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Osteoarthritis and Cartilage

RHEB gene therapy maintains the chondrogenic characteristics and protects cartilage tissue from degenerative damage during experimental murine osteoarthritis

S. Ashraf † [a], B.J. Kim ‡ [a], S. Park §, H. Park † [**], S.-H. Lee ‡ [*]

† School of Integrative Engineering, Chung-Ang University, Seoul, Republic of Korea
‡ Department of Medical Biotechnology, Dongguk University, Seoul, Republic of Korea
§ Department of Biomedical Science, CHA University, Seoul, Republic of Korea

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SUMMARY

Objective: Osteoarthritis (OA) is characterized by cartilage degeneration resulting from hypertrophic changes in chondrocytes caused by altered gene expression. The involvement of Ras homolog enriched in brain (RHEB) in OA regulation is unclear.

Methods: Human knee articular cartilage samples - were analyzed for structural and biological changes by histology, immunohistochemistry, real time PCR and western blotting. OA-mouse model developed by surgical destabilization of the medial meniscus (DMM) were treated with adenovirus harboring *Rheb* gene to analyze onset and progression of OA. Histological scoring, immunohistochemistry, and TUNEL assay was performed to assess cartilage damage across the entire joint.

Results: Human and mouse OA cartilage is degenerated and has markedly reduced levels of RHEB. Human OA-degenerated chondrocytes (DC) exhibited a fibroblastic phenotype and 80 % of degenerative cartilage were senescent, with higher levels of reactive oxygen species (ROS). Gene expression analysis of DC revealed almost no *COL2A1* expression and reduced *SOX9* and *RHEB* expression. Transient transfection of *RHEB* rescued the DC phenotype and reduced senescence and ROS levels markedly. *RHEB* overexpression also increased *COL2A1* and *SOX9* expression. In an OA-mouse model, the Rheb protein level decreased as the severity of OA increased. Ectopic expression of *Rheb* using adenovirus in mouse-OA cartilage suppressed surgically-induced OA pathogenesis accompanied by modulation of Adamts5, Mmp 13, Col 10, and Col2a1 expression. *Rheb* induction significantly reduced apoptosis in OA-cartilage.

Conclusion: RHEB plays an important role in maintaining the chondrogenic characteristics of chondrocytes, and has potential in preventing progression of OA in the destabilize the medial meniscus (DMM) mouse model of OA.

Introduction

Osteoarthritis (OA) is the most common joint disabling condition and distresses the articular cartilage[1]. Chondrocytes synthesize the matrix molecules needed to maintain physical function of the cartilage, such as aggrecan and collagen type 2 (Col2a1)[2]. healthy chondrocytes (HCs) are the key to the cartilage tissue's maintenance, and chondrocytes deterioration accompanies matrix degradation[3]. The massive destructive structural changes in the cartilage eventually lead to the initiation and progression of OA[4].

Regenerative therapies aimed at repairing cartilage in OA include autologous chondrocyte implantation (ACI) and gene therapy[5]. ACI is a promising approach, but it is challenging to obtain adequate numbers of clinical-grade chondrocytes for successful therapy[2,6,7]. HCs undergo senescence or dedifferentiation during *in vitro* culture and produce higher reactive oxygen species (ROS) resulting in deviation from their normal functions[2,6]. These

* Address correspondence and reprint requests to: S.-H. Lee, Department of Medical Biotechnology, Dongguk University, Seoul, Republic of Korea. Tel: 82-31-961-5153; Fax: 82-31-961-5108.
** Address correspondence and reprint requests to: H. Park. School of Integrative Engineering, Chung-Ang University, Seoul, Republic of Korea. Tel: 82-2 820-5940; Fax: 82-2 814-2651.
E-mail addresses: heyshoo@cau.ac.kr (H. Park), soohong@dongguk.edu (S.-H. Lee).
[a] Equally contributed.

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senescent chondrocytes exhibit irreversible growth arrest, phenotypic changes, decreased expression of chondrogenic markers like aggrecan, Col2a1, and higher expression of hypertrophic markers like collagen type X (COL10)[2,4,6,8];. However, gene therapy by adenoviruses offers a radically different approach for OA treatment. Because, adenovirus carries and directly transfer genes of interest into articular chondrocytes to modulate the structure and function of OA cartilage[1].

Oxidative stress and senescence are also important factors which are associated with aging and contribute to the progression of OA[9,10]. Mechanical stress and inflammatory mediators are implicated in ROS production in chondrocytes[11]. Excessive ROS are cytotoxic to chondrocytes, resulting in senescent and dedifferentiated chondrocytes that show decreased survival[2,6,12]. Senescence and ROS are also involved in the inhibition of proteoglycan synthesis and degeneration of the ECM, ultimately having detrimental effects on cartilage[4,11,13,14]. Targeting the senescence and oxidative stress can reduce the progression of OA.[10,15]

Ras homolog enriched in brain (RHEB) belongs to the Ras superfamily which regulates cell growth, cell proliferation, and differentiation[16]. Previous functional studies have revealed that *Rheb*$^{-/-}$ mice die at the midgestation embryonic stage, most likely because of impaired development of the cardiovascular system[17]. Importantly, heart tissue originates from the same mesodermal stem cell lineage that gives rise to cartilage and bone connective tissue[18]. Taking this into account, previously, we found that RHEB is involved in maintaining the native characteristics of chondrocytes and the regeneration of cartilage[2,19]. RHEB also reduces the senescence and oxidative stress via upregulation of MCL1 during prolonged culture of chondrocytes, and plays an important role in cartilage formation from chondrocytes implanted under the skin of nude mice and during *in vitro* 3D culture.[2]

Our previous findings about important role of RHEB in chondrocytes prompted us to investigate its yet unexplored role in OA. In the present study, we hypothesized that RHEB may play a regulatory role in the reversal of the phenotypic and functional characteristics of OA-chondrocytes. Second, in well-established murine OA model generated by surgical destabilization of the medial meniscus (DMM), forced expression of *Rheb* gene via adenoviruses would be sufficient to mitigate OA progression.

Materials and methods

Human articular chondrocyte isolation and cell culture

Human cartilage tissue was taken from the knees of patients during a total-knee replacement after receiving informed consent. Ethical approval was taken from the institutional review board of Seoul St. Mary's Hospital (IRB No. 2014–097). Chondrocytes were isolated from white-colored and apparently normal-looking areas of cartilage from OA joints following a previously described protocol[2]. Special care was given to exclude subchondral bone.

Isolated cartilage tissues from each patient had approximate weight of 4–5 g. Cartilage was washed extensively with Hank's Balanced Salt Solution (HBSS) buffer and chopped into small pieces. Chondrocytes from minced cartilage were isolated enzymatically with overnight treatment of 0.5 mg/mL collagenase type II in Dulbecco's Modified Eagle Medium low glucose (DMEM-LG) (Gibco BRL, Gaithersburg, MD, USA) without serum at 37°C. Then next day tissue was filtered using a 40 mm filter and harvested cells were centrifuged, washed three times with PBS, and re-suspended in DMEM-LG. Chondrocytes isolated were cultured for expansion in DMEM-LG supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in humidified air with 5 % (v/v) CO_2 at 37°C or preserved in liquid nitrogen. The initial seeding density was 2×10^4 cells/cm^2.

Cell morphology and cell area

Cellular morphology was visualized by differential interference contrast (DIC) microscopy (DIC). DIC images were used to determine cell area. Briefly, cells area was measured from 100 cells/donor using ImageJ software and average area from each donor was plotted in dot bot.

Protein and RNA extraction from human cartilage tissue

Healthy cartilage devoid of any damage in the femoral condyles and tibial plateau joint, and degenerative cartilage showing clear damage, was collected from the knee of an OA patient. Almost 50 mg of cartilage frozen in liquid nitrogen was pulverized using a mortar and pestle. Total protein was extracted using PRO-PREP buffer (iNtRON Biotechnology, Seongnam-Si, Korea), according to the manufacturer's instructions. Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA).

RHEB gene delivery in chondrocytes

Human *RHEB* gene (NM_005614.3) cloned in pEGFP-N1 vector that also contained the gene sequence for green fluorescent protein (GFP) was obtained from Enzynomics, Daejeon, Korea. pEGFP-N1 vector without *RHEB* gene sequence was used as a Mock/control. For transfection, microporator (Neon™, Invitrogen) was used as previously described[2]. Efficiency of transfection was assessed by visualizing GFP in chondrocytes using a microscope (Nikon, Eclipse 55i, Kanagawa, Japan) and quantified by fluorescence activated cell sorting (FACS) using BD FACSCalibur system (San Jose, CA, USA) after 24 h.

Quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). cDNA was synthesized using RT-PreMix (Bioneer, Daejeon, Korea) and quantitative real-time PCR (qPCR) was performed using the Power SYBR Green Master Mix (Life Technologies, UK) on Step One Plus PCR system (AB Applied, Life Technologies) as described previously[2]. Gene expression data was normalized with *GAPDH* housekeeping gene. The primers used are described in Supplementary Table 1.

Western blotting

Protein was harvested from the cells using radioimmunoprecipitation assay buffer (Sigma) and concentration was measured by bicinchoninic acid assay (Pierce). Approximately 25 μg of total protein was used in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting, as described previously[6]. RHEB protein was quantified in Western blot bands using imageJ software and normalized with β-Actin protein bands. Anti-RHEB, anti-SOX9, anti-MMP-13, and anti-COL10 antibodies were obtained from Abcam (Cambridge, MA). Anti-COL2A1 antibody was purchased from Millipore (Billerica, MA). Goat anti-mouse and anti-rabbit IgG antibodies conjugated with Alexa 594 were purchased from Molecular Probes (Eugene, OR).

SA-β-galactosidase staining

Senescence associated β-galactosidase (SA-β-Gal) was detected by a staining kit (Cell Signaling Technology, Danvers, MA, USA).

Briefly, cells were cultured for 2–3 days and subconfluent cells were stained according to the manufacturer's directions. Any blue-stained cells were considered positive for senescence. All of the cells in three different fields were counted and the percentage of senescent cells was calculated. The experiment was performed using cells from three independent tissue harvests.

Detection of ROS

Cells were cultured on coverslips (Thermo Scientific, NJ, USA) in 12-well plates. At 80–90% confluency, a pre-warmed HBSS/Ca/Mg solution that contains 3mM of the MitoSOX Red (M36008, Invitrogen) probe and 100 nM of the MitoTracker Green (MP 07510, Invitrogen) probe was added and incubated for 15 min at 37°C. Nuclear staining was done by 0.2 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) in the dark. After washing with PBS, partially dried coverslips were mounted on glass slides with mounting medium. Fluorescence was observed by confocal microscope (Leica, Wetzlar, Germany).

Osteoarthritis animal model

Male C57BL/6 mice (9 weeks old) purchased from Orient-bio, Seungnam, Korea, were anesthetized with Zoletil (50 mg/kg) and Rompun (10 mg/kg). OA was induced by dissecting the medial meniscus-ligament to destabilize the medial meniscus (DMM) in the right knee joint of the hind limb during day time, as described previously[20]. Mice in the sham group were surgically treated as above, but without dissecting the medial meniscus ligament. Five mice were housed together in a cage and were raised at 55–65% humidity at a controlled temperature of 24 ± 3°C with a 12-h light/dark cycle and cared according to the institutional protocol. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University (IACUC150023).

Experimental groups and adenovirus injection

Two sets of experiments were designed using the OA mice. In the first experiment, mice ($n = 24$) were divided into the sham group (8 weeks) and OA groups at 2, 4, and 8 weeks ($n = 6$/time point) as shown in Table Ia. In second experiment, mice ($n = 28$) were divided into sham group ($n = 7$) and three OA groups with adenovirus injection overexpressing GFP (Mock) and *Rheb* ($n = 7$/group) as shown in Table Ib. The adenovirus (1×10^9 PFU/10 μL) was injected directly into the synovial cavity once a week for 3 weeks after inducing OA[21]. The samples from the second experiment were analyzed at 8 weeks.

Table Ia
Experimental groups details

Experimental groups	Mice sacrificed at (No. of weeks)	Number of mice (male)
Sham (Normal cartilage)	8	6
DMM (OA)	2, 4, 8	18 (6 for each time point)

Table Ib
Experimental groups details. All mice in this group were sacrificed at 8 weeks after surgery

Experimental groups	Injection (μL/mice)	Time point of injections	Number of mice (male)
Sham (Normal cartilage)	–	–	7
DMM (OA) + saline	10	0, 2, 4 weeks	7
DMM (OA) + adenovirus-GFP (Mock)	10	0, 2, 4 weeks	7
DMM (OA) + adenovirus-Rheb	10	0, 2, 4 weeks	7

Histology

Full-thickness human cartilage was fixed in 4 % paraformaldehyde for 3 days. Whole joint tissue was decalcified in 5 % nitric acid for 48 h at room temperature and embedded in paraffin wax. Tissue sections were obtained at a thickness of 4 μm.

In the animal experiments, the mice were euthanized with carbon dioxide at 2, 4, and 8 weeks postoperatively and the right knee joints were fixed in 4 % paraformaldehyde for 24 h. Whole joint tissue was decalcified in 5 % nitric acid for 10 h at room temperature and embedded in paraffin wax. Approximately seven coronal sections with a thickness of 4 μm were prepared from an entire joint tissue at 100 μm intervals. The sections were stained with safranin-O/fast green to examine the amount of sulfated glycosaminoglycans (GAGs), as described previously[22].

Osteoarthritis scores

The safranin-O/fast green staining images were used to evaluate the progression of OA semi-quantitatively using the OA scoring system in the double blind manner as reported previously[23]. Histological scoring was performed on the medial femoral condyles and tibial plateaus. Each knee yielded 1–3 slides with an area of 700–800 μm of an entire joint tissue for scoring. Data was presented in histograms as mean with ±standard deviation from six individual mice.

Immunohistochemistry and apoptosis assay

Immunohistochemical staining for RHEB (Abcam, ab25873), ADAMTS5 (Abcam, ab41037), MMP-13 (Abcam, ab39012), type II collagen (Millipore, CP-18), and type X collagen (Abcam, ab58632) was performed as described previously[2]. The primary antibodies were used at 1/200 dilution for 2 h at room temperature. Signals were detected using biotinylated anti-rabbit or anti-mouse antibodies, and a peroxidase-conjugated avidin system, according to the manufacturer's instruction (Golden Bridge International, Mukilteo, WA, USA). The immuno-stained samples were counter-stained with Harris' hematoxylin (Sigma, MO, USA) before microscopic examination using a Nikon E600 instrument (Nikon Co., Tokyo, Japan). Immuno-stained sections were quantified for intensity of immunostaining using imageJ software.

Apoptosis of chondrocytes in articular cartilage tissue was observed in the 8-week time point groups using a TdT-FragEL™ DNA Fragmentation detection kit, according to the manufacturer's instructions (Calbiochem, La Jolla, CA, USA). The number of apoptotic chondrocytes in relation to the total number of cells was quantified in the region of interest (ROI).

Statistical analysis

In vitro experiments in each condition were performed in triplicate. The results were expressed statistically as the mean ± S.D. For the animal study, the two-tailed unpaired Student's *t* test was used, while analysis for multiple samples was carried out by the one-way analysis of variance (ANOVA) using Tukey's post-hoc test using SPSS 12.0.1 (SPSS, Inc.). *P*-values < 0.05 were considered statistically significant.

Results

Human OA cartilage exhibited reduced RHEB levels

Hypertrophy of chondrocytes in articular cartilage during OA has been implicated in the pathogenesis of cartilage degradation[24]. We observed morphological differences in human healthy looking

compared to OA cartilage, and Safranin-O staining showed decreased GAG matrix in the OA cartilage [Fig. 1(A)]. We observed the focal area in OA cartilage and found that the intermediate layer of the chondrocytes was almost absent. In HC, the RHEB level was higher in chondrocytes of the superficial and intermediate layers, its level decreased in the deep layer, and was almost absent in the hypertrophic cells of the deep layer. However, in OA cartilage RHEB expression was barely detectable [Fig. 1(A)]. Protein analysis of DC samples from four donors revealed reduced RHEB expression [Fig. 1(B)]. These findings indicated the critical role of RHEB in maintaining cartilage structural integrity.

OA chondrocytes show phenotypic and molecular variations

To determine the morphological and functional differences among HCs and degenerated chondrocytes (DCs), we isolated chondrocytes from healthy-looking or damaged cartilage from the same OA knee [Fig. 2(A)]. HCs showed a typical polygonal phenotype similar to that reported previously in many studies[2,4,6]; however, the degenerated chondrocytes (DC)s were elongated and resembled fibroblastic-like chondrocytes [Fig. 2(B)]. DCs were much larger and fibroblastic than the HCs, which is a property of dedifferentiated chondrocytes[25,26]. Dot blot of cell area measured in healthy and degenerative chondrocyte isolated from different donors also revealed a significant difference [Fig. 2(B)]. Interestingly, more than 80 % of DCs isolated from five different patients also showed higher expression of SA-β-Gal [Fig. 2(C)]. SA-β-Gal is a distinct marker of senescence, which represents a gradual deterioration of the functional characteristics of cells[27]. Furthermore, elevated ROS levels were detected in DCs. Green and red fluorescence upon treatment with MitoTracker and MitoSOX dyes indicate the presence of mitochondrial superoxides (predominantly ROS) [Fig. 2(D)]. We also found decreased mRNA and protein expressions of COL2A1, SOX9, and RHEB in DCs compared with those in HCs [Fig. 2(E)–(F)]. Taken together, the results demonstrated that chondrocytes isolated from degenerated cartilage from OA knee joints have abnormal characteristics. Moreover, the decreased mRNA level of *RHEB* in DC might be a marker for the reversal of the chondrogenic properties of DC.

Reversal of chondrogenic properties of DCs by RHEB overexpression

To confirm the association of RHEB with the phenotype and molecular functions of DCs, we transfected DCs transiently with the *RHEB* gene. Surprisingly, *RHEB*-overexpression altered the phenotype of DCs, causing a reversal towards smaller, polygonal cells [Fig. 3(A)]. *RHEB*-overexpression not only recovered the morphology of the DCs, but also reduced senescence by 35–40% [Fig. 3(B)]. *RHEB*-overexpression also decreased ROS levels [Fig. 3(C)]. Importantly, *RHEB*-overexpression increased the level of COL2A1 [Fig. 3(D)–(E)], which was almost negligible in non-transfected DCs [Fig. 2(E)–(F)]. SOX9 expression also increased with *RHEB*-overexpressing DCs [Fig. 3(D)–(E)]. These results showed that RHEB has a distinct role in the reversal of non-chondrogenic properties in DCs isolated from OA cartilage and might have a role in the reversal of OA *in vivo*.

Expression pattern of Rheb in the articular cartilage of OA mice

The expression pattern of RHEB was first examined in a mouse OA model. Progression of OA was evident at 0, 2, 4, and 8 weeks in the articular cartilage, as revealed by Safranin-O staining (Fig. 4(A), $P < 0.01$) and OA scoring (Fig. 4(B), $P < 0.01$), showing massive destruction of the cartilage surface, the loss of chondrocytes, and GAGs content over time. The expression of RHEB was high in the sham (normal cartilage) mice, but markedly decreased in the OA cartilage at 4 weeks and thereafter [Fig. 4(A) and (C)]. This result showed that the expression of RHEB was down regulated upon induction of OA.

Contribution of Rheb-overexpression in OA regulation in mice

To understand the effect of Rheb on the progression of OA, adenoviruses over-expressing the *Rheb* gene were injected into the synovial cavity of the OA joint in the mouse model. As shown by Safranin-O staining, the OA group with GFP expression (Mock) showed increased structural destruction and loss of GAG content [Fig. 5(A)]. With *Rheb* overexpression, the articular cartilage did not show significant signs of OA progression, except for minor irregular structures on the surface at 8 weeks. The semi-quantitative OA scores also showed that overexpression of *Rheb* inhibited OA progression at 8 weeks, with statistical significance ($P < 0.001$) [Fig. 5(B)].

The results of the histological observation were further confirmed by the expression pattern of OA related proteins, including Adamts5, Mmp 13, and Col 10 at 8 weeks [Fig. 5(A) and (C)]. None of these proteins were detected in the sham group (8

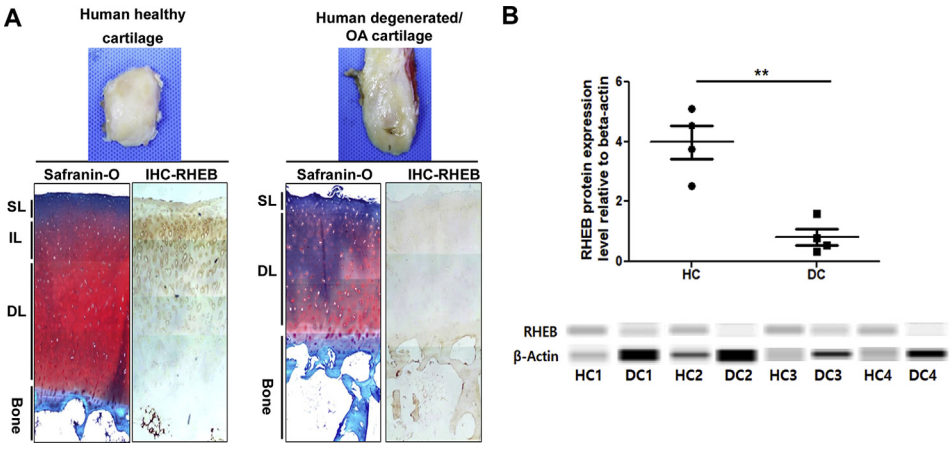

Fig. 1. Ras homolog enriched in brain (RHEB) expression decreases in human osteoarthritis (OA) cartilage **(A)** Human healthy cartilage shows a clear white appearance, while OA cartilage looks degenerated. Paraffin sections of human healthy and OA cartilage were processed for Safranin-O staining and immunohistochemistry (IHC) for RHEB. Multiple pictures were acquired from each Safranin-O or IHC-RHEB stained sample and used to generate mosaic images (SL: Superficial layer, IL: Intermediate layer, DL: Deep layer, Scale bar: 100 μm) **(B)** Protein harvested from the four HC and four degenerative cartilage samples from different donors were subjected to western blotting. Graph of relative protein concentration was plotted (**$P < 0.01$).

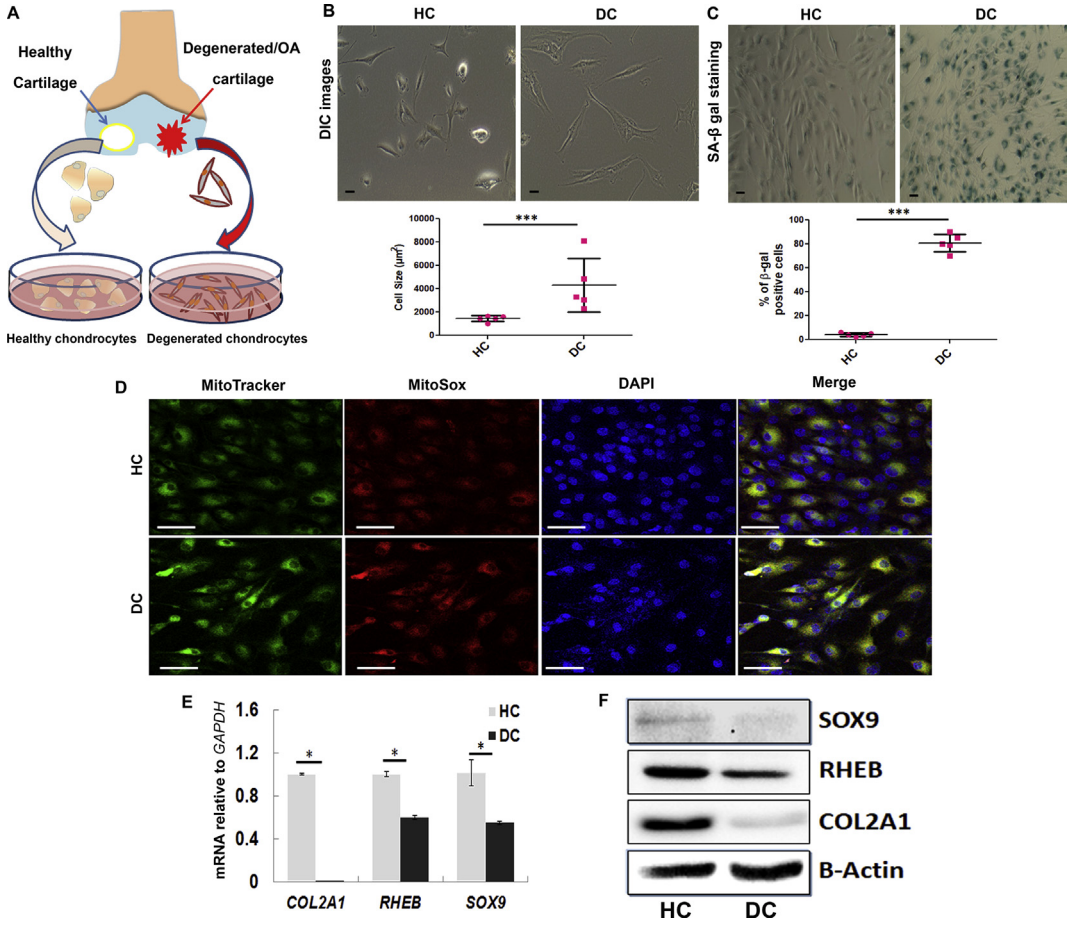

Fig. 2. Osteoarthritis (OA) chondrocytes showed phenotypic variations, higher senescence, increased reactive oxygen species (ROS) level, and decreased chondrogenic potential **(A)** Schematic of chondrocytes isolation from healthy and OA cartilage **(B)** Upper panel shows **c**ellular morphology of the healthy chondrocytes (HC) and degenerated chondrocytes (DC) and lower panel represent Dot plot of the cell area. Each dot represents one donor (***, $P < 0.001$) **(C)** Upper panel shows SA-β-Gal staining. Blue stained cells are indicative of senescence. Lower panel represents the percentage of senescent cells in a dot plot. Each dot represents one donor (***, $P < 0.001$) **(D)** Oxidative stress was observed by MitoTracker Green and MitoSox Red dyes. DAPI was used to stain nuclei **(E)** Gene expression was determined by real time reverse-transcription-PCR (RT-PCR). Data is shown for a representative experiment from multiple donor samples ($n = 3$; *, $P < 0.05$) **(F)** Protein expression as observed by Western blot ($n = 3$) (Scale bar in panel B–D = 100 μm).

weeks), but all were detected clearly in the DMM group. Notably, they were still detected in the Mock group, but disappeared in the ad-Rheb group at 8 weeks [Fig. 5(A) and (C)]. Meanwhile, high levels of the chondrogenic proteins Col2a1 and Sox 9 were detected in the sham and ad-Rheb groups [Fig. 5(A) and (C)]. Together with the histological observations, these results suggested that expression of *Rheb* in the synovial joint could inhibit OA progression.

Rheb inhibits chondrocyte apoptosis during OA progression in mice

Apoptosis in the chondrocytes was markedly increased in the DMM mice group at 8 weeks. Apoptosis in chondrocytes was reduced in all layers of the tibial cartilage in the ad-Rheb group compared with that in the DMM and Mock groups [Fig. 6(A)]. Quantification of TUNEL-stained cells (brown color) showed significantly fewer apoptotic chondrocytes in the ad-Rheb group compared those in the DMM and Mock groups ($P < 0.005$) [Fig. 6(B)].

Discussion

Despite numerous studies being published about OA over the last decade, the mechanism of cartilage destruction and the pathogenesis of OA remain unclear, limiting the advancement of new treatments that prevent the development and progression of OA[2,21]. To gain an insight into osteoarthritic chondrocytes, we applied several techniques to identify the degenerative mechanisms that cause functional abnormalities and subsequently lead to chondrocyte degeneration and OA development.

Human healthy and OA cartilage showed a different pattern of chondrocyte layers: the intermediate layer of chondrocytes was almost absent in the OA cartilage where RHEB is expressed predominantly. Interestingly, RHEB expression was very low in pre-hypertrophic chondrocytes and almost absent in hypertrophic chondrocytes [Fig. 1(A)]. This might explain the reduced RHEB mRNA and protein expressions in the DCs isolated from OA cartilage, which are mostly hypertrophic in nature. The isolated primary chondrocytes from human OA cartilage also exhibited a dedifferentiated phenotype, and such chondrocytes are unable to regenerate normal articular cartilage[2,4,6]. Previously, we reported that the main cause of these phenotypic changes, which lead to dedifferentiation of chondrocytes, is higher production of ROS[2,4,6]. When ROS production exceeds the antioxidant capacities of the cell, oxidative stress occurs, leading to structural and functional cartilage damage, such as chondrocyte death and matrix degradation[13]. Moreover, ROS is also responsible for suppression of anabolic pathways and upregulation of catabolic pathways, resulting lower expression of ECM-forming genes, like *COL2A1*, and upregulation of

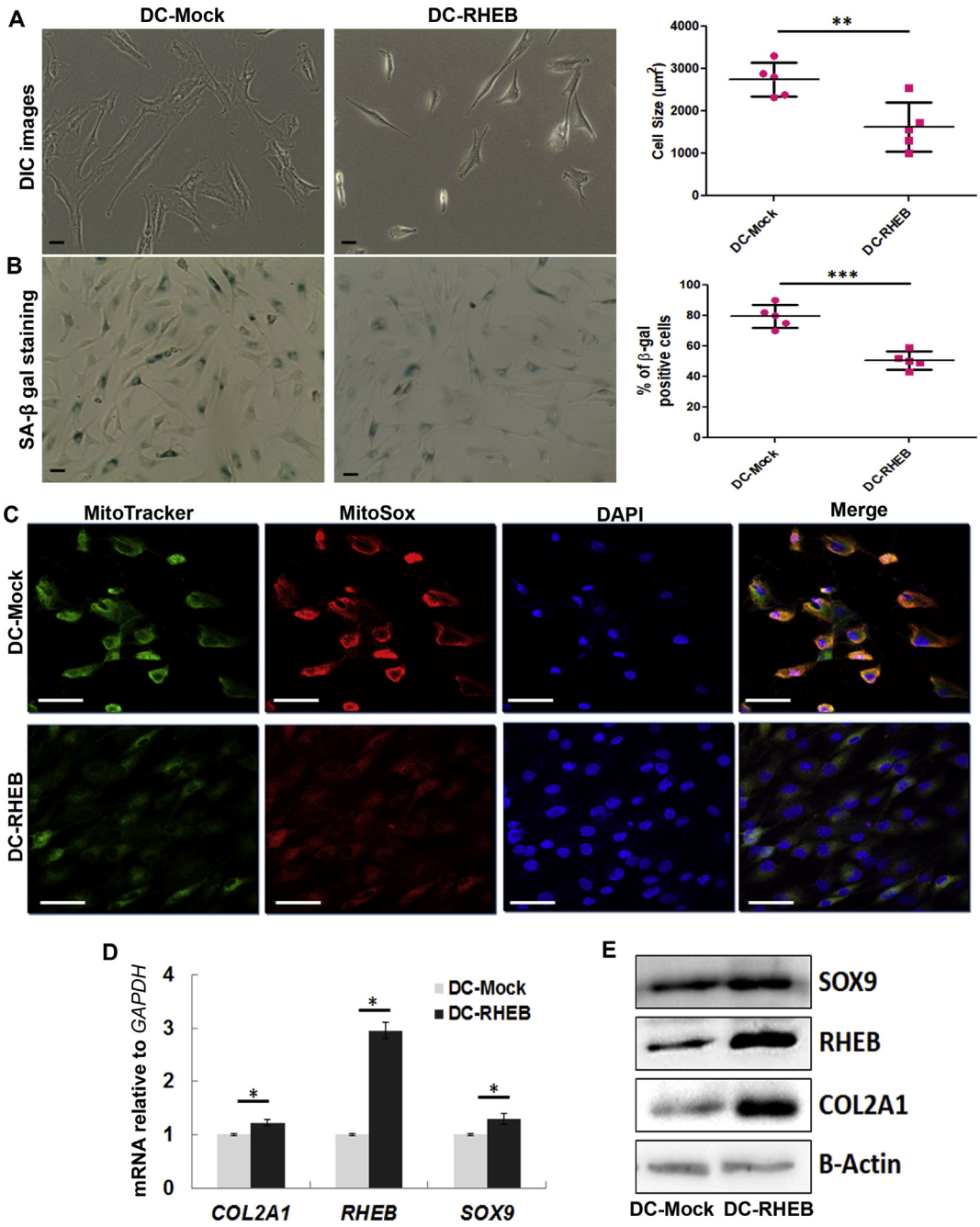

Fig. 3. Reversal of phenotypic and molecular characteristics of degenerated chondrocytes (DCs) by RHEB-overexpression **(A)** Cellular morphology of the DCs, transfected with empty vector (DC-Mock) and RHEB vector (DC-RHEB) was visualized by differential interference contrast (DIC). Dot plot of the cell area after transfection from five donors is shown on the right side. Each dot represents one donor (**, $P < 0.01$) **(B)** SA-β-gal staining for senescence. The percentage of senescent cells is shown in a dot plot from five donor's samples (***, $P < 0.001$) **(C)** ROS detection by staining with MitoTracker Green and MitoSOX Red dyes. DAPI was used to stain nuclei **(D)** Gene expression was determined by real time reverse-transcription-PCR (RT-PCR). Data is shown for a representative experiment from multiple donor samples **(E)** Protein expression as observed by Western blot (Scale bar: 100 μm; $n = 3$; *, $P < 0.05$).

ECM degradation genes like *MMP13*.[6] Transient overexpression of RHEB in DCs was sufficient to rescue the dedifferentiated phenotype, reduce senescence and ROS production, as well as promote *COL2A1* and *SOX9* production (Fig. 3). These results were also consistent with our previous study in which RHEB maintained the COL2A1 and SOX9 levels in primary chondrocytes during prolonged culture up to passage 6.[2]

One mechanism in which RHEB may be acting is through MCL1 which we have shown in a previous study is upregulated by RHEB[2]. Several studies have reported the link between senescence, ROS, and MCL1. Bolesta *et al.*[28] have shown that MCL1 localizes to mitochondria and prevents senescence and loss of phosphorylation of retinoblastoma (RB) protein, which is followed by inhibition of ROS production[29]. MCL1 is also involved in the downregulation of p27 expression, a critical inducer of senescence[29], and decreases ROS production and senescence via NOX4 during chemotherapy[30]. This can be one possibility of downregulation of senescence and ROS in osteoarthritic chondrocytes in the present study.

This study demonstrated that RHEB is not only capable of increasing chondrogenic markers in HCs, but also in degenerated

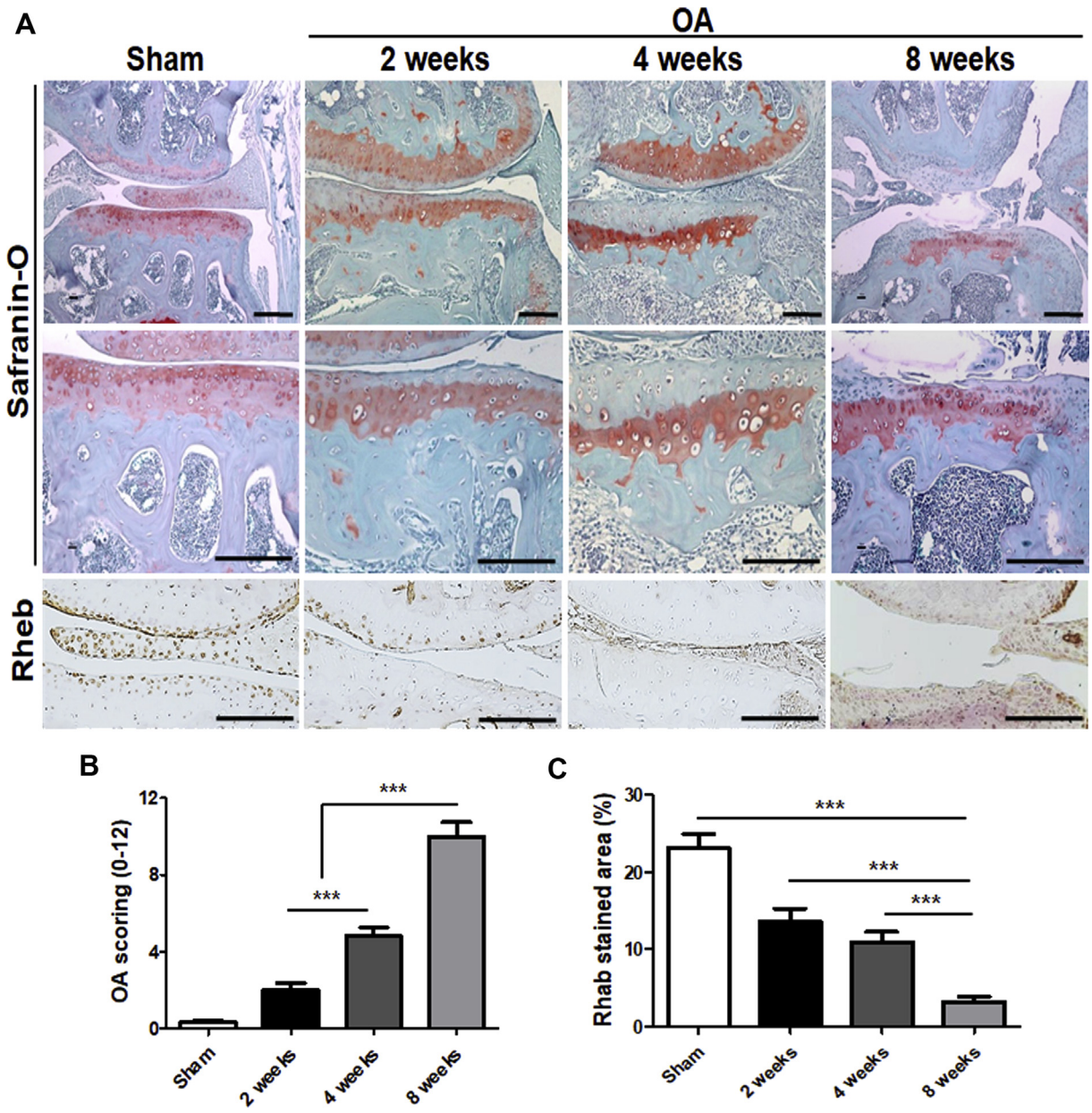

Fig. 4. Destabilization of the medial meniscus (DMM) osteoarthritis induction in mouse knee joints and evaluation of RHEB expression at 0, 2, 4, and 8 weeks after the destabilize the medial meniscus (DMM)-osteoarthritis (OA) development (A) Safranin-O staining (Upper two panels). The lower panel shows immunohistochemistry (IHC) for RHEB (B) Osteoarthritis scoring. For more details, see the materials and methods (C) Quantification of the Rheb IHC stained areas (Scale bar: 200 μm; $n = 6$/time point; ***, $P < 0.001$).

OA chondrocytes, revealing its importance in maintaining chondrocyte biology. According to Baar *et al.*[31] targeted apoptosis of senescent cells is beneficial in response to chemotoxicity and aging. However, one of the limitation of our study is that it is unclear whether RHEB potentially reverses the senescent cells to non-senescent cells or if it increases the proliferation of non-senescent cells. Another limitation is that the present study did not use controls from healthy donors.

Finally, we explored the effects of Rheb on surgically-induced OA. Rheb expression decreased markedly as cartilage destruction increased with OA progression demonstrating that Rheb has an intricate role in cartilage maintenance. Ectopic *Rheb* expression in DMM-induced OA attenuated cartilage destruction notably and maintained a near to normal cartilage structure. Rheb's role is not limited to maintaining cartilage integrity, but it also markedly suppressed the cartilage destruction enzymes Adamts5 and Mmp 13, suppressed the dedifferentiation marker Col 10, and upregulated the expression of the chondrogenic marker Col2a1. Among these, Adamts5 and Mmp13 are crucial effectors of OA cartilage destruction and Col2a1 is crucial for the composition of hyaline ECM[21,32]. Rheb is located upstream of mTORC1[16,17]; which is known to regulate cartilage formation via Sox 9 expression during mesenchymal condensation[33] In a study by Zhang *et al.*, mTORC1 inhibition by Rheb deletion was shown to enhance collagenase-induced osteoarthritis by M2-macrophages polarization in mice[34]. In contrary, some studies reported that mTOR is a positive regulator of OA and mTOR ablation protects cartilage and progression of OA[35,36]. From these conflicting reports we can assume that Rheb might be working independent of mTOR signaling in cartilage. Second, based on

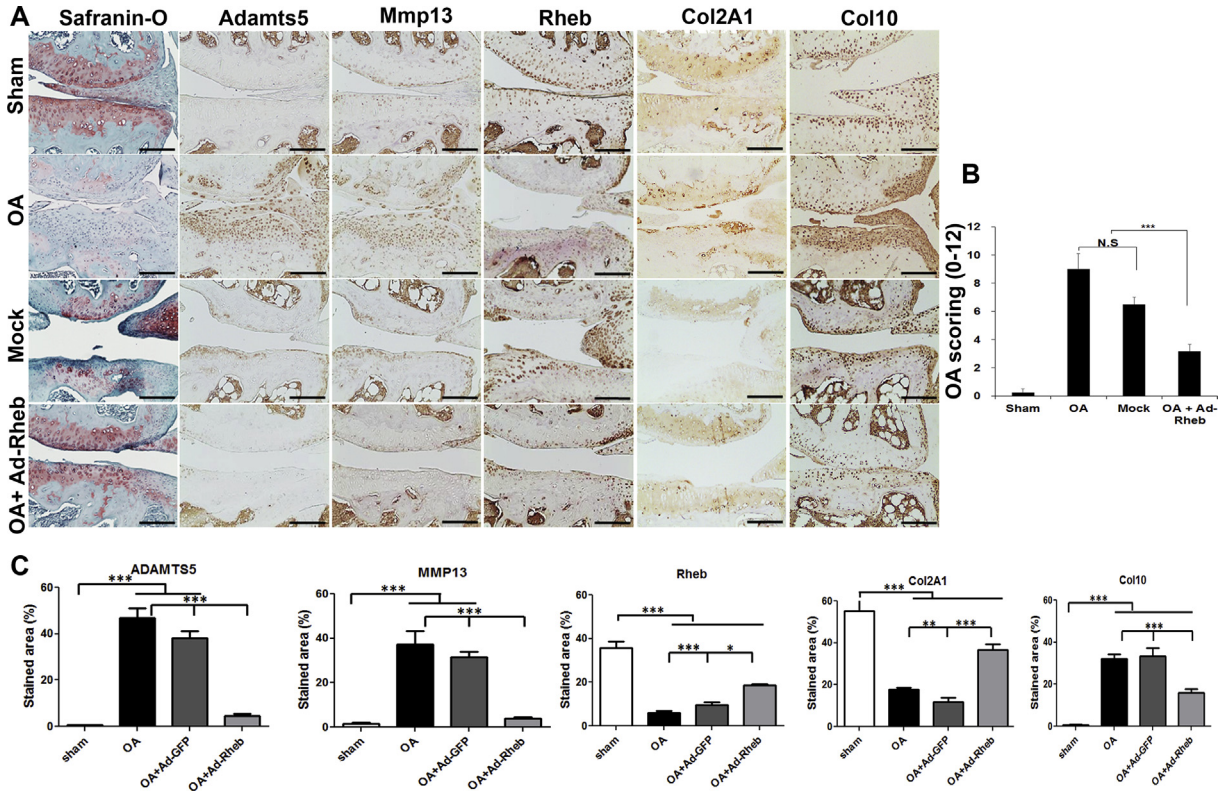

Fig. 5. Effect of RHEB overexpression on the inhibition of osteoarthritis (OA) progression and downregulation of OA-related proteins in OA knee joint cartilage (A) Safranin-O staining and immunohistochemistry at the 8-week time point (B) Osteoarthritis scoring (C) Quantification of the IHC stained sections representative of panel (A) (Scale bar: 200 μm; $n = 6$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

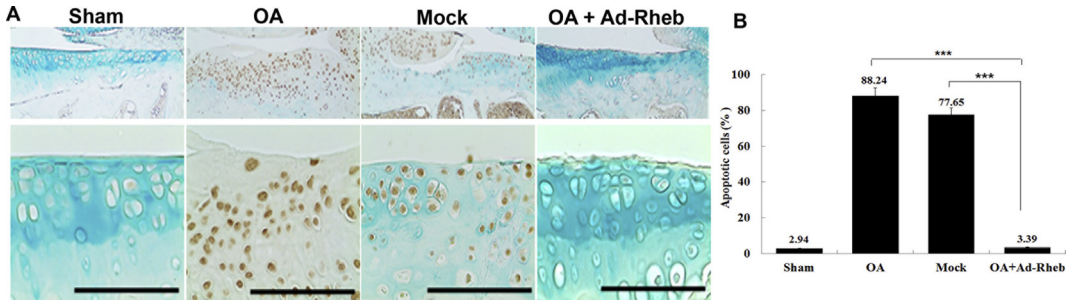

Fig. 6. RHEB gene therapy inhibited apoptosis after DMM-osteoarthritis (OA) mice knee cartilage (A) Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay (B) Percentage of apoptotic cells counted in the represented figures shown in panel (A) (Scale bar: 200 μm; $n = 6$; *, $P < 0.05$).

our data, Rheb works as up-regulator of Sox9 in HCs[2] and in OA-chondrocytes (present study) suggesting the role of Rheb in cartilage maintenances through Sox9.

Apoptosis evaluation in the DMM-induced OA mouse model revealed that RHEB inhibited apoptosis in articular chondrocytes. The significance of RHEB in chondrocyte apoptosis *in vivo* was suggested by the results of our previous study in which RHEB upregulated MCL1, an antiapoptotic marker[2]. In this study we did not observe in reduction of apoptosis after *RHEB* gene transfection in degenerative chondrocytes during *in vitro* (data not shown), but marked differences were observed in the reduction of apoptosis *in vivo*. This might suggest that adenoviral gene therapy into synovium also effects the synovial fibroblasts that potentially influence cartilage destruction as previously reported[37]. In addition, RHEB was identified as a cell cycle regulating protein that may inhibit apoptosis pathways in articular chondrocytes[16]. Collectively, our *in vitro* and *in vivo* data indicated that RHEB is a crucial negative regulator of cartilage destruction.

In summary, this study demonstrates that osteoarthritic chondrocytes exhibited de-differentiation, senescence and oxidative stress that might compromises their suitability for use in ACI applications. Upregulating RHEB may assist with recovering chondrocytes morphology and reducing senescence and oxidative stress in monolayer culture. Importantly, *Rheb* gene therapy during experimental murine osteoarthritis protects cartilage tissue from degenerative damage. Further studies will be required to elucidate Rheb mechanisms inhibiting progression of OA in murine OA model.

Statement of author contributions

The idea of this study was conceived and brought in to write up form by Sajjad Ashraf and Byoung-Ju Kim. Sung Hyun Park helped

during mice surgery. All authors contributed to make the final form of manuscript. Hansoo Park and Soo-Hong Lee critically reviewed and edited the manuscript. Whole study was done under the supervision of Hansoo Park and Soo-Hong Lee.

Conflict of interest

The authors declare that there is no conflict of interest associated with this study.

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Supplementary data

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