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HMGB1 can activate cartilage progenitor cells in response to cartilage injury through the CXCL12/CXCR4 pathway

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Abstract

Introduction: Recent studies have suggested that cartilage progenitor cells (CPCs) could be activated and differentiated into chondrocytes to produce matrix and to restore the integrity of damaged cartilage after injury. However, the mechanism involved in CPC activation upon damage is still unclear. This study aims to investigate the role of high mobility group box chromosomal protein 1 (HMGB1) in both activation and migration of CPCs during cartilage injury.

Material and methods: Explants harvested from mature bovine stifle joints were used for impact injury. The proliferation and migration of CPCs were examined via confocal imaging. Gene and protein expression of Hmbg1, Cxcl12, and Cxcr4 was also examined by quantitative polymerase chain reaction (qPCR), ELISA, and western blot. Each experiment was repeated 3 times. ANOVA and Student's t-test were performed for statistical analysis.

Results: HMGB1 released from dead and damaged chondrocytes after an impact injury could activate CPCs in the superficial zone of cartilage and promote their migration and proliferation to injury sites. However, the block of HMGB1 activation with its specific binding inhibitor glycyrrhizin inhibits the proliferation and migration of CPCs. Further investigations demonstrate that HMGB1 promotes CPCs migration through the pathway of C-X-C motif chemokine 12 (CXCL12) and its receptor CXCR4. Quantitative analysis of HMGB1 in cell culture medium also indicates that CPCs may have a self-activation property after the HMGB1 released from dead cells has been exhausted.

Conclusion: HMGB1 is a pivotal factor that could enhance the migration and proliferation of CPCs through the CXCL12/CXCR4 pathway after cartilage injury, which could provide useful information for cartilage repair and osteoarthritis treatment.

Key words: *HMGB1; CXCL12/CXCR4 pathway; cartilage injury; cartilage progenitor cells*

Introduction

Although damaged tissue may be repaired through the activation and differentiation of tissue-specific progenitor cells, progenitor cells are usually in a quiescent status in normal tissue [1, 2]. To re-enrol into a cell cycle, the quiescent progenitors have to be activated by some given factors, such as high mobility group box chromosomal protein 1 (HMGB1) [1]. HMGB1 is a ubiquitously expressed, highly conserved nuclear protein that plays important roles in chromatin organization and transcriptional regulation [3, 4]. HMGB1 can be passively released from dead cells or actively secreted from activated immune cells, and possibly some other types of cells under given conditions. And HMGB1 can bind to chemokine (C-X-C motif) ligand 12 (CXCL12) and induce the migration of inflammatory cells like monocytes and bone marrow stromal cells through chemokine (C-X-C motif) receptor 4 (CXCR4) [5–7]. Moreover, extracellular HMGB1 can induce several responses, including the release of proinflammatory cytokines, cell proliferation, and cell migration, unlike intracellular HMGB1 [7, 8]. Therefore, HMGB1 released from dead and damaged cells during cartilage injury could be considered as an important factor for the activation of CPCs.

To explore the role of HMGB1 involved in the mechanisms of CPCs activation, migration, and proliferation after an injury, this study aims to do the following: (1) observe the CPCs' activation and migration in response to the impact damage of osteochondral explants using a confocal microscope, agarose gel migration, and quantitative transwell migration under the conditions of HMGB1 and its specific chemical inhibitor glycyrrhizin [9, 10]; (2) to investigate the quantitative polymerase chain reaction (qPCR) expression of CXCL12/CXCR4 in CPCs and its effect on HMGB1 on CPCs migration; and (3) to evaluate the progenitor cell properties and multipotent differentiation abilities of CPCs isolated from the superficial zone of cartilage.

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Material and methods

Reagents

CXCL12, CXCR4 primary antibodies, and goat anti-rabbit IgG-Per-CP-Cy5.5 secondary antibodies were obtained from Santa Cruz Biotechnology (CA, USA), HMGB1 was from OriGene (Beijing, CHINA), ELISA HMGB1 Detection Kits were from Chondrex Inc. (Redmond, WA, USA), 10%ITS+ Premix and TGF-β3 were purchased from R&D Systems (Minneapolis, MN, USA), and the 6.5 mm diameter Corning polyester Transwells were from Corning Incorporated (Corning, NY, USA).

This research was approved by the Internal Review Boards of the authors' affiliated institutions.

Explant harvest, injury, and culture

Osteochondral explants (20 mm × 20 mm) were prepared from the lateral tibial plateau of mature bovine stifle joints. Three explants were used for each treatment group. The explants were cultured in a growth medium (DMEM/F-12 and 10% FBS) and incubated in 5% oxygen. After 2 days of neutralization culture, explants were impacted with a drop tower (15 J/cm²), and the damage-associated molecular pattern molecules (DAMPs) could be released from damaged cartilage. The impacted explants were continually cultured in a basic serum-free medium (DMEM/F-12, containing ITS-insulin transferrin selenium) or basic medium with different treatment factors (a. 10 nm HMGB1, b. 25 μM glycyrrhizin, c. 10 nm HMGB1 + 25 μM glycyrrhizin) and incubated in a lower oxygen condition (5% oxygen) for another 7 days.

Damaged cartilage was able to activate CPCs on the surface of the cartilage and induce their proliferation and migration to the injured site. This procedure takes 6–8 days [11].

Confocal imaging for CPC migration in the explants

After 7 days of culture, osteochondral explants were then stained with 1 μg/mL of calcein AM for live cells and 1 μM of ethidium homodimer-2 (EHD) for dead cells in equilibrated phenol red-free culture medium for 30 min. Stained osteochondral explants were scanned under a confocal microscope (Nikon). The morphology and number of CPCs at the impact sites and surrounding area were observed and compared among different treatment groups.

CPCs harvested from explants

CPCs were harvested by a surface digestion with 0.25% trypsin/EDTA buffer. Specifically, cartilage explants were put upside down into a 100-mm culture dish containing 5 mL of 0.25% trypsin/EDTA buffer (only the cartilage surface was submerged in the digestion buffer) for 10 min, followed by flushing the surface of the cartilage. Digestion buffer containing CPCs was centrifuged (500 g, 5 min). CPC pellets were resuspended in growth medium. The number of CPCs was normalized to the surface size of the cartilage and compared among different treatment groups. After collection of the CPCs, the rest of the cartilage was used for non-CPC chondrocyte (NC) harvesting.

Quantitative assay of CPCs migration

Under agarose gel, migration and quantitative transwell migration assay were performed to examine the effects of HMGB1 on the migration of CPCs. After 30 min of cooling under sterile conditions, 3 wells (diameter 6 mm, distance between each well 3 mm) per plate were punched on the gel by using a 60-mm biopsy punch. 5 mL DMEM were added onto the gel and incubated for 2 hours to neutralize the gel before seeding the cells. 1×10^4 CPCs were seeded in the middle well of each plate. Basic serum-free medium with/without HMGB1 (or plus glycyrrhizin) was added to the sides separately. The CPCs were cultured for 5 days.

For transwell migration assay, 6.5 mm diameter Corning polyester Transwells were used for CPC migration quantitative assay. Following the manufacture's protocol, 0.6 mL of condition medium (serum-free medium without or with 1 nM, 5nM, 10 nM, and 20 nM of HMGB1) was added into each well. 1×10^5 CPCs in 0.1 mL growth medium were seeded into each transwell insert. CPCs were cultured for 24 hours, and cells that migrated out of the transwell into the plate well were lysed with 100 uL RLT buffer. The number of cells was quantified by measuring the DNA content using a high-sensitivity Quant-iT™ dsDNA Assay Kit.

qPCR for gene expression

qPCR was set up using SYBR Green Master Mix following the manufacture's protocol. Primers were designed individually with Primer3 software based on the mRNA sequence of bovine cartilage. The primer sequences are listed in Supplementary File — Table S1.

Immunofluorescence staining

CPCs and NCs were cultured on chamber slides in growth medium for 48 hours. The cells were incubated overnight at 4°C with primary antibodies of CXCL12, CXCR4, and Notch1 antibody individually at a ratio of 1:200. The cells were mounted with Fluoro-Gel II DAPI (Electron Microscopy Sciences) and observed under a confocal microscope (Nikon).

Chondrogenic, osteogenic, and adipogenic differentiation of CPCs

For chondrogenic differentiation, 1×10^6 of CPCs were pelleted by centrifugation in a 15-mL centrifuge tube and cultured in chondrogenic medium (DMEM-high glucose, 4 mg/mL L-proline, 2.5 mg/mL ascorbate-2-phosphate, 100 nM dexamethasone, 10% ITS + Premix Tissue Culture Supplement, 100 ug/mL sodium pyruvate, 2mM L-glutamine, pen/strep bio-antibiotics, and TGF-β3 10 ng/mL) for 14 days. Safranin O staining was performed for 10 um thickness frozen pellet sections to evaluate the chondrogenic differentiation.

For osteogenic differentiation, 5×10^4/cm² CPCs per well were cultured in osteogenic differentiation medium (αMEM, 10% FBS, 10 mM β-glycerol phosphate, 50 μM ascorbic acid, and 10 nM dexamethasone) for 14 days. Osteogenic differentiation was examined by alkaline phosphatase (ALP) and alizarin red staining.

For adipogenic differentiation, 5×10^4/cm² of CPCs were cultured in adipogenic differentiation medium (PromoCell) for 14 days. Adipogenic differentiation was examined by oil red O staining.

ELISA and western blot assay of HMGB1 in cell culture medium

CPCs were harvested from explants as described above. NCs were harvested by collagenase and Pronase digestion [12]. 1×10^5 of freshly harvested CPCs or NCs were cultured for 24 hours, and the growth medium was then replaced for 24 hours. The medium was harvested and the HMGB1 concentration in the medium was assayed by using the ELISA HMGB1 Detection Kit.

10 ug total protein was loaded in each well for western blotting. Separated protein bands were then transferred to a PDVF membrane. HMGB1 primary rabbit antibody and Beta-Actin primary mouse antibody were used for immunoblotting.

Statistical analysis

Each experiment was repeated 3 times. All data are expressed as mean ± SD. CPC proliferation assays with base serum-free medium, HMGB1, and glycyrrhizin treatments were analysed by analysis of variance (ANOVA). Student's t-test was performed for the CPC transwell migration assay, qPCR gene expression, and HMGB1 concentration measurements. Statistical significance was set as a p value less than 0.05.

Results

HMGB1 activates CPCs and promotes their migration

The surface images of the osteochondral explants scanned from the confocal microscope (Fig. 1) showed that large amounts of CPCs were activated and migrated towards the impact sites in the 10 nM HMGB1-treated group compared to the serum-free control group. Migrating CPCs showed an elongated fibroblast-like shape (Fig. 1BE). The number of migrating CPCs decreased dramatically after being treated with 25 μM HMGB1 inhibitor glycyrrhizin (Fig. 1CF). In serum-free control medium, activated CPCs with less elongated shape at the impact site of the cartilage were also observed. Although there was no HMGB1 added in the serum-free control medium, the impact itself can cause the release of HMGB1 from dead and damaged cells [13, 14]. Images of the under-agarose migration experiments (Fig. 2) also illustrated that more CPCs migrated towards the wells with HMGB1. Without the attracting factor of HMGB1, fewer CPCs migrated towards the serum-free medium control wells. The migration of CPCs towards HMGB1 plus its inhibitor glycyrrhizin wells was similar to that of the serum-free control. All the results indicated that HMGB1 can enhance the migration of CPCs. Transwell migration assay by the measurement of DNA content from migrated CPCs quantitatively showed that 10 nM HMGB1 significantly increased the migration of CPCs through the Transwell membrane.

HMGB1 improves the proliferation of CPCs

The numbers of CPCs harvested from cartilage surface were compared among different treatment groups (Fig. 3). After 7 days of culture, more CPCs were harvested from the surface of cartilage with the stimulation of 10 nM HMGB1 compared to the serum-free control medium and HMGB1 inhibitor glycyrrhizin-treated group. The impact itself also lightly promoted the proliferation of CPCs, but the increase was not significant.

Expression of CXCL12 and CXCR4 in CPCs and NCs

Because CXCL12 and CXCR4 are necessary for HMGB1-induced progenitor cell migration, it is important to confirm their expression in CPCs. The qPCR results in Fig. 4 show that the expression of both CXCL12 and its receptor CXCR4 of CPCs were significantly higher than the NCs. CXCL12 expression in CPCs was 4.3-fold higher compared to its expression in NCs (p 0.05). CPCs also had 3.2-fold higher expression of CXCR4 compared to NCs (p 0.05). Gene expression was also confirmed with regular reverse transcription
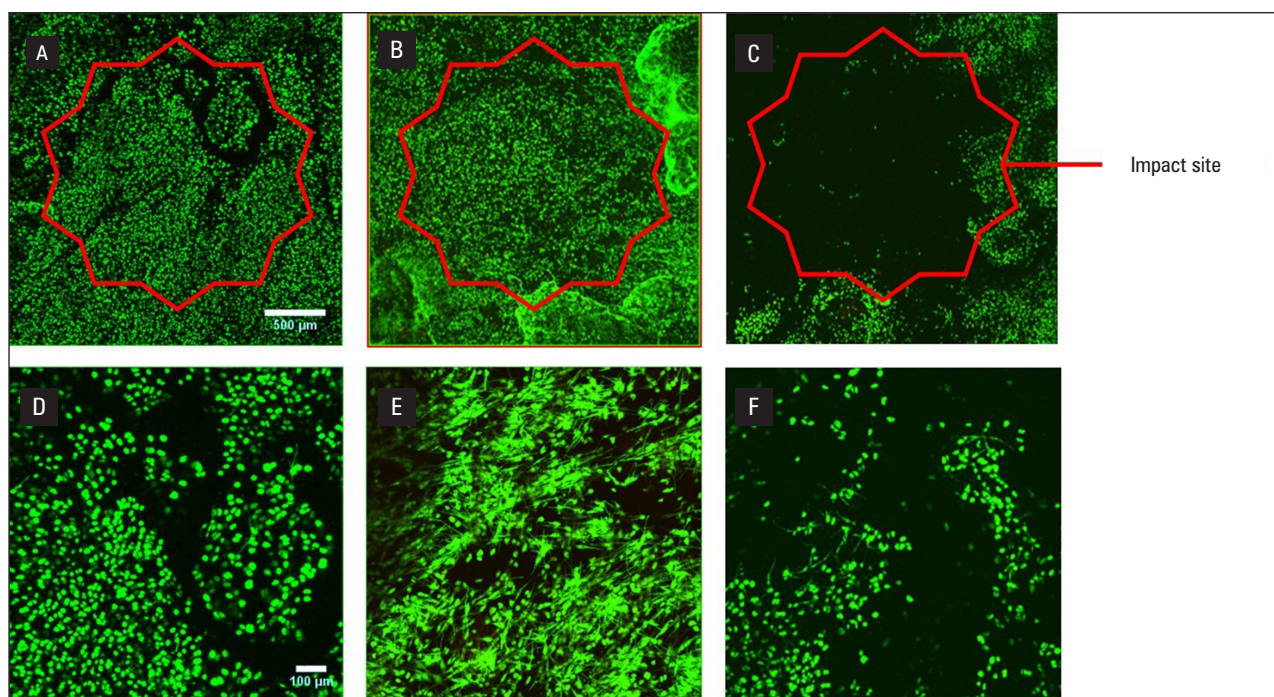
Figure 1. *Confocal microscopy imaging. Cartilage progenitor cells (CPCs) activated by high mobility group box chromosomal protein 1 (HMGB1) released from dead cells (**A**, **D**). Impact cartilage explants cultured in serum-free medium plus 10 nM (**B**, **E**) or 25 nM (**C**, **F**) HMGB1; 4× magnification (**A**, **B**, **C**); 10× magnification (**D**, **E**, **F**)*
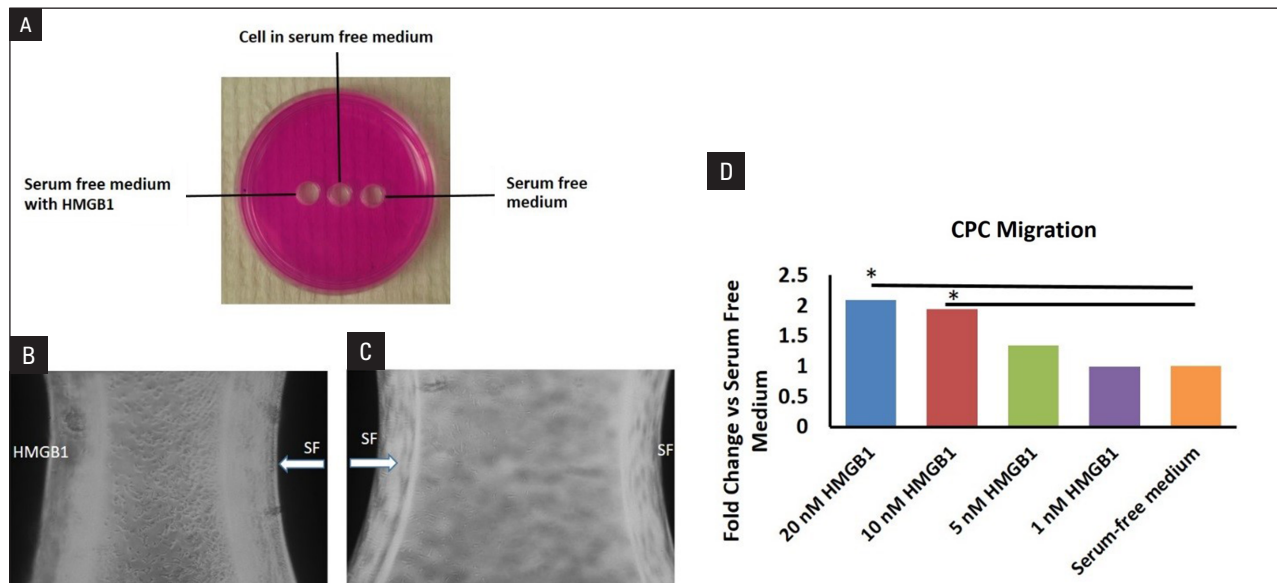
Figure 2. *Cartilage progenitor cell (CPC) migration under 1% low melting point agarose gel and in Transwells. Cell migration toward the well with 10 nM high mobility group box chromosomal protein 1 (HMGB1)* **(A)**. *Cell migration toward the well with serum free medium* **(B)** *or 10 nM HMGB1* **(C)**. *Transwell cell migration assay* **(D)**
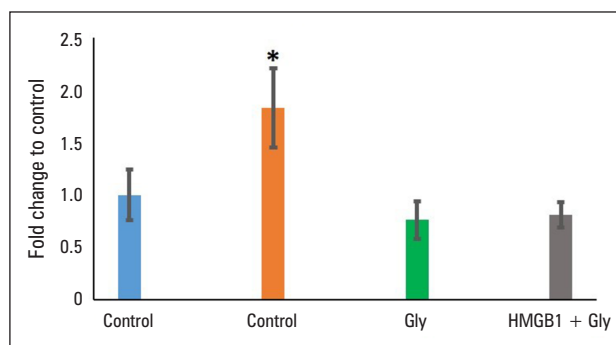

Figure 3. *Cartilage progenitor cells (CPCs) harvested from impacted cartilage explants. The number of cells is shown as the fold change compared to serum-free control medium. The number of CPCs harvested from the culture medium with high mobility group box chromosomal protein 1 (HMGB1) were significant higher than the control and inhibitor treatment. p < 0.05*

polymerase chain reaction (RT-PCR). 1.5% agarose gel electrophoresis of the products from RT-PCR showed that CPCs had higher expression of both CXCL12 and CXCR4 compared to NCs (Supplementary File — Fig. S1). Also, the higher gene expression of cartilage progenitor cell select marker Notch 1 was found in CPCs compared to NCs.

Immunofluorescence staining of CXCL12, CXCR4, and Notch 1 in CPCs and NCs
In addition to the examination of gene expression at the mRNA level, the expression of CXCL12, CXCR4, and Notch 1 was also confirmed by immunofluores-

cence staining at the protein level. Immunofluorescence images under confocal microscope in Figure 5 showed the bright positive stain of both CXCL12 and CXCR4 in CPCs, but the expression of CXCL12 and CXCR4 in NCs showed very weak staining. The cartilage progenitor select marker Notch 1 showed stronger staining in CPCs, as can be seen in Figure 6. Protein expression results from the immunofluorescence staining were consistent with the qPCR results of gene expression. CPCs had a higher level of expression of migration-related protein and progenitor cell select marker.

Multipotent differentiation of CPCs
CPCs were cultured in the inductive media of chondrogenesis, osteogenesis, and adipogenesis to verify the properties of multipotent differentiation of CPCs. Safranin O staining of frozen sections of CPC pellets, as in Figure 7A, demonstrate that the characteristics of cartilage was obvious with the presence of proteoglycan-rich extracellular matrix. After 2 weeks of osteogenic induction, the images in Figure 7B showed histological evidence of increased alkaline phosphatase (ALP) activity, and matrix mineralization was also detected by the alizarin red staining. Also, oil red O staining of adipogenic induction of CPCs (Figure 7C) also showed obvious evidence of intracellular lipid droplet accumulation. These results confirmed the multipotent progenitor properties of CPCs.

Expression and release of HMGB1 by CPCs
HMGB1 concentration in the culture medium was assayed by ELISA. Figure 8A shows that HMGB1 released
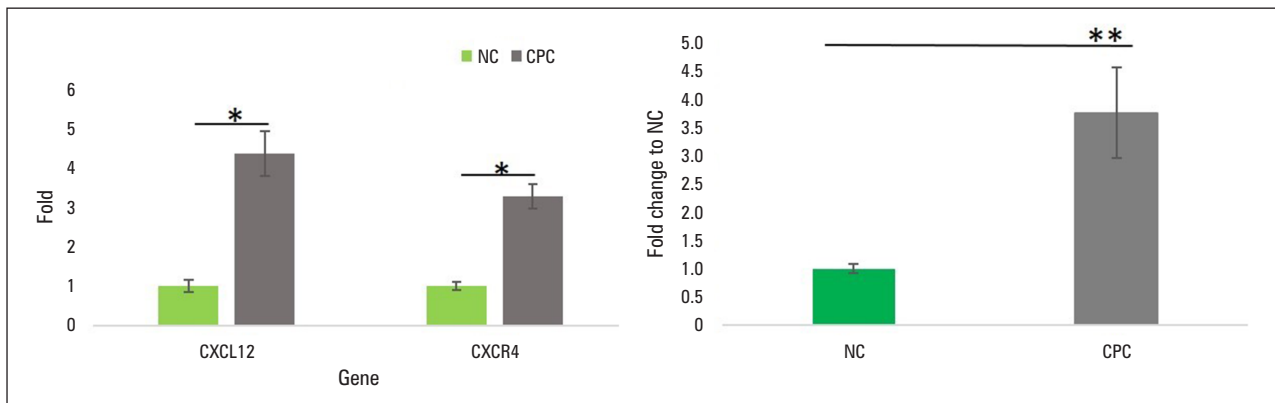
Figure 4. *Quantitative polymerase chain reaction (qPCR) for gene expression.* **A.** *Expressions of C-X-C motif chemokine 12 (CXCL12) and its receptor (CXCR4) were significantly higher in cartilage progenitor cells (CPCs);* **B.** *Gene expression of cartilage progenitor cell select marker Notch 1 were significantly higher in CPCs.* $*p < 0.05$, $**p < 0.01$
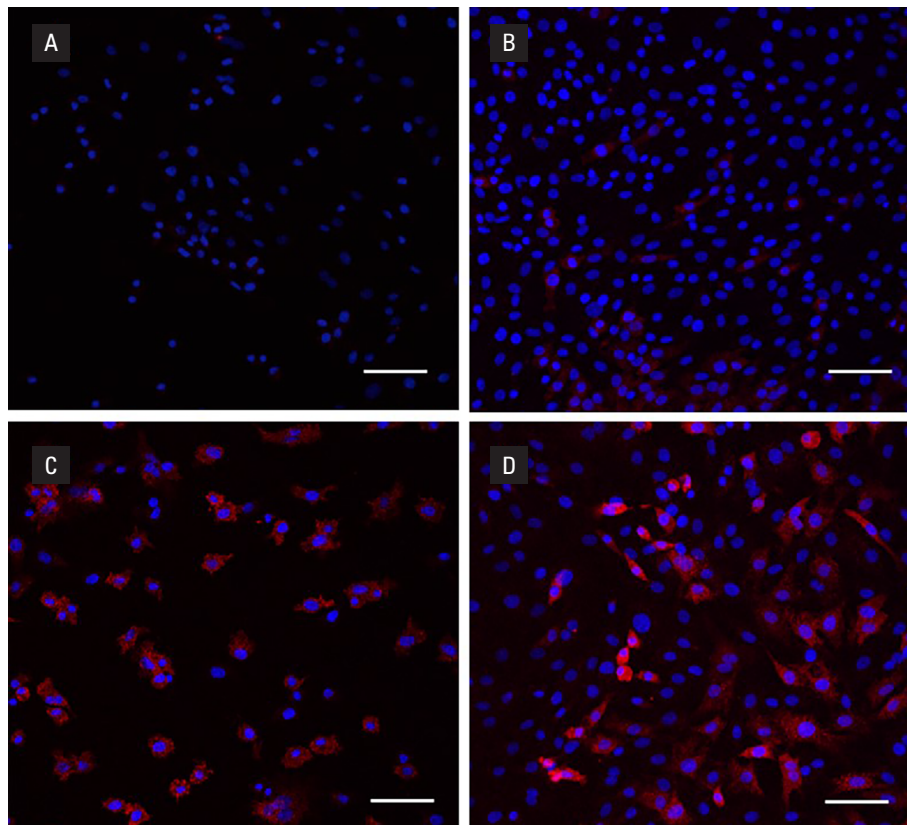

Figure 5. *Immunofluorescence staining of C-X-C motif chemokine 12 (CXCL12) and its receptor (CXCR4). Both CXCL 12 and its receptor CXCR4 showed higher expression in cartilage progenitor cells (CPCs);* **A.** *CXCL12 in CPCs;* **B.** *CXCR4 in CPCs;* **C.** *CXCL12 in non-CPC chondrocytes (NCs);* **D.** *CXCR4 in NCs. Bar scale 100 um*

by CPCs and NCs in serum-free culture medium was 351 ng/mL/1×10^6 cells and 156 ng/mL/1×10^6 cells, respectively. And the western blotting results in Figure 8B illustrate the higher protein expression of HMGB1 in CPCs compared to NCs. These results indicate that CPCs may have a self-activation property by the expression and release of HMGB1.

Discussion

Adult tissue progenitor cells have similar properties to stem cells with the abilities of self-renewal and multipotent differentiation. Those cells can often transdifferentiate into different types of tissues [15]. Tissue stem cells are typically set aside during development
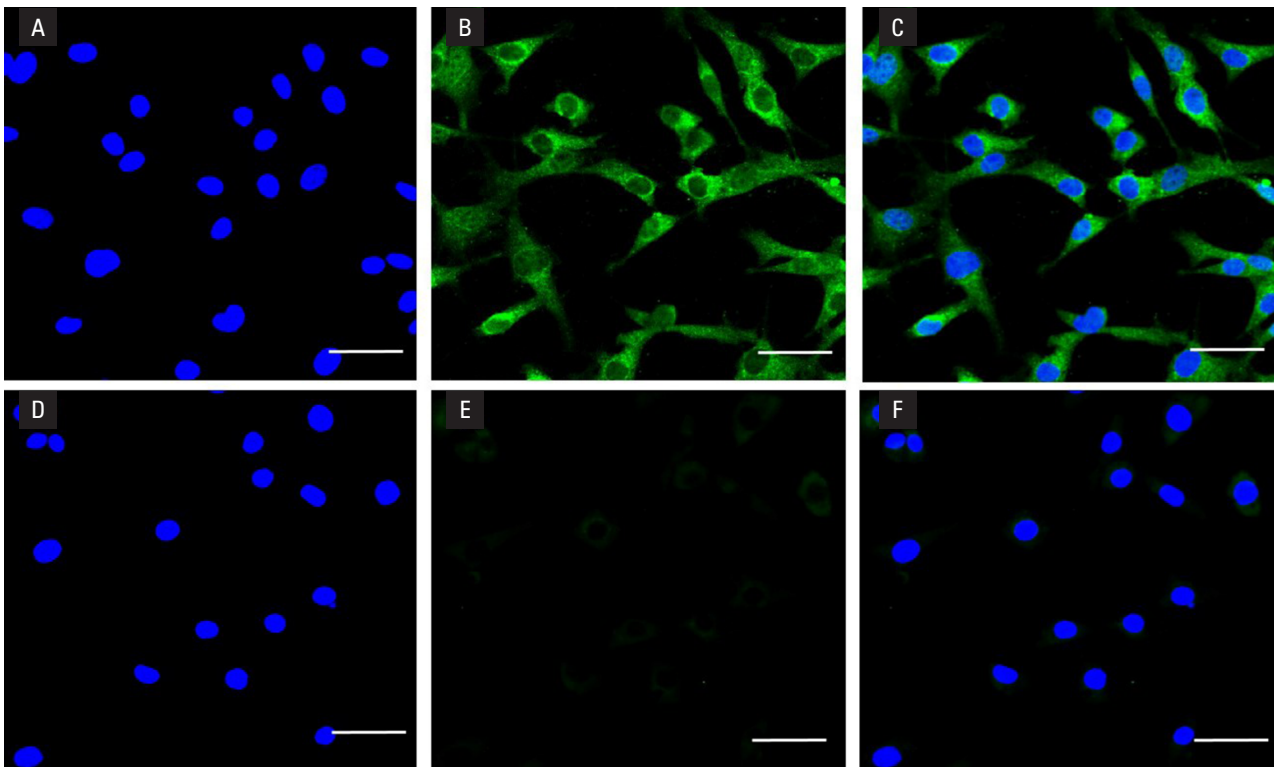
Figure 6. *Immunofluorescence stain of progenitor cell select marker Notch 1. The expression of Notch 1 in cartilage progenitor cells (CPCs) (**A.** DAPI staining; **B.** Notch 1 staining; **C.** Merged image) and NCs (**D.** DAPI staining; **E.** Notch 1 staining; **F.** Merged image). Bar scale 50 um*
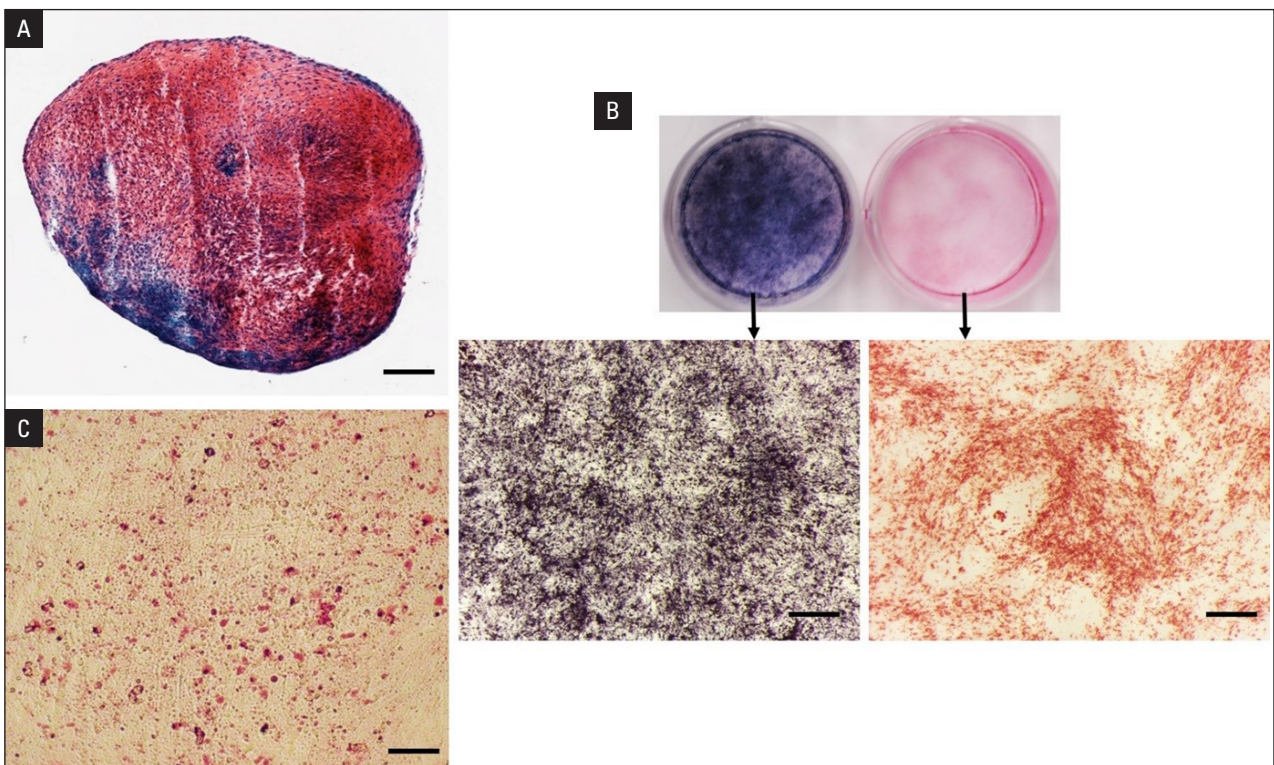

Figure 7. *Multipotential differentiation of cartilage progenitor cells (CPCs). **A.** Frozen section from CPC pellets. Safranin O staining showed proteoglycan-rich extracellular matrix; **B.** Alkaline phosphatase (ALP) and alizarin red staining showed positive mineralization induced by osteogenic differentiation; **C.** Staining of oil red O showed intracellular lipid droplet accumulation*
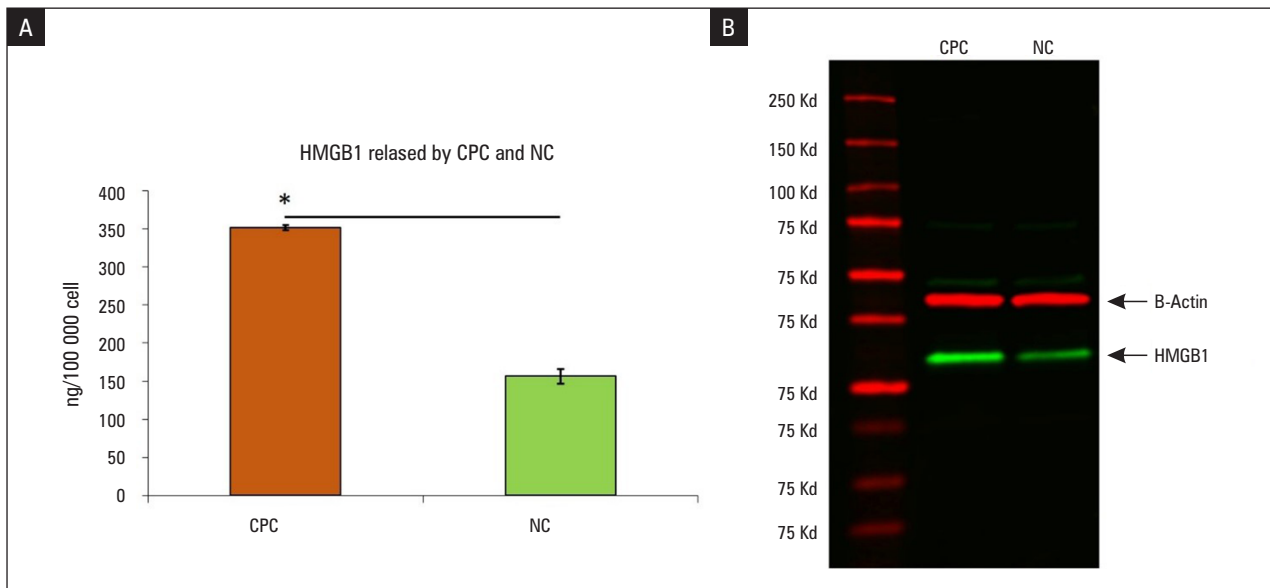
Figure 8. *Elisa assay of high mobility group box chromosomal protein 1 (HMGB1) in serum-free medium, and protein expression of HMGB1 in cartilage progenitor cells (CPCs) and non-CPC chondrocytes (NCs).* **A.** *Concentration of HMGB1 in CPCs medium was much higher than that in NC culture medium, $p < 0.05$;* **B.** *Western blotting analysis of HMGB1*

to provide a source for replenishment of tissue over time in response to damage [15]. Adult stem cells have been found in most major organ systems. Cartilage progenitor cells, the stem cells of articular cartilage, have also been found in both the superficial zone and deep zone [16, 17]. In our studies, CPCs were successfully isolated from the superficial zone of bovine cartilage. It was also confirmed that CPCs have high multipotent abilities of chondrogenic, osteogenic, and adipogenic differentiation, and they exhibit high expression of the stem cell marker Notch 1.

Regular amounts of CPCs could maintain the surface of articular cartilage in response to daily wear and tear. After injury, it is reported that large amounts of CPCs could be activated and start to expand and migrate to the injury sites [18], but the mechanism of CPC activation remains unclear. With an impact injury model on bovine articular cartilage explants, we found that some elongated cells migrated towards the damaged site within the superficial zone after 7 days of in vitro culture. Moreover, cell lysates can increase the migration of CPCs, and HMGB1 within cell lysates is one of the key factors for the activation of CPCs. Instead of cell lysates, purified HMGB1 can also attribute to the migration of CPCs and induce their migration. However, the migration of CPCs can be inhibited by glycyrrhizin, a HMGB1-specific inhibitor. These findings indicate that HMGB1 is a key activator of CPCs during cartilage injury. When an acute cartilage injury occurs, large numbers of dead cells within damaged cartilage are produced, and HMGB1 is negatively released from the dead cells in the short

term. With the stimulation of HMGB1, CPCs can be activated and then migrate into damaged areas of cartilage to initiate the repair process.

It has demonstrated that CPCs start to proliferate and migrate to the injury sites due to the activation of HMGB1; however, most of the damage-associated molecular pattern molecules (DAMPs), including HMGB1, could be removed within days or even hours after the injury [19]. As an avascular organ, there is no infiltration of inflammatory cells in cartilage after injury. Therefore, the main question for cartilage is how to keep the activated status of CPCs after the damage. It has been reported that stimulated astrocytes could release HMGB1 protein into the extracellular medium during brain injury [20, 21]. In cartilage injury, it is not known whether a given population of cells in cartilage can also release HMGB1 upon their activation. To answer that question, HMGB1 expression and concentration were measured in the culture medium and culture cells in our study. It was found that CPCs can release large amounts of HMGB1 compared to NCs. All these findings illustrate that the self-released HMGB1 might provide a source for CPC activation during cartilage repair without the involvement of inflammatory cells in vitro. Also, articular cartilage injury in the in vivo environment is usually accompanied by synovial membrane inflammation. Initially released HMGB1 from dead chondrocytes may induce the infiltration of inflammatory cells to the synovial membrane and cause acute joint inflammation. Infiltrated macrophages and monocytes can positively release HMGB1 [22], which may also play a role in the activation of CPCs.

Receptor for advanced glycation end products (RAGE) is the main receptor of HMGB1 [22, 23]. However, it has been reported that the ability of HMGB1 to induce the recruitment of inflammatory cells and mesenchymal stem cells is RAGE receptor independent [24]. HMGB1 can recruit bone marrow stem cells to the injured tissue even if the RAGE receptor is disabled [17, 25]. Cell-derived factor-1/CXCL12 is a strong attractant for inflammatory cell and stem cell recruitment during tissue injury [26], and HMGB1 is required for the CXCL12-dependent migration of inflammatory cells [26, 27]. HMGB1-induced recruitment of inflammatory cells depends on CXCL12. HMGB1 and CXCL12 can form a heterocomplex and act exclusively through CXCR4, but not through other HMGB1 receptors. The mechanism of CPC migration upon induction of HMGB1 requires further investigation. In our study, the results show that, in addition to the release of HMGB1, CPCs also had high expressions of CXCL12 and CXCR4. These findings indicate that the HMGB1, CXCL12, and CXCR4 system may play an important role in CPCs migration and self-activation without the requirement of an external factor after the acute phase of cartilage injury.

Although our research indicates that HMGB1 is an important factor for the activation and migration of CPCs through the CXCL12/CXCR4 signalling pathway in vitro, damaged cartilage in clinics cannot be repaired by itself without the help of interventional treatment like chondrocytes or BMSCs (bone morrow mesenchymal stem cell) implantation. In situ autologous CPCs activation, proliferation, and migration may be a better option for cartilage injury repair. More experiments related with the level and time slot of HMGB1 expression in the joint after cartilage injury are required to determine the function of HMGB1 in vivo. Also, previous studies have found that OA cartilage exhibited unique migratory CPCs, and the CPCs have strong chondrogenic capacity [17, 28]. The HMGB1 level also increased in synovial tissue and synovial fluid of OA patients, and it may be involved in the process of OA [28, 29]. Hence, the role of HMGB1 in CPCs activation and migration in OA cartilage should be investigated in future studies.

Conclusion

The results of this research highlight the importance of the role of HMGB1 in CPC activation and migration in response to cartilage injury. We demonstrated that HMGB1 could activate CPCs and induce their migration towards injury sites through the CXCL12/CXCR4 pathway. These findings also indicate that HMGB1 is a potential novel therapeutic target for cartilage repair and OA treatment in clinics.

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