Rapid Cartilage Regeneration of Spheroids Composed of Human Nasal Septum-Derived Chondrocyte in Rat Osteochondral Defect Model

Jung Ho Jeon¹ · Byeong Gon Yun¹ · Min Jae Lim¹ · Seok Jung Kim² ·
Mi Hyun Lim¹ · Jung Yeon Lim¹ · Sun Hwa Park¹ · Sung Won Kim³

Received: 11 September 2019 / Revised: 14 November 2019 / Accepted: 22 November 2019 / Published online: 25 January 2020
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Abstract

BACKGROUND: Cell-based therapies have been studied for articular cartilage regeneration. Articular cartilage defects have little treatments because articular cartilage was limited regenerative capacity. Damaged articular cartilage is difficult to obtain a successful therapeutic effect. In additionally these articular cartilage defects often cause osteoarthritis. Chondrocyte implantation is a widely available therapy used for regeneration of articular cartilage because this tissue has poor repair capacity after injury. Human nasal septum-driven chondrocytes (hNCs) from the septum show greater proliferation ability and chondrogenic capacity than human articular chondrocytes (hACs), even across different donors with different ages. Moreover, the chondrogenic properties of hNCs can be maintained after extensive culture expansion.

METHODS: In this study, 2 dimensional (2D) monolayer cultured hNCs (hNCs-2D) and 3 dimensional (3D) spheroids cultured hNCs (hNCs-3D) were examined for chondrogenic capacity *in vitro* by PCR and immunofluorescence staining for chondrogenic marker, cell survival during cultured and for cartilage regeneration ability *in vivo* in a rat osteochondral defect model.

RESULTS: hNCs-3D showed higher viability and more uniform morphology than 3D spheroids cultured hACs (hACs-3D) in culture. hNCs-3D also showed greater expression levels of the chondrocyte-specific marker Type II collagen (COL2A1) and sex-determining region Y (SRY)-box 9 (SOX9) than hNCs-2D. hNCs-3D also expressed chondrogenic markers in collagen. Specially, in the osteochondral defect model, implantation of hNCs-3D led to greater chondrogenic repair of focal cartilage defects in rats than implantation of hNCs-2D.

CONCLUSION: These data suggest that hNCs-3D are valuable therapeutic agents for repair and regeneration of cartilage defects.

Keywords Spheroid culture · Cartilage · Chondrocyte · Osteochondral defect model

✉ Sung Won Kim
kswent@catholic.ac.kr

¹ Department of Biomedicine and Health Science, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Republic of Korea

² Orthopedic Department, Uijeongbu St. Mary's Hospital, 271 Cheonbo-ro, Uijeongbu-si, Gyeonggi-do 11765, Republic of Korea

³ Department of Otolaryngology-Head and Neck Surgery, College of Medicine, Seoul St. Mary's Hospital, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Korea

1 Introduction

Autologous chondrocyte implantation (ACI) has been widely used for regeneration of articular cartilage defects [1]. Various drugs and surgical treatments have been developed to prevent cartilage defects, and hACs are used for treatment of ACI. In this cell-based technique, hACs are isolated from biopsies of healthy articular cartilage from patients undergoing arthroscopic surgery. However, hACs from isolated cartilage have disadvantageous characteristics that may influence the reproducibility of clinical

results [2]. First, hACs exhibit limited redifferentiation ability after expansion in culture [3, 4]; in addition, hACs of aged donors exhibit decreased chondrogenic potential [5–8]. Finally, obtaining a biopsy causes injury to a healthy area of articular cartilage [9].

Some researchers have supported the use of hNCs from the septum as an alternative to overcome the disadvantages of hACs for ACI treatment and to potentially improve treatment outcomes. The use of hNCs instead of hACs could be advantageous [10]. When hNCs are exposed to different environments, they can react physically in a manner similar to that of hACs *in vitro*. In addition, for this treatment strategy, cartilage biopsies can be obtained with less invasive procedures than removal of tissue from a specific articular area, decreasing donor site morbidity due to biopsy [11]. Moreover, studies have shown that compared to hACs, hNCs has several other advantages; for example, hNCs are known to exhibit better cell proliferation and differentiation capacity than hACs, and the quality of the effects on isolated tissues have been shown to be less donor age-dependent for hNCs than for hACs [12, 13].

Long expansion cultures time and serial passaging increase dedifferentiation of chondrocytes [14]. Chondrocyte dedifferentiation has been shown to cause progressive loss of specific markers such as extracellular matrix (ECM) proteins, COL2A1, and aggrecan (ACAN). At the same time, chondrocyte dedifferentiation increases the levels of markers associated with the fibroblastic phenotype, such as type I collagen (COL1A1) [15]. Generally, chondrocyte dedifferentiation occurs during expansion culture [16]. Dedifferentiated chondrocytes cannot produce articular cartilage ECM; instead, they form fibro-like cartilage ECM. The fibro-like cartilage ECM of dedifferentiated chondrocytes cannot withstand the chemical and mechanical stresses of articular cartilage [17].

Some studies have presented methods to decelerate chondrocyte dedifferentiation, such as changing of culture medium supplements or cell densities or mimicking of the cartilage microenvironment with culture conditions. Several studies have reported successful redifferentiation of dedifferentiated chondrocytes through regulation of culture conditions. The results of these studies have shown that chondrocytes can recover the differentiated chondrocyte phenotype and the expression of chondrocyte marker molecules [18]. In addition, cell behavior in spheroid culture can differ from that in expansion culture [19]. Similarly, spheroid culture can be used to maintain the chondrocyte phenotype and support redifferentiation of dedifferentiated chondrocytes [19–21].

Several different types of natural scaffolds have been tested in experimental animals for articular cartilage repair [22, 23]. Spheroid culture is performed by embedding cells in scaffolds or collecting them into

spheroids by centrifugation [24]. The major reason for embedding the cells in scaffolds during spheroid culture is to increase stability at implantation. Collagen scaffolds are the most popular because collagen is a constituent of articular cartilage. Collagen is used to build spheroid scaffolds and has been used in biocompatible scaffolds [25, 26]. Recently, the sizes of spheroid cultures have decreased because of advances in cartilage production processes [27–29]. The aim of this study was to develop an injectable therapeutic agent for articular cartilage defects. For this purpose, hACs and hNCs were isolated from individual tissues and expanded, and spheroid cultures of the cells were characterized and compared after 7 days. The characteristics of the two cell types were also compared at the RNA level. Specifically, we evaluated cell viability and the expression of chondrocyte-specific markers of expansion for spheroids cultured in a collagen mixture or expansion medium. The effects of the cells were confirmed after transplantation of the cells into osteochondral defect models.

2 Materials and methods

2.1 Cell isolation and culture

hACs were obtained from the catholic university of Korea, Uijeongbu St. Mary's Hospital. The experiments utilizing hACs were conducted in compliance with the Institutional Review Board of the Catholic Medical Center Clinical Research Coordinating Center (UC14CNSI0150) and after obtaining written informed consent from the donors themselves. hNCs were obtained from Seoul St. Mary's Hospital. The experiments utilizing hNCs were also conducted in compliance with the Institutional Review Board of the Catholic Medical Center Clinical Research Coordinating Center (KC08TISS0341) and after obtaining written informed consent from the donors themselves. Harvested human nasal septum cartilage samples were sliced into 2–3 mm pieces in DPBS (Thermo Fisher Scientific, Waltham, MA, USA). The cartilage pieces were washed twice with medium and treated using Collagenase type 2 (Gibco-BRL, Grand Island, NY, USA) at 0.02 g/ml in medium overnight at 37 °C in incubator. The culture medium consisted of low-glucose DMEM (Gibco-BRL), supplemented with 1% antibiotic/antimycotic solution (Gibco-BRL) and 10% fetal bovine serum (Gibco-BRL). Each cell suspension was filtered through a 40 mm cell strainer (BD Falcon, San Jose, CA, USA), and the suspension was centrifuged to recover the cell spheroid. The supernatant was discarded, the spheroid was washed and resuspended in medium, and the cells were seeded in 100 mm culture dishes (Thermo Fisher Scientific).

2.2 Spheroid culture

Spheroid cultures were initiated by adding 1.2×10^6 hNCs or hACs in 1 ml of low-glucose DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) with 1% antibiotic/antimycotic solution (Gibco-BRL) to each well of the micromolds (Prodizen, Gyeonggi-do, Korea). Cells were seeded onto each substrate and were subsequently cultured for 3 days to investigate cellular behavior on each substrate. Cell aggregation and spheroid formation with each method were observed daily under a microscope.

2.3 Cell viability and proliferation

The viability of the cells cultured in the concave molds was assessed using a live/dead assay kit (Invitrogen, Carlsbad, CA, USA). Briefly, 1.2 μ l of calcein AM solution (Invitrogen) and 4 μ l of ethidium homodimer-1 solution (Invitrogen) were dissolved in 1 ml of DPBS (Gibco-BRL), which was then added to the cells. The cells were then incubated at 37 °C for 20 min. The proliferation of the cells was analyzed after culture in 4-well slide dishes (Thermo Fisher Scientific) for 3 days. The cultured cells were incubated with BrdU antibodies (1:250, Abcam, Cambridge, UK) overnight. The cells were counterstained with DAPI (1:1000, Sigma-Aldrich Co., St. Louis, MO, USA) for 30 min. The stained cells were observed using a confocal laser scanning microscope (LSM 5 Exciter, Carl Zeiss, Oberkochen, Germany), and the resulting acquired images were analyzed using ZEN software (Carl Zeiss). To formulate a cell-laden collagen complex, we purchased collagen (Ubiosis, Seongnam, Korea) at a concentration of 30 mg/ml. hNCs-2D were detached from the tissue culture plate with 0.25% trypsin solution, and a cell suspension was prepared. The cell suspension was mixed with the collagen solution. The total cell concentration was 10^6 cells/ml. hNCs-3D were harvested from micromolds and mixed with the collagen solution.

2.4 Chondrocyte gene expression

We used PCR to analyze the expression of several chondrocyte-related genes (Table 1). The two types of human chondrocytes were cultured in 2D and 3D for harvesting of RNA. The samples were incubated with TRIzolTM (Thermo Fisher Scientific), and RNA was extracted. A RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize cDNA. The normalized relative levels of the PCR products were determined for each target gene. The results were analyzed using a Gel Doc XR (Bio-Rad, Hercules, CA, USA).

Table 1 Primer sequences used in PCR assays

SOX9	Forward	gcgacgtcatctccaacatc
	Reverse	tggtcgggtagtcgtactg
ACAN	Forward	tcccaacagatgcttccat
	Reverse	gaacatcattccactcgccc
COL1A1	Forward	caggctggtgtgatgggatt
	Reverse	aaacctctctcgctcttgc
COL2A1	Forward	cttaggcccgagagaagg
	Reverse	gcgtaggaggtcatctgga

2.5 Osteochondral defect model and treatment

Ten-week-old (~ 250 g) male Sprague–Dawley rats were anesthetized using Zoletil/Rompun (50 mg/kg and 5–10 mg/kg, respectively) administered by intraperitoneal (IP) injection in an IACUC-approved animal laboratory facility. After shaving the right knee of each rat and sterilizing it using povidone-iodine swabs, a medial parapatellar incision was made on the knee, and the patella was deflected laterally to expose the trochlear surface. A hole 1.5 mm in diameter and 1 mm deep was drilled into the center of the trochlea using a High-Speed Rotary Micro-motor Kit (Seashin, Daegu, Korea) with a 1.5 mm trephine (Fine Science Tools, Foster City, CA, USA) to induce an osteochondral defect. The experimental animals were divided randomly into four groups: the normal group ($n = 5$), the collagen treatment group ($n = 5$), the collagen with hNCs treatment group ($n = 5$) and the collagen with hNCs-3D treatment group ($n = 5$). The animals were housed in pairs for the duration of the experiment. After defect creation, hNCs-2D or hNCs-3D encapsulated in collagen was implanted by injection using a 1 ml syringe (BD Biosciences, Franklin Lakes, NJ, USA) to achieve full defect filling. The gel usually hardens within 10 min, and hNCs-2D or hNCs-3D encapsulated in collagen was fitted in the defect site. At 4 and 8 weeks, the animals were double euthanized using CO₂ gas and cervical dislocation, and the knees were harvested. The samples were fixed in 10% formalin for 24 h for analysis. The left knees (uninjured) were also harvested as animal-specific internal controls for observation.

2.6 Histological and immunohistochemical analyses

The fixed knees were decalcified in EDTA solution (DyneBIO, Seongnam, Korea) for 21 days (with replacement of the EDTA solution every 2 days), washed for 1 h in running deionized water and then washed in 10% formalin (Sigma-Aldrich Co.) for 30 min. The samples were

serially dehydrated through ethanol/xylene and embedded in paraffin with the medial side of the knee facing down. The embedded samples were sectioned at 7 mm thickness. The sections on cover slides were rehydrated through xylene/ethanol/water and stained with Harris modified hematoxylin and eosin Y (H&E, Sigma Aldrich, St. Louis, MO, USA), Masson's trichrome (Sigma Aldrich). Standard protocols were followed for all stains. Immunofluorescence (IF) staining was performed for COL1A1 (Abcam), COL2A1 (Abcam) and SOX9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections on cover slides were rehydrated through xylene, ethanol and water. The sections were then permeabilized with 0.1% Triton X-100 for 5 min, blocked with 10% normal goat serum (Invitrogen) for 30 min at room temperature, and stained with primary antibodies raised against COL1A1 (1:500, Abcam), COL2A1 (1:500, Abcam) and SOX9 (1:500, Santa Cruz Biotechnology, Inc.) in 1% BSA/PBS at 4 °C overnight. Secondary antibody detection was performed using Alexa Fluor 546-conjugated goat anti-rabbit IgG (Thermo Fisher) for 60 min at room temperature in the dark. After each staining step, the slides were washed 3 times with PBS for 5 min each. Nuclei were counterstained with DAPI (Thermo Fisher) for 5 min at room temperature in the dark.

2.7 Statistical analysis

All experiments contained 4 biological replicates ($n = 4$). The studies were repeated using hNCs derived from four donors. The data are presented as the mean $\pm$ SD or as the mean $\pm$ SEM from at least three independent experiments. All statistical analyses were performed in SPSS 13.0 (SPSS Inc., Chicago, IL) using one-way ANOVA with Fisher's Least Significant Difference (LSD) post hoc test. Probability values less than 0.05 were considered to indicate statistical significance.

3 Results

3.1 ECM expression characterization of hNCs and hACs

We first evaluated the ECM RNA expression of chondrocytes in 2D monolayer and 3D spheroid *in vitro* cultures after three passages. hNCs and hACs were isolated from individual tissues, and *in vitro*-cultured hNCs and hACs were analyzed for expression of ECM genes (Fig. 1). The hNCs-3D expressed higher levels of the COL2A1 and SOX9 genes than the other groups (Fig. 1A). COL1A1 levels were higher in the 2D monolayer cultured hACs

(hACs-2D) than in the other groups. These differences were statistically significant (Fig. 1B).

3.2 Proliferation and viability of hNCs and hACs

After isolation from cartilage, chondrocytes were expanded through monolayer culture. After three passages of *in vitro* culture, cell proliferation capacity was evaluated using BrdU staining. hNCs-2D showed higher proliferation rates than hACs-2D according to BrdU staining, and these results were confirmed by DAPI and BrdU quantification. Immunostaining of cells after BrdU administration revealed that hNCs-2D had approximately 1.5-fold greater proliferation rates than hACs-2D (Fig. 2A, B). The viability of the cells was confirmed by live/dead staining. After three passages, cells were cultured to form 3D spheroids. The 3D spheroids survived to the 7th day. Overall, hNCs formed tighter 3D spheroids than hACs (Fig. 2C). When we assessed the viability of hNCs in collagen, we were able to confirm that hNCs-2D and hNCs-3D survived for up to 7 days (Fig. 2D). Moreover, hNCs-2D showed growth, and hNCs-3D also showed internal growth. In the case of the hNCs-3D, it was confirmed that the cells spread and moved over time.

3.3 ECM expression characterization of hNCs in collagen

After confirming that the hNCs were viable in collagen, we confirmed that the expression patterns of ECM proteins were consistent with chondrocytes by immunostaining the cells with chondrocyte-specific antibodies. Immunostaining for COL2A1, ACAN and SOX9 showed that hNCs-2D in collagen continued to express the ECM proteins for 7 days (Fig. 3A). Encapsulation of the hNCs-2D in collagen as single cells increased the expression of the chondrocyte-specific proteins during the 7 days period. Similarly, compared to hNCs-2D, hNCs-3D in collagen exhibited increased ECM expression over the 7 days period (Fig. 3B).

3.4 Injection of the treatments into osteochondral defect models

We investigated the therapeutic potential of hNCs for cartilage regeneration. Animal models were developed to evaluate the effects of encapsulated hNCs in collagen *in vivo*. The schema shows the design of the hNCs-2D and hNCs-3D in collagen (Fig. 4A). Articular cartilage defects were created by drilling holes 1.5 mm deep and 1 mm in diameter. hNCs-2D or hNCs-3D were implanted into the defects with collagen.

Fig. 1 Characterization of hNCs and hACs. **A** Quantitative PCR analysis of type II collagen (COL2A1), sex-determining region Y (SRY)-box 9 (SOX9), Aggrecan (ACAN), and type I collagen

(COL1A1). **B** The intensities of the PCR bands were quantified by densitometry. *Denotes significance versus individual glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intensity

Fig. 2 BrdU cell proliferation assay. **A** Confocal microscopic images of hNCs-2D and hACs-2D stained with BrdU dye in a cell proliferation assay. **B** DAPI and BrdU staining quantification.

C Staining of live and dead hNCs-3D and hAC-3D in culture. **D** Staining of live/dead hNCs-2D, hNCs-3D in collagen. Scale bars: 100 μ m

3.5 Implantation of hNCs and hNC-3D spheroids into cartilage defect models

To examine the therapeutic effects of these strategies for cartilage regeneration in an osteochondral defect model, hNCs-2D, hNCs-3D and collagen were implanted into

osteochondral defect model rats (Fig. 5A). After 8 weeks, H&E staining showed that the hNCs-2D and hNCs-3D had infiltrated into the defect area (Fig. 5A). hNCs-3D infiltrated tissues exhibited articular-like surfaces. The hNCs-3D surfaces were smooth compared to the hNCs-2D and collagen group surfaces (Fig. 5B).

Fig. 3 Characterization of hNCs-2D and hNCs-3D in collagen. Images of hNCs stained with type II collagen (COL2A1), sex-determining region Y (SRY)-box 9 (SOX9) and Aggrecan (ACAN) antibodies are shown. **A** hNCs-2D in collagen. **B** hNCs-3D in collagen. Scale bars: 100 μ m

Fig. 4 Schematic of the osteochondral defect model. **A** Overview of the preparation of cells for injectable treatments. **B** Macroscopic findings of the femur at 4 and 8 weeks after operation. Scale bars: 1 mm

4 Discussion

The pathogenesis of OA is complex. Various types of stress are believed to stimulate articular chondrocyte metabolism, providing a mechanism for cartilage to adapt to

physiological needs [30]. There are various contributing factors for OA, including genetic predisposition, cumulative cartilage load, and genetic changes in chondrocytes [31–33]. Changes in chondrocyte metabolism caused by the disease weaken the structure of cartilage. Additionally,

Fig. 5 *In vivo* transplantation of hNCs-2D, hNCs-3D into osteochondral defect models. **A** H&E staining of cartilage repair tissue. **B** Masson's trichrome staining for proteoglycan visualization in cartilage repair tissue. Scale bars: 250 μm

imbalance between cartilage synthesis and degradation due to various stimuli, such as injury and stress, can cause OA by inducing tissue collapse [34]. Normal articular cartilage is composed of ECM proteins such as COL2A1 and ACAN. This composition contributes to the stiffness of cartilage tissue [35]. Chondrocytes are the only differentiated cells of articular cartilage and are responsible for the maintenance and production of the ECM [36]. However, articular chondrocytes are not suitable for treatment because they do not normally express their phenotype in culture. Most donors have experienced arthritis. Pathological changes in cartilage tissues can negatively affect chondrocytes [37]. Additionally, chondrocytes lose their phenotype during the culture process; dedifferentiation happens, and the cells change shape [38]. These changes make chondrocytes difficult to use for treatment purposes. The stability of the chondrocyte phenotype depends on the shape and the density of the cells [39]. Chondrocytes in high densities maintain their phenotype [40]. The

differentiation ability of chondrocytes is important for cartilage tissue reconstruction. Dedifferentiated chondrocytes do not produce hyaline cartilage and thus cannot heal articular cartilage damage [41]. The aim of this study was to develop an injectable treatment for OA. For this purpose, hNCs and hACs were isolated from individual tissues. The characteristics and RNA expression of the cell types were compared, and the findings were confirmed by transplantation of the cells into osteochondral defect models.

Spheroid culture was first described in 1998 [42]. Traditionally, spheroid culture methods involve the aggregation of $1\text{--}5 \times 10^5$ mesenchymal stem cells (MSCs) in chondrogenic differentiation medium. However, one issue associated with spheroid culture is the steep diffusion gradient caused by the large diameter of each spheroid (2–3 mm). As a result, necrotic spheroid cores are sometimes observed [43]. This issue can be addressed by reducing the sizes of the spheroids. Reductions in spheroid diameter enhance metabolite and molecule diffusion,

enable greater homogeneity and reduce the size to an injectable scale [44]. Generally, spheroid culture methods allow MSCs to differentiate for 14–28 days [45]. Our primary focus was to identify the early chondrogenic potential of cells grown with spheroid culture methods without differentiation of hNCs. Chondrocyte differentiation is more likely to occur at later time points than at earlier time points during culture. We decided to culture the spheroids for 7 days. Experiments were performed using hACs as controls. It was expected that the spheroids cultured by both 3D spheroid culture methods would express chondrogenic markers such as COL2A1, ACAN and SOX9 but would express relatively low levels of dedifferentiation genes. The 2D culture method was compared with the spheroid culture methods. We indeed found expression of the abovementioned chondrogenic markers. In RNA analysis, hNCs-2D showed higher levels of SOX9 and lower levels of COL1A1 than hACs-2D (Fig. 1A). COL2A1 levels were similarly expressed between the cell types in 2D culture. As expected, the 3D spheroids showed higher RNA levels than the 2D cultured cells. The 3D spheroids exhibited lower COL1A1 RNA levels but higher SOX9 and COL2A1 RNA levels than the 2D cultured cells. This finding suggests that the 3D spheroid culture method supports a more chondrocyte phenotype at the RNA level than 2D culture and that hNCs show therapeutic potential for articular cartilage.

Cell proliferative capacity was evaluated through BrdU staining (Fig. 2A). Cell proliferation is an important characteristic with regards to procurement of cells for treatment. Cells with sufficiently high proliferation rates can be provided to a patient stably as a remedy, enabling the patient to receive adequate treatment. Additionally, a suitable cell supply is economically beneficial for a patient. The cell proliferative capacity was confirmed by assessing the number of proliferating cells compared to the total number of cells through BrdU and DAPI staining. hNCs-2D had higher proliferation rates than hACs-2D (Fig. 2B). Constant size of spheroids during 3D culture is an important feature for injectable drugs. Traditionally, cartilage spheroid culture methods have attempted to aggregate large numbers of cells into a single spheroid and differentiate them into cartilage. These spheroids form sharp radial diffusion gradients and form necrotic cores. To alleviate this issue, we reduced the diameters of the spheroids. This adjustment eliminated the necrotic cores and facilitated metabolite and signaling molecule diffusion. In general, smaller spheroids can more easily pass through needles and can be developed as injectable treatments. Cell viability was confirmed in the 3D cultures through live and dead cell staining. In 3D culture, it was confirmed that hNCs-3D formed tighter spheroids than hACs-3D

(Fig. 2C). hNCs are more advantageous than hACs in terms of supply and stability in 3D culture. We assessed the ability of the cells to grow and survive in collagen (Fig. 2D), and hNCs were able to grow, migrate and survive in collagen.

The hNCs expressed characteristic chondrocyte markers when cultured in collagen (Fig. 3). In addition, it was confirmed that the hNCs expressed chondrocyte markers even when they were mixed with collagen after 2D culture and were subsequently grown in 3D culture (Fig. 3A). In particular, chondrocyte marker expression was higher in 3D spheroids cultured in collagen than in cells subjected to 2D culture in collagen. These results were similar to those of the RNA analysis. The ability of the cells to be used as therapeutic agents for cartilage regeneration was evaluated by filling cartilage defects with a mixture of hNCs and collagen.

We studied the therapeutic potential of hNCs implantation for the treatment of articular cartilage defects. To confirm the efficient cartilage regeneration potential of hNCs, we established an osteochondral defect model in rats by drilling holes to a depth of 2 mm and with a diameter of 2 mm (Fig. 4A). At 4 and 8 weeks after transplantation, some cartilage tissues still exhibited defects in each group (Fig. 4B).

Through various types of staining, we confirmed the ability of the hNCs-3D to regenerate cartilage (Fig. 5). We identified hyaline cartilage-specific metachromasia and ECM accumulation through these staining methods.

Our data demonstrate that hNC transplantation promotes cartilage regeneration in rats with osteochondral defects. In particular, cartilage regeneration occurred more clearly in models transplantation with hNCs-3D than the hNCs-2D. These results were also confirmed 8 weeks after transplantation in the hNCs-3D group. Overall, our findings suggest that hNCs-3D have several properties that make them well suited for regenerative medicine targeting osteochondral diseases.

Acknowledgements This work was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (NRF-2019M3E5D5064110, NRF-2019M3A9H2032424), Institute for Information & communications Technology Promotion (IITP) grant funded by the Korea government (MSIP) (No.2017-0-00953), and the Korea Health Industry Development Institute funded by the Ministry of Health and Welfare (HI14C3228). This work was also supported by the Institute of Clinical Medicine Research of Bucheon St. Mary's Hospital, Research Fund, 2018. The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical statement The study protocol was approved by the institutional review board of the Catholic Medical Center Clinical Research Coordinating Center (IRB No. KC08TIS0341, UC14CNSI0150). Informed consent was confirmed (or waived) by the IRB. IACUC and Department of Laboratory Animal (DOLA) in Catholic University of Korea, Songuei Campus accredited the Korea Excellence Animal laboratory Facility from Korea Food and Drug Administration in 2017 and acquired AAALAC International full accreditation in 2018. All of surgical interventions and presurgical and postsurgical animal care were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the IACUC (Institutional Animal Care and Use Committee) in school of medicine, The Catholic University of Korea. (Approval Number: CUMS-2017-0040-04).

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