational potential of this article: Together, our findings demonstrate that JD-312 is a promising new therapeutic molecule for cartilage regeneration with clinical potential.

1. Introduction

Osteoarthritis (OA) is a common debilitating disease characterized by degeneration of articular cartilage [1,2]. The pathogenesis of OA is multifactorial, and in addition to cartilage degradation, is marked by subchondral bone sclerosis, osteophyte formation and muscle weakness

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[3]. Globally, the prevalence of OA has increased by 48 % over the last three decades and is a major public health concern [4]. OA has a significant impact on the quality of life of patients, imposes a heavy economic burden on both patients and society, and hinders social development, causing substantial morbidity and disability [5]. In fact, OA resulted in over 18 million years of healthy life lost to disability (YLD) globally which accounts for 2.2 % of all YLDs [6].

Whilst current treatment options improve joint function and reduce joint pain, they capacity to regenerate and repair the articular cartilage is limited and remains an unmet clinical need. Mesenchymal stem cell (MSC) based cellular therapy is a promising new strategy for regeneration and repair of cartilage defects, which has implications for slowing and even reversing the progression of OA [7–9]. As multipotent cells with the ability to differentiate into chondrocytes, MSCs can be obtained from a variety of autologous tissues including bone marrow (hBMSCs), adipose tissue derived (hADMSCs), peripheral blood (PB-MSCs) and umbilical cord-derived (hUCMSCs), with minimal donor site morbidity [10].

Intra-articular injections of MSCs have been increasing tested for treatment of OA, both in pre-clinical models and clinical trials. Indeed, in animal models of OA ranging from mouse to goats, transplantation of MSCs has shown to reduce pain, prevent and even repair cartilage degradation [11–14]. In a phase I/II clinical trial for knee OA (NCT02580695), intra-articular injection of MSCs were safe, reduced pain from baseline but did not show any structural improvements via MRI at 6 and 12-month follow-up [15]. In another clinical study, hBMSCs administered with platelet rich plasma (PRP) as a co-adjuvant demonstrated no additional regenerative effect at 12 months compared to PRP alone [16]. Whilst these studies may require a longer follow-up to assess any improvements in cartilage regeneration, it is evident that the efficacy of MSCs-based therapy is inconsistent.

Despite the promising outlook for the use of MSCs in cartilage repair, there are several challenges involved in its use. One of the major issues associated with MSC-based therapy is the inconsistency in the differentiation of cells to hyaline cartilage phenotype of chondrocytes, with some studies showing development of ossification in pre-clinical models [17]. Other challenges facing the use of MSC are the heterogeneity in MSC cell populations and the uncertainty regarding the preservation of the MSCs integrity in the inflammatory environment of the OA joint [18].

One of the strategies to overcome the challenges of MSC-based therapy is to pre-differentiated MSCs into chondrocytes prior to administration. However, a study by Jia et al. (2018) observed that articular cartilage defects treated with pre-differentiated MSCs resulted in the formation of less resilient fibrocartilage in rabbit model of OA, whilst hyaline cartilage was detected with undifferentiated MSCs treatment [19]. This was also consistent with the findings of a more recent study in rats which showed that transplanting pre-differentiated hUCMSCs using chondrogenic media resulted in an inferior cartilage repair outcomes compared to undifferentiated hUCMSCs [20]. Recently, kartogenin (KGN) a small-molecule drug has shown to promote chondrogenic differentiation of MSCs by upregulating collagen II (Col2), aggrecan (ACAN) and SRY-box transcription factor 9 (SOX-9) expression [21]. However, the hydrophobic nature of the molecule makes efficient intracellular delivery challenging and make require the use of toxic organic solvents, or nanocarriers [22,23]. Furthermore, it has been reported that KGN alone is only moderately effective in increasing glycosaminoglycans (GAG) and lubricin production in hBMSCs and require co-treatment with TGF-β1 and BMP-7 [24].

In this study, we identify JD-312 as small molecule compound that has a more pro-chondrogenic effect on MSCs compared to KGN. We also show that transient induction of MSCs towards chondrogenic differentiation by JD-312 prior to transplantation gives superior cartilage repair and regenerative abilities in pre-clinical models of OA. These findings provide novel insights into the clinical potential of JD-312 in promoting MSC mediated cartilage regeneration.

2. Materials and methods

2.1. Animals

Sprague Dawley rats and C57BL/6 mice were obtained from the Laboratory Animal Center (LAC) of East China Normal University (ECNU) and all animal experiments were approved by the ECNU Animal Care and Use Committee.

2.2. Cell isolation and culture

mBMSCs were obtained from the bone marrow cavity of 8-week-old male C57BL/6 mice, and cultured in α-MEM (Gibco, USA) supplemented with 10 % fetal bovine serum (Gibco, USA), 100U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, USA). Primary chondrocytes were obtained from the knee-joint cartilage of newborn mice by digesting the tissue with 0.1 % type-2 collagenase overnight and culturing the cells in DMEM/F12 medium (Corning, USA) supplemented with 10 % fetal bovine serum, 100U/mL penicillin and 100 μg/mL streptomycin.

hUCMSCs and hBMSCs (ScienCell, USA) were grown in Mesenchymal Stem Cell Basal Medium (Dakewe, China) with 5 % EliteGro-Advanced (EliteCell, USA), 100U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37 °C with 5 % CO_2.

2.3. Preparation of compound JD-312

4-Chloro-2-nitrophenol (1.74 g, 10 mmol), 2-bromoethyl methyl ether (4.17 g, 30 mmol), potassium carbonate (4.14 g, 30 mmol) was dissolved in N, N-dimethylformamide (30 mL), and the reaction was carried out at 80 °C for 2 h. The solution was then extracted with ethyl acetate. The organic phase was washed twice with distilled water, once with saturated brine, and dried over anhydrous sodium sulfate. After the organic phase evaporated, the compound 4-chloro-1-(2-methoxyethoxy)-2-nitrobenzene (2.2 g, 95 %) was separated and purified by column chromatography.

4-Chloro-1-(2-methoxyethoxy)-2-nitrobenzene (2.2 g, 9.5 mmol) was dissolved in ethanol (30 mL), and iron powder (2.66 g, 47.5 mmol) and ammonium chloride (5.08 g, 95 mmol) were added, and the mixture stirred at 50 °C for 2 h. After the reaction was complete, it was filtered through celite using suction and the filtrate concentrated. The compound was then separated and purified by column chromatography to obtain the compound 5-chloro-2-(2-methoxyethoxy) aniline (1.35 g, 71 %).

5-Chloro-2-(2-methoxyethoxy) aniline (202 mg, 1 mmol), thiophene-2-carboxylic acid (141 mg, 1.1 mmol), DIEA (388 mg, 3 mmol) and HATU (418 mg, 1.1 mmol) were dissolved in 3 mL of N, N-dimethylformamide and the reaction was stirred at room temperature for 6 h. After the reaction was complete, the solution was extracted with ethyl acetate, and the organic phase dried over anhydrous sodium sulfate. The product was then isolated and purified by column chromatography to obtain N-(5-chloro-2-(2-methoxyethoxy) phenyl) thiophene-2-carboxamide (compound **JD-312**, 246 mg, 79 %).

^{1}H NMR (500 MHz, Chloroform-d) δ 8.74 (s, 1H), 8.56 (d, J = 2.6 Hz, 1H), 7.67–7.63 (m, 1H), 7.59–7.54 (m, 1H), 7.16–7.11 (m, 1H), 7.04–6.99 (m, 1H), 6.90 (d, J = 8.7 Hz, 1H), 4.24–4.18 (m, 2H), 3.76–3.70 (m, 2H), 3.42 (s, 3H). HR MS (ESI) calcd. for C14H14ClNO3S $[M+Na]^+$ *m/z* 334.0281, found: 334.0275.**2.4. Alcian Blue staining**.

The cells were washed twice with PBS and then fixed with 4 % paraformaldehyde for 10 min. Subsequently, they were stained with 1 % alcian blue in a solution of 3 % glacial acetic acid for 5 h or overnight. Excess stain was washed off with PBS and the stained cells imaged.

2.5. Migration assays

The migration and invasion assays were performed using 8 μm

Transwell™ inserts (Corning, USA). A total of 1×10^4 cells were seeded in the upper Transwell chamber in 200 μL media, and 600 μL media containing different concentrations of JD-312 was added to the bottom chamber. After 24 h, the migrated cells in the lower chamber of the Transwell were fixed with 4 % paraformaldehyde and washed with PBS three times. The cells were then stained with 0.1 % crystal violet for 10 min. The non-migrating cells in the upper layer were carefully removed using a cotton swab. The number and appearance of the migrated cells were observed and recorded under a microscope. The cell count was determined using ImageJ software.

2.6. 3D culture and proteoglycan detection

MSCs (hUCMSCs or mBMSCs)were seeded into a 15 mL centrifuge tube at a concentration of 250,000 cells/2 mL, and centrifuged at 400 g for 7 min. The tube cap was slightly loosened to ensure sufficient oxygen and then placed in a cell culture incubator. On the third day, 3D cell spheroids were formed at the bottom of the tube. Fresh culture medium containing 10 μM KGN or JD-312 was replaced every 3 days. The tube was gently shaken every day to prevent the spheroids from adhering to the bottom. The weight of the spheroids was measured after continuous suspension culture for 21 days. The GAG (polyprotein) content in the medium was detected by adding dimethylmethylene blue (DMMB) dye solution to the supernatant and measuring the absorbance at a wavelength of 525 nm.

2.7. qRT-PCR

Total mRNA extraction, cDNA conversion, and quantification of mRNA were performed as previously described [25]. Total mRNA was extracted using Trizol (Invitrogen, USA) and cDNA obtained using the 2 × Hifair® II. SuperMix (Yeasen, China) reverse transcription system.

The qRT-PCR reaction was performed according to manufacturer's protocol using SYBR chemistry (Yeasen, China).

The following primers were used for the reactions:

Human GAPDH: 5'-ACAACTTTGGTATCGTGGAAGG-3' (forward).
and 5'-GCCATCACGCCACAGTTTC-3' (reverse).
Human Col2a1: 5'-TGGACGCCATGAAGGTTTTCT-3' (forward).
and 5'-TGGGAGCCAGATTGTCATCTC-3' (reverse).
Human ACAN: 5'-GTGCCTATCAGGACAAGGTCT-3' (forward).
and 5'-GATGCCTTTCACCACGACTTC-3' (reverse).
Mouse GAPDH: 5'-AGGTCGGTGTGAACGGATTTG-3' (forward).
and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse).
Mouse Col2a1: 5'-CCTCCGTCTACTGTCCACTGA-3' (forward).
and 5'-ATTGGAGCCCTGGATGAGCA-3' (reverse).
Mouse ACAN: 5'-CCCTCGGGCAGAAGAAAGAT-3' (forward).
and 5'-CGCTTCTGTAGCCTGTGCTTG-3' (reverse).

2.8. Western blotting

Cell samples use RIPA lysis to extract protein. Protein concentration is measured with the BCA kit (Thermo, USA). SDS-PAGE electrophoresis isolates protein samples and transfers them to nitrocellulose membranes. Block treatment with 5 % BSA. Antibody incubation is then performed. Detailed antibody information is as follows: anti-Col2a1 ((Servicebio, China), anti-Acan (Proteintech, USA), anti-GAPDH (CST, USA). Following overnight incubation at 4 °C. After being washed with TBST three times, the membranes were incubated with secondary antibodies without light for 1 h and washed with TBST three times again. Odyssey Infrared Imaging System (LI-COR, USA) was used to obtain images.

2.9. RNA-seq and analysis

RNA sequencing was conducted to understand the molecular mechanisms that were altered in hUCMSCs by JD-312 treatment (10 μM) for 7 days. Briefly, 1×10^6 cells for each sample were used to isolate total RNA by the standard TRIzol protocol. Subsequently, the gene expression library was constructed by Illumina TruseqTM RNA Sample-Prep Kit, and sequenced on the Illumina HiSeq platform. After initial quality control, the sequence data were mapped to the human reference genome, and differentially expression genes between sample groups were evaluated by DESeq2 ($P < 0.05$, $\log2(FC) > 2$). The volcano, heatmap and enrichment analysis (KEGG and GSEA) were performed on the normalized gene expression data.

2.10. CCK-8 assay

The CCK-8 reagent is a commonly used method for measuring cell viability. The process involves seeding the cells in a 96-well plate and incubating them for 48 h. After aspirating the media, 100 μL of the premade CCK-8 solution (10 %) is added to each well and incubated at 37 °C for 1 h. Subsequently, the absorbance was measured at a wavelength of 450 nm using a spectrophotometer. It is important to protect the entire operation from light to ensure accurate results.

2.11. Animal studies

All surgeries were performed in a sterile environment and the experimental instruments were sterilized. This study used 8-week-old male SD rats which were randomly allocated to Sham, PBS, MSC, MSC + KGN or MSC + JD-312 group. In this study, the hUCMSCS were pre-differentiated towards the chondrogenic lineage using KGN and JD-312 at concentrations of 10 μM respectively for 7 days prior to treatment.

For destabilization of medial meniscus (DMM), rats were injected intraperitoneally with 4 % chloral hydrate (1 mL/100 g) to anesthetize them. The knee joint and the joint cavity was exposed by cutting the skin and the underlying muscles. The meniscus was sectioned using a microsurgical blade and wound was sutured. Subsequently, the joint cavity was injected with MSCs, pre-differentiated stem cells or PBS control 3 days and 4 weeks post-surgery. At 8 weeks post-surgery, the rats were euthanized by carbon dioxide asphyxia, tissues collected and macroscopic photos of the cartilage repair status of each sample were obtained through a digital camera.

For the murine studies, 10–12 week old male C57BL/6 mice were randomly allocated to Sham, Control, KGN or JD-321 group. The mice were then anesthetized with 1.25 % avertine (2,2,2-tribromoethanol) and their meniscus sectioned similar to the rat studies described above. At 3 days post-surgery, the mice were treated with vehicle or small-molecules (10 μM) corresponding to their groups by injection into the joint cavity, once a week for 8 weeks. At the end of the study, the mice were euthanized, and knee joints collected for histological analyses.

2.12. Measurements of thermal hyperalgesia

The paw withdrawal latency (PWL) and threshold (PWT) of thermal were measured in mice using the Ugo-Basile 37370 plantar test (Comerio, Italy). The test works by exposing the soles of the mouse's feet to a heat stimulus, and the time taken for the mouse to withdraw its paw is recorded. The mice were placed on a glass plate of constant temperature for 30 min before each test. Thermal thresholds of the ipsilateral hind paws were assessed three times with a 10 min interval between trials. The time(s) from irradiation to paw withdrawal was recorded as the thermal withdrawal latency. The intensity of the infrared generator was adjusted to produce withdrawal latencies of approximately 10 s (25 infrared intensity).

2.13. Mechanical threshold sensory test

A simplified up-down method (SUDO method) using von Frey nylon filaments was employed to test the hind-paw reflex sensitivity to

punctate static mechanical stimuli. Mice were habituated to the wire mesh bottom cages for 30 min before the test. Von Frey filaments (Aesthesio von Frey Kit, Ugo Basile, Varese, Italy) were applied from underneath the mouse to the mid-plantar surface of the hind paws and size of the filament that elicited a positive response after 5 tests with different filament types. After each pain measurement, the mouse was allowed to rest for at least half an hour. Three measurements were taken for each mouse, and the average was calculated from the recorded values.

2.14. Immunofluorescence

Knee joints of mice were fixed for 24 h in 4 % paraformaldehyde (wt/vol), dehydrated into 100 % ethanol, and decalcified for one week in 0.5 M EDTA pH 8.0 and dehydrated. Paraffin-embedded tissue was sectioned (7 μm) and stained with Safranin O according to standard procedures. The OARSI histologic scoring was used to measure the cartilage and synovial damage of OA [26]. Immunofluorescence staining for Col2a1 (Abcam, Ab34712) and ACAN (Proteintech, 13880-1-AP) were routinely performed. Briefly, paraffin sections were de-waxed and antigen retrieval performed using proteinase K (20 μg/mL) at 37 °C for 30 min. The sections were permeabilized with 0.1 % Triton-100 for 10 min and blocked with 2 % goat serum for 1 h. The sections were incubated with the primary antibody overnight at 4 °C, followed by incubation with the fluorescent secondary antibody at room temperature for 1 h. The sections were stained with DAPI (10 μg/mL) for 5 min and then imaged using Olympus BX53 microscope. The images were quantitated by ImageJ.

2.15. Statistical analysis

All data were analyzed with GraphPad Prism 8.3 (GraphPad, San Diego). One-way ANOVA followed by Tukey's t-tests multiple comparisons was used to compare the groups. Two-tailed Student's t-test was used to detect statistically significant treatment effects when only two groups were compared. $P < 0.05$ were considered as statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Quantification was performed from at least three independent experimental groups and presented as mean ± s.d.

3. Results

3.1. Identification of JD-312 as compound that promotes cartilage differentiation

We screened a small molecule library of 110 compounds against hUCMSCs using transcript-level expression of chondrogenic markers (Col2 and ACAN) as a primary outcome (Supplementary Fig. S1). After screening for small molecules that can induce better Col2 and ACAN expression than KGN (a positive control), cell toxicity studies in hUCMSCs were performed for all candidate molecules (Supplementary Fig. S2). JD-312 elicited an upregulation in expression of Col2 and

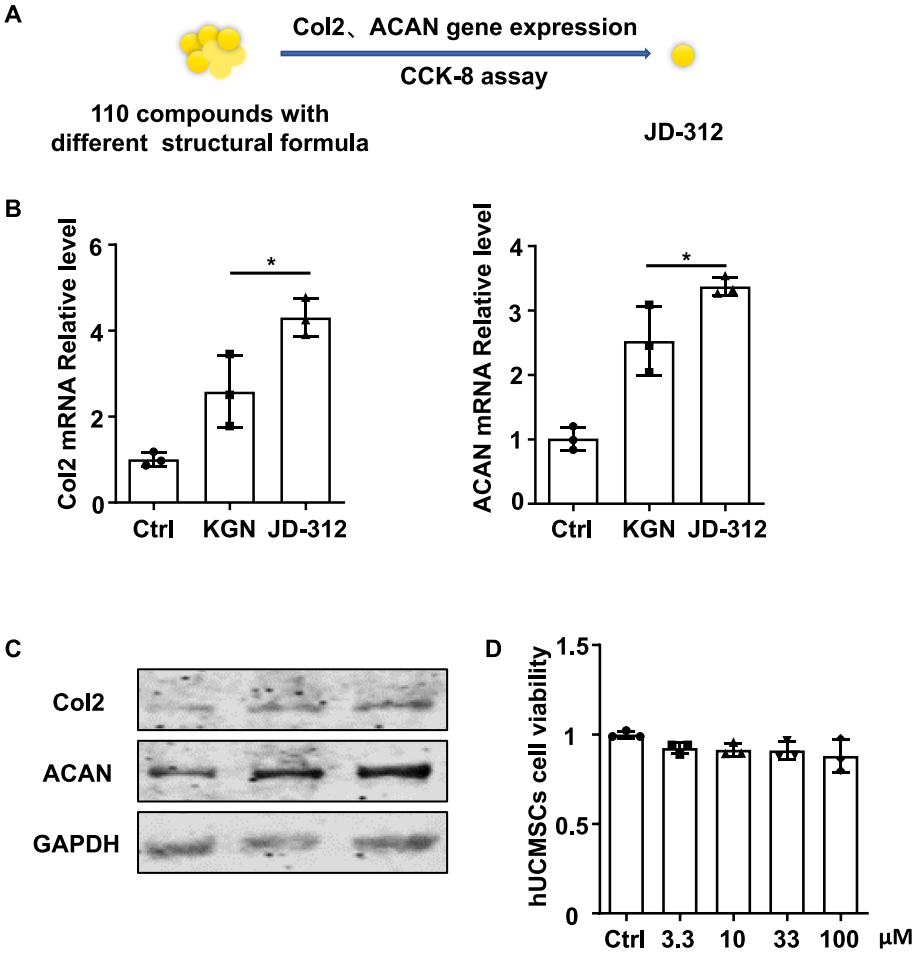

Figure 1. Identification of JD-312 as compound that promotes cartilage differentiation
A) Schematic of drug screening strategy. **B)** JD-312 increases chondrocyte-specific gene expression in hUCMSCs (JD-312, 10 μM. KGN, 10 μM). Cells were incubated for 7 days to detect mRNA expression levels via RT-qPCR (n = 3). Type II Collagen, *Col2a1*. Aggrecan, *Acan*. **C)** The protein levels of Col2 and ACAN in hUCMSCs were analysed by western blotting. D) Effect of JD-312 on cell viability of hUCMSCs detected by CCK8 assay (n = 3).

ACAN compared to KGN ($P < 0.05$) (Fig. 1A–C), and not exhibit any toxic effect on cell viability unlike other compounds (Fig. 1D). Taken together, these results suggest that JD-312 may have therapeutic potential for as a pro-chondrogenic small molecule for treating cartilage related diseases.

3.2. JD-312 promotes the chondrogenic differentiation of hUCMSCs in vitro

We used a micromass culture of hUCMSCs to validate the effectiveness of JD-312 in inducing chondrogenic differentiation. We observed a

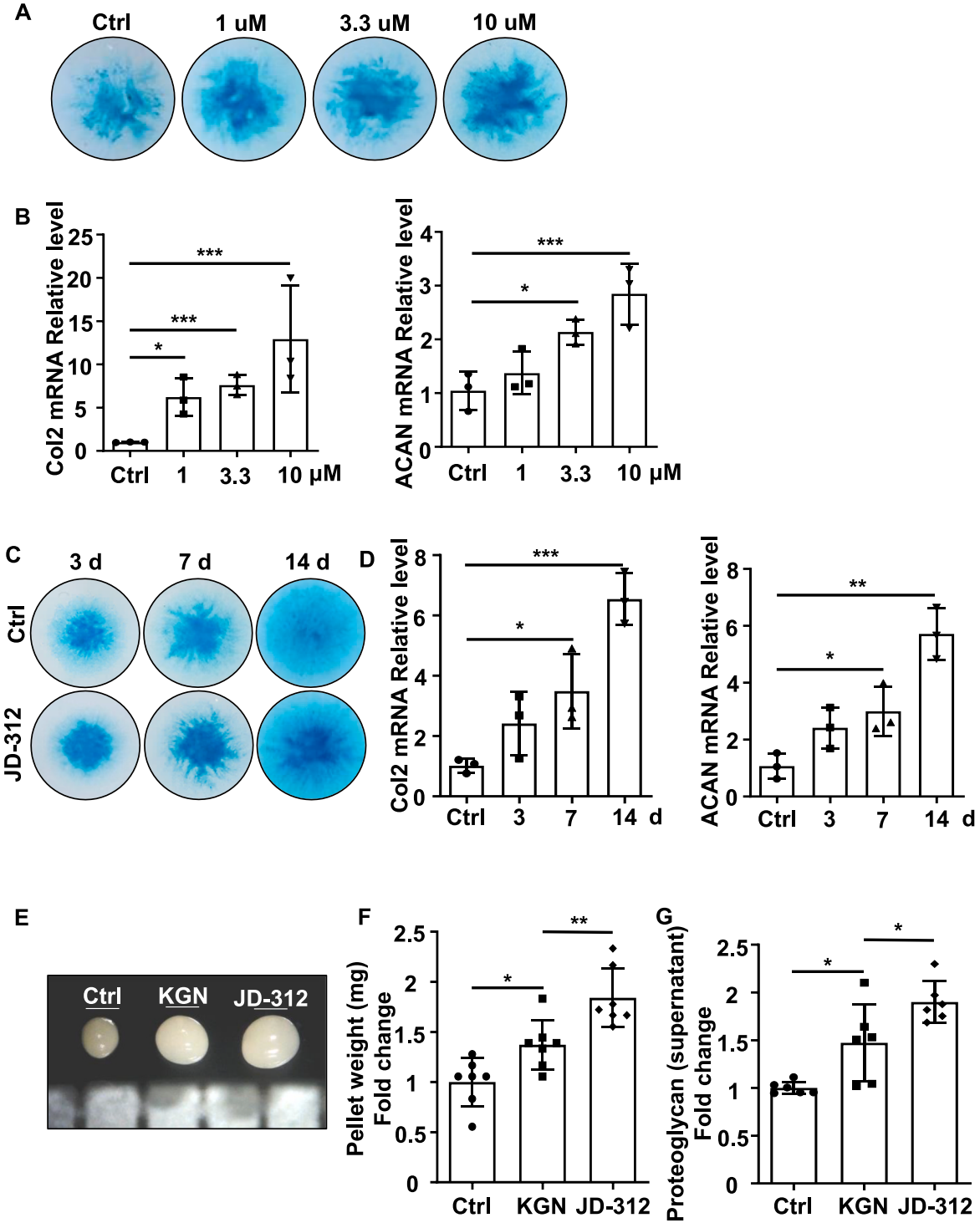

Figure 2. JD-312 promotes the chondrogenic differentiation of hUCMSCs *in vitro*.
A) Alcian blue staining showing the effect of JD-312 on chondrogenic differentiation of hUCMSCs. **B)** RT-qPCR analysis of *Col2a1* and *Acan* mRNA expression in hUCMSCs treated with varying concentrations of JD-312. **C)** Alcian blue staining showing the effect of JD-312 (10 μM) on chondrogenic differentiation of hUCMSCs over time. **D)** RT-qPCR analysis of *Col2a1* and *Acan* mRNA expression over time in hUCMSCs treated with JD-312. **E)** Macroscopic images of 3D micromass cultures of hUCMSCs treated with kartogenin (KGN) and JD-312. **F)** illustrates the weight of 3D micromass cultures shown in E. **G)** The concentration of glycosaminoglycan (GAG) released in the culture supernatant of the 3D micromass cultures at 21 days. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dose-dependent increase in alcian blue staining with JD-312 treatment (0–10 μM, Fig. 2A) of hUCMSCs, which suggests increased GAG matrix synthesis and thus chondrogenic differentiation. This was accompanied by increased mRNA expression of chondrogenic markers Col2 and ACAN (Fig. 2B, $P < 0.05$). Furthermore, JD-312 (10 μM) exposure over 14 days resulted in time-dependent increase in alcian blue staining and chondrogenic differentiation of hUCMSCs as measured by Col2 and ACAN expression (Fig. 2C and D).

In addition to the 2D *in vitro* culture, we used a 3D suspension culture system with hUCMSCs to assess the chondrogenic differentiation. We observed a significantly larger pellet weight for the JD-312 (10 μM) treated cells compared to the controls as well as KGN (10 μM) ($P < 0.01$, Fig. 2E and F). This was associated with an increased proteoglycan content in the culture supernatant with JD-312 (10 μM) treatment ($P < 0.05$; Fig. 2G).

3.3. JD-312 promotes chondrogenic differentiation and migration of BMSCs in vitro

The capacity to repair cartilage damage can be boosted by the colonization of BMSCs at the site of injury, and the chondrogenic differentiation of these cells can be facilitated through the use of small molecules [27]. Next, we investigated the ability of JD-312 to induce chondrogenic differentiation in BMSCs. We observed that treatment of human BMSCs with JD-312 (10 μM) resulted in chondrogenic differentiation and GAG matrix synthesis to a similar extent as KGN (Fig. 3A). In the 3D suspension culture system, we observed that both KGN and JD-312 significantly increased the pellet size formed by hBMSCs compared to the control group (Fig. 3B). The proteoglycan content in the culture supernatant after a 21-day culture was also higher for KGN and JD-312 treated hBMSCs compared to the untreated controls, with no significant difference between the two treatments (Fig. 3C). Expression of chondrogenic differentiation markers (Col2 and ACAN) was significantly higher for KGN and JD-312 treated cells compared to untreated controls, with JD-312 inducing significantly more Col2 expression than KGN ($P < 0.01$; Fig. 3D).

The migration of MSCs to the site of cartilage injury and subsequently differentiate into articular chondrocytes is potential strategy for regeneration and repair of cartilage defects [28–30]. JD-312 promoted migration of hBMSCs in Transwell culture system in a dose-dependent manner (Fig. 3E and F). This dose-dependent pro-migratory effect of JD-312 was also observed for murine BMSCs (mBMSCs, Fig. 3G and H).

3.4. JD-312 treated hUCMSCs enhances cartilage repair after DMM surgery in SD rats

Given that JD-312 has shown chondrogenic potential *in vitro*, we next evaluated its ability to repair articular cartilage *in vivo*. hUCMSCs were pre-treated with 10 μM JD-312 (MSC + JD-312), KGN (MSC + KGN) or vehicle (MSC) for 7 days and then injected into the joint cavity of SD rats which have undergone DMM. SHAM surgery and PBS injected group served as controls. After 8 weeks, the cartilage injury and glycosaminoglycan loss observed in the PBS control group was repaired in all treatment groups, with MSC + JD-312 showing the most improvement (Fig. 4A). The Osteoarthritis Research Society International (OARSI) scores were also significantly lower for the MSC + KGN and MSC + JD-312 group compared to MSC treatment alone (Fig. 4B). Moreover, the expression of chondrogenic differentiation factors Col2 and ACAN was also higher for the MSC-JD-312 group compared to MSC only control (Fig. 4A–C).

3.5. JD-312 can promote postoperative cartilage repair in DMM mice model

Next, we used a murine model of DMM to determine the effect of JD-312 treatment on cartilage degeneration following injury. Our results show that the damage of articular cartilage and glycosaminoglycan loss post-DMM was significantly lower following intra-articular treatments with JD-312 and KGN compared to the PBS controls, with no differences in the OARSI score between the two small-molecule treatments (Fig. 5A and B). Furthermore, the expression of cartilage anabolic factors Col2 and ACAN was also higher in the KGN and JD-312 treatment groups compared to the control with JD-312 having more Col2 positive cells than KGN (Fig. 5A and D, $P < 0.05$).

Treatment with JD-312 also showed a higher paw withdrawal threshold in von Frey assay and significantly increased thermal withdrawal latency in a thermal hyperalgesia test when compared to the control and KGN treated groups (Fig. 5C). These results demonstrate that JD-312 is effective in reducing DMM surgery-induced cartilage degeneration and pain.

3.6. Identifying the molecular mechanisms that govern JD-312's chondrogenic effect in hUCMSCS

Lastly, we performed transcriptome-wide expression analyses to understand the molecular mechanisms that govern the physiological effects of JD-312. hUCMSCs were treated with JD-312 for 7 days and the changes in gene expression compared with untreated controls. We observed an upregulation of 823 transcripts and a downregulation of 788 transcripts which is illustrated in a volcano plot (Fig. 6A). KEGG pathway enrichment and GSEA analyses showed that upregulated genes associated with focal adhesion, PI3K-Akt signaling and the ECM-receptor interaction pathway were the most enriched (Fig. 6B and C). Interestingly, cartilage oligomeric matrix protein (COMP) was the most upregulated gene ($log2FC = 7.88$, Fig. 6D, Supplemental Fig. 4), and is enriched in all three of the above pathways. COMP is known to promote the expression of SOX9 and induce the formation of cartilage matrix and it is plausible that JD-312 mediates in pro-chondrogenic effects in MSCs via COMP.

4. Discussion

Currently, there are no effective therapies for articular regeneration clinically, making it an unmet need. Here, we show that JD-312 is a novel non-toxic small molecule promotes chondrogenesis in human MSCs by upregulating expression of anabolic markers (*Col2a1* and *Acan*). Furthermore, pre-treating of MSCs with JD-312 to induce chondrogenic differentiation prior to intra-articular injection showed remarkable regeneration of cartilage in rat models of OA. Direct intra-articular injection of JD-312 in murine model of OA showed reduced loss of articular cartilage and improved pain parameters. Lastly, we identified that the effects of JD-312 are at least in part mediated via upregulation of COMP.

Cartilage tissue is avascular with limited ability to repair and self-renew, and without effective treatments, a small cartilage defect can lead to progressive tissue deterioration, joint dysfunction and pain and eventually the degenerative disease of OA. Conventional treatment strategies include medication for pain relief, microfractures, osteochondral autografts, autologous chondrocyte implantation and joint replacement surgeries. Whilst these methods may ease pain symptoms, they are not creative as they do not restore the damaged cartilage [31]. MSC-based cell therapy is an up and coming alternative as they have the ability to differentiate into mature chondrocytes that can repair and regenerate cartilage lesions [32]. However, this modality faces several challenges that need to be overcome, such as maintaining the chondrogenic potential of the cells and migration of the cells to non-target tissues [33]. In fact, a post-surgical prognosis study observed that 76 % of patients who received hADMSC transplantation had abnormal or severely abnormal repair of cartilage lesions [34].

There are multiple strategies that are currently being explored to improve the outcomes of MSC-based cell therapy. They range from using of microrobots to improve targeting of MSCs to lesions [35], to using

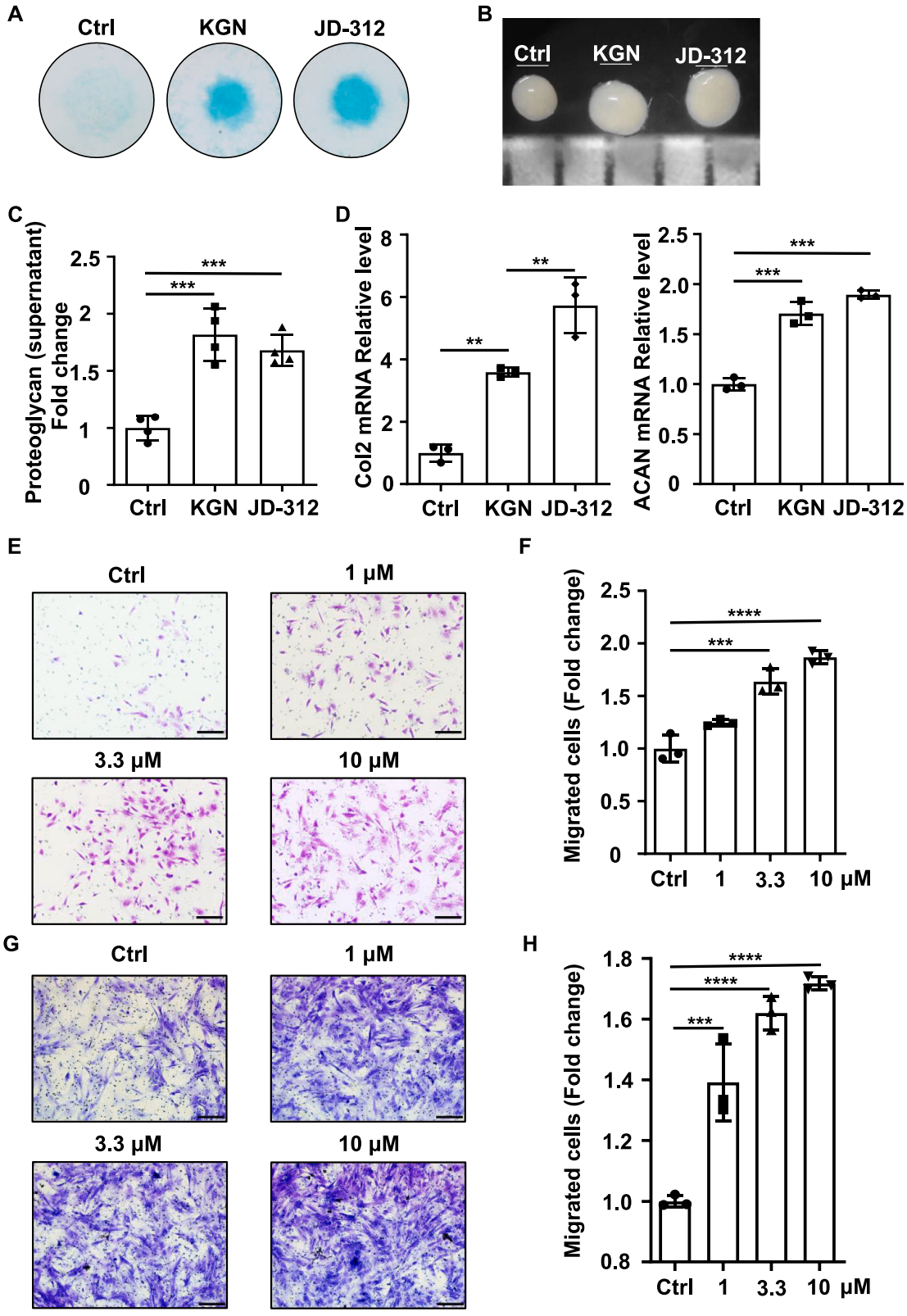

Figure 3. JD-312 promotes chondrogenic differentiation and migration of BMSCs in *vitro*

A) Alcian blue staining showing the effect of JD-312 on chondrogenic differentiation of hBMSCs. **B)** Macroscopic images of 3D micromass cultures of hBMSCs treated with kartogenin (KGN) and JD-312. **C)** The concentration of glycosaminoglycan (GAG) released in the culture supernatant of the 3D micromass cultures of hBMSCs treated with KGN or JD-312. **D)** RT-qPCR analysis of *Col2* and *Acan* mRNA expression in hBMSCs treated with KGN or JD-312 at day 14. **E)** Crystal violet stained mBMSCs that have migrated across the transwell membrane following treatment with JD-312 and **F)** the quantification of the images. **G)** Crystal violet stained hBMSCs that have migrated across the transwell membrane following treatment with JD-312 and **H)** the quantification of the images. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

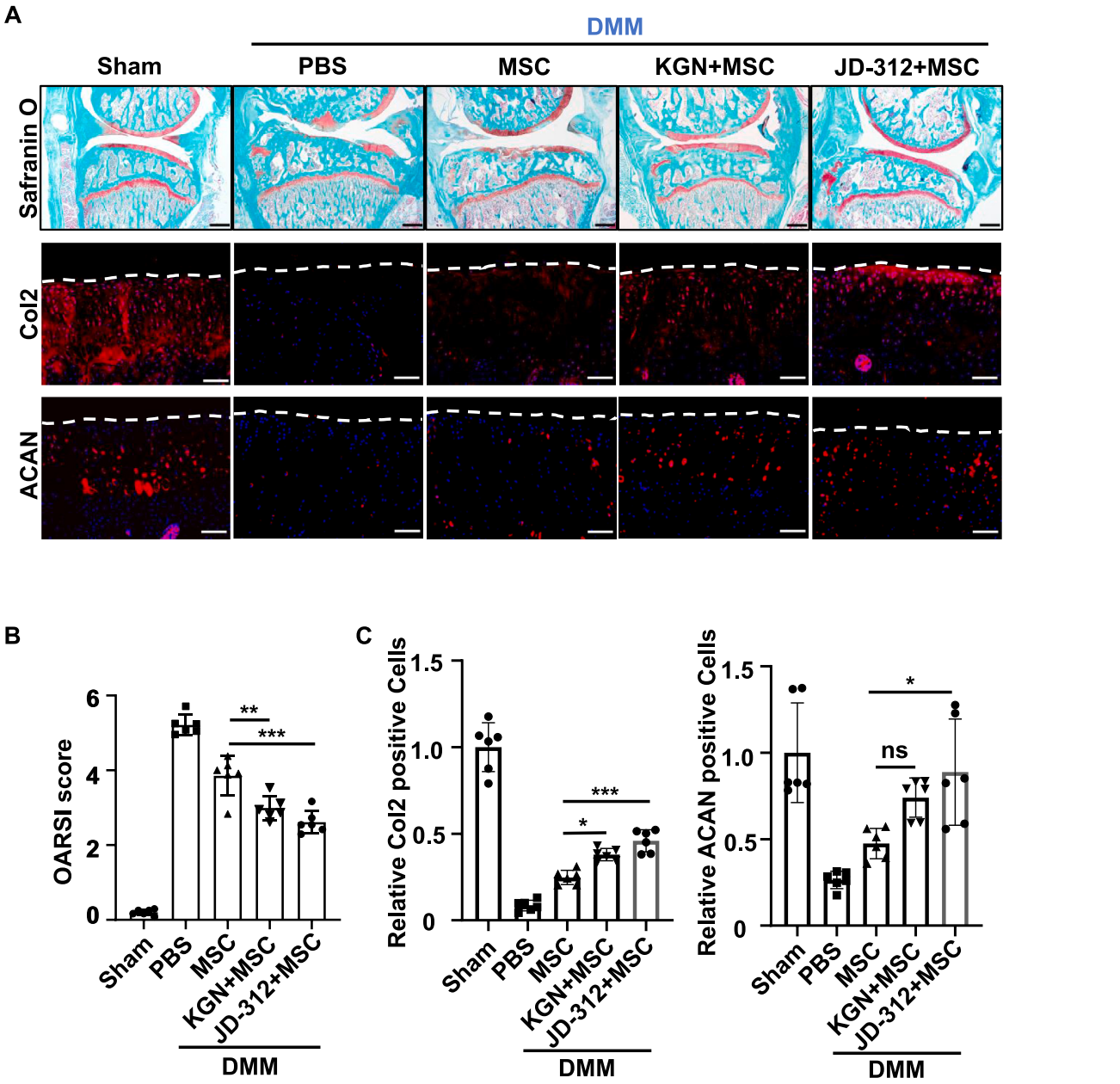

Figure 4. JD-312 treated hUCMSCs enhances cartilage repair after DMM surgery in SD rats
A) Representative images of Safranin-O staining of knee joints following sham operation or DMM in rats which were treated with hUCMSCs (MSC), or hUCMSCs transiently induced with KGN (KGN + MSC) or JD-312 (JD-312+MSC), with PBS as control. Scale bar = 400 μm. The OARSI scores for the sections are reported in **B)**. Knee joint sections were stained with antibodies against Col2 and ACAN and the expression is quantified in **C)**. n = 6 per group. Red, Col2a1, ACAN; Blue, DAPI. (*P < 0.05, **P < 0.01, ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

MSC extracellular vesicles to accelerate the restoration of cartilage [36]. Here we show induction of hUCMSCS with our novel chondrogenic compound JD-312 has more favorable outcomes than a well-established chondroprotective agent (KGN) *in vitro*, with higher Col2 and ACAN expression, and a larger 3D chondrocyte pellet with increased GAG expression.

We also show that a transient pre-differentiation of MSCs towards chondrogenesis prior to injecting may have better regeneration outcomes for MSC-based therapy. In a DMM rat model, whilst we observed cartilage regeneration with MSC treatment alone, MSCs pre-treatment with KGN had better outcome with the most pronounced effect observed following JD-312 pre-treatment. This is in contrast to a majority of studies that aim pre-differentiate MSCs into mature chondrocytes prior to administration which have shown limited success [37–39]. This might partially be due to ability of MSCs to undergo hypertrophic chondrogenic differentiation, which hampers the formation of hyaline cartilage *in vivo* [40]. Indeed, MSC differentiation to chondrocytes is a complex process governed by a balance of factors including cytokines, extracellular matrix, physical stimuli and interactions with other cells in the microenvironment [39]. Transient induction of MSCs by JD-312 towards chondrocyte lineage prior to administration may allow the cells to continue differentiating in this complex *in vivo* environment, preventing them from undergoing hypertrophy, and thereby improving repair outcomes as observed in *this* study.

Although direct administration of JD-312 into the joint cavity of mice following DMM showed no further improvement in articular cartilage regeneration compared to KGN, the pain parameters were significantly better. Mechanistically, KGN is reported to regulate core-

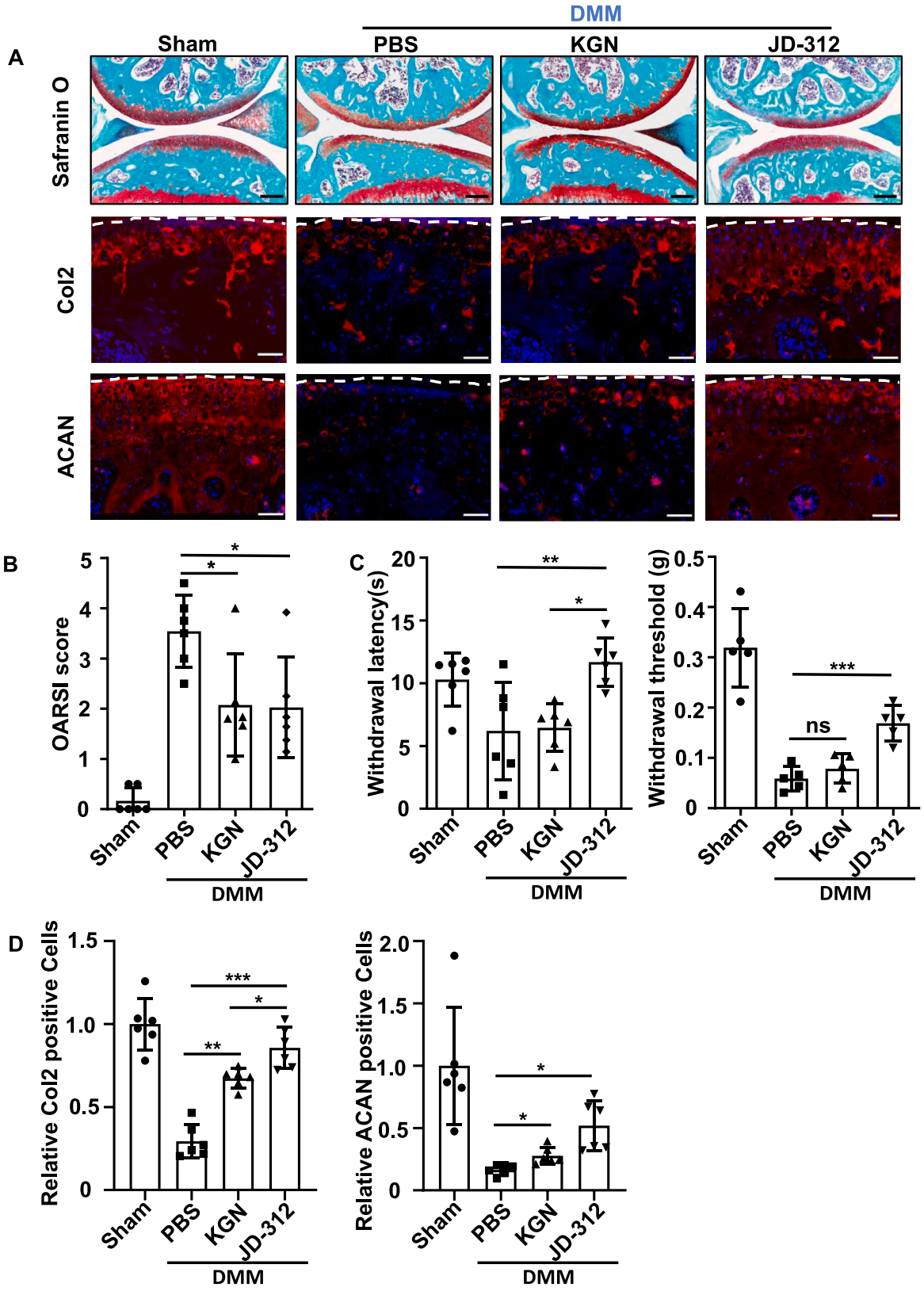

Figure 5. JD-312 promotes postoperative cartilage repair in DMM mice model

A) Representative images of Safranin-O staining of knee joints following sham operation or DMM in mice which were treated with KGN (10 μM) or JD-312 (10 μM), with PBS as control. Scale bar = 150 μm. The OARSI scores for the sections are reported in **B)**. Knee joint sections were stained with antibodies against Col2 and ACAN and the expression is quantified in **D)**. Pain responses was measured using the von Frey assay and the thermal paw withdrawal test which are quantified in **C)**. n = 6 per group. Red, Col2a1, ACAN; Blue, DAPI. (*P < 0.05, **P < 0.01, ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

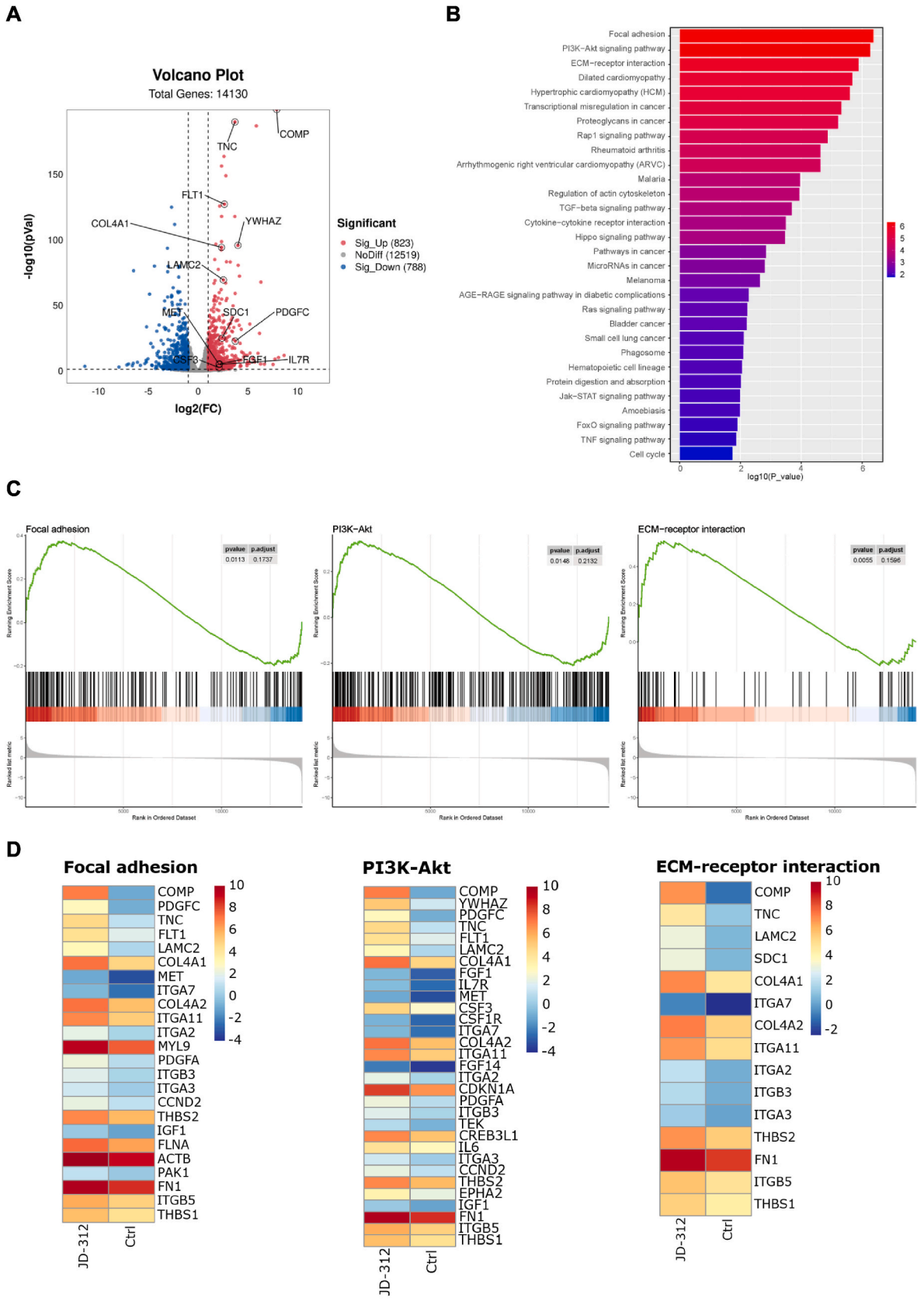

Figure 6. Identifying the molecular mechanisms that govern JD-312's chondrogenic effect in hUCMSCS
A) Volcano plots depicting the differentially expressed genes between hUCMSCs treated with JD-312 (10 μM) after 7 days. **B** and **C)** KEGG and GSEA enrichment analysis of the differentially expressed genes. **D)** Heatmap depicting the fold changes of differentially expressed genes associated with the Focal adhesion, PI3K-Akt, and ECM-receptor interaction pathways highlighted.

binding factor β (CBF-β) which binds to RUNX proteins and forms a transcriptional complex that regulates multiple genes [41]. Thermal and neuropathic pain have been shown to be regulated via Runx, with mice lacking *Runx1* having delayed reaction time to pain stimuli [42]. Thus, it is possible that despite of having anabolic effects on cartilage, KGN upregulates RUNX1 and mediates increased pain nociception in mice compared to JD-312 [41]. Here, we identify the predominant molecular mechanisms that mediate the effects of JD-312 to be genes associated with the focal adhesion, PI3K-Akt and the ECM-receptor interaction pathways with COMP being the most upregulated gene. COMP is known the stimulate chondrocyte proliferation, chondrogenesis and matrix homeostasis [43–45]. Based on the gene signature, we believe that the effects of JD-312 are largely anabolic in nature, enhancing cartilage regeneration and thus reducing pain.

In conclusion, this study identified JD-312 as novel small molecule which can induce chondrogenic differentiation in MSCs and is effective in mediating cartilage repair and regeneration. Our findings demonstrate that JD-312 can be a promising therapeutic molecule for MSC based cartilage regeneration strategies and has clinical potential.

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Declaration of competing interest

No confict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2023.11.007.

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