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Growth Factor-free Chondrogenic Differentiation from Induced Pluripotent Stem Cells using Minicircle Vectors

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Running Title: Chondrogenesis using minicircle vectors and induced pluripotent stem cells

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Abstract

The human degenerative cartilage has low regenerative potential. Chondrocyte transplantation offers a promising strategy for cartilage treatment and regeneration. Currently chondrogenesis using human pluripotent stem cells are accomplished using human recombinant growth factors. Here, we differentiated human induced pluripotent stem cells (hiPSCs) into chondrocytes and cartilage pellet using minicircle vectors. Minicircles are used as a non-viral gene delivery system for gene therapy in various diseases. Non-viral gene delivery can produce growth factors without integrating into the host genome. Minicircle vectors containing bone morphogenetic protein 2 (BMP2) and transforming growth factor, beta 3 (TGFβ3) were successfully generated and delivered to hiPSC-derived outgrowth (OG) cells. Cell pellets generated using minicircle-transfected OG cells successfully differentiated into chondrogenic lineage. Chondrogenic pellets transfected with growth factor-encoding minicircles effectively recovered osteochondral defect in rat models. Taken together, this work shows the potential application of minicircles in cartilage regeneration using hiPSCs.

Keywords: Minicircle; Induced pluripotent stem cells; Chondrogenesis; Chondrocyte; Bone morphogenetic proteins; Transforming growth factors;

1. Introduction

The poor recovery of damaged cartilage has prompted researchers to develop a define regeneration strategy [1]. The repair technique used for articular cartilage is mostly done using various cell sources with limited propagating ability. Chondrocytes are usually obtained autologously or generated *in vitro* from adult stem cells (i.e., adipose-derived stem cells and mesenchymal stem cells) [2, 3]. However, primary chondrocytes and adult stem cells are in short supply and easily lose its characteristics under *in vitro* culture conditions. It is also reported that the chondrogenic potential of the adult stem cells depends on the pathological status of the donor.

Over the past decade, human induced pluripotent stem cells (hiPSCs) have shown boundless possibilities in tissue regeneration. hiPSCs expand significantly and maintain their pluripotency for several passages [4]. The

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use of hiPSCs in cell-based therapy can be promising for damaged tissues with low regenerative abilities. Current differentiation protocols of hiPSCs towards chondrocytes are done with several growth factors, such as bone morphogenetic proteins (BMPs) and transforming growth factors (TGFs) [5, 6]. BMPs were originally known to be critical in the development of bone and cartilage [7, 8]. The deletion of BMP2 resulted in severe defects during the endochondral bone development [9]. It revealed the role of BMP2 in chondrocyte survival and proliferation. TGFβ superfamily proteins control the architecture in various tissues by contributing in processes such as proliferation, differentiation and apoptosis. Between all TGFβ proteins, three isoforms (i.e. TGFβ1, TGFβ2 and TGFβ3) are reported to induce chondrogenesis [10-14]. Most researches are done with TGFβ1 or TGFβ3 [15]. TGFβ3 is reported to have higher differentiation ability than TGFβ1 [16]. It was also reported that the isotype was not a critical issue after a certain time point [15]. Combining several growth factors for chondrogenesis was reported to have enhancing effect on the differentiation process. The combination of BMP2 and TGFβ3 in three-dimensional culture system enhanced the chondrogenic differentiation of bone marrow-derived MSCs [17].

The use of recombinant human growth factors is now considered as a critical factor in the field of tissue engineering for regenerative medicine. However, the frequent addition of growth factor molecules during differentiation is cost-effective. Overexpression of several growth factors by gene delivery was efficient in chondrogenesis. Retroviral delivery of TGFβ1 in synovial-derived MSCs enhanced proliferation of cells and accelerated chondrogenic differentiation [18]. The overexpression of SOX9 enhanced differentiation in mouse mesenchymal stem cells (MSCs) and umbilical cord blood-derived MSCs [19, 20]. Increased collagen type II expression was confirmed in mouse embryonic stem cells after human SOX9 overexpression [21].

Non-viral gene delivery is promising for safe *in vivo* gene transfer strategy. Yet, commercial plasmid DNA vectors contain bacterial sequences that may induce immune responses by producing antibodies against bacterial proteins [22]. The gene expression in the host cell can alter by the antibiotic resistance marker and the immune responses [23]. Minicircles are vectors with eliminated bacterial backbones and transcription units including the antibiotic resistance gene. Therefore, it has a relatively small size than other commercial vectors. The small size and the ability to avoid immune reactions lead to high expression of the foreign gene *in vitro* and *in vivo*. Minicircles also showed the possibility in pre-clinical gene therapy research [24].

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Safe and efficient gene transfer is promising for the use of gene-modified stem cells in therapeutic applications [25]. The effect of minicircle vectors encoding human proteins was confirmed through our preliminary researches [26-28]. The combination of minicircle vectors and stem cells can suggest a new regenerative tool for clinical application. In this study, we differentiated hiPSCs using minicircles encoding the cDNA of BMP2 and TGFβ3. Outgrowth (OG) cells were induced from hiPSC-derived embryoid bodies (EBs). Minicircles were transfected in hiPSC-derived OGs. Transfected OG cells were generated into chondrogenic pellets and maintained for 30 days. Chondrogenic pellets were further characterized using various assays. Minicircle-induced chondrogenesis using hiPSCs suggests a new approach for future application in tissue engineering and regenerative medicine.

2. Results

2.1 Generation of minicircles encoding human growth factors

Human growth factor-encoding minicircle expression plasmid vectors were generated by synthesizing the codon optimized cDNA of human BMP2 and TGFβ3. The cDNA of each growth factor was sub-cloned into the parental plasmid vector downstream to the CMV promoter (Figure 1A). The insert sequences are cloned into the BamHI and XbaI restriction sites in the multiple cloning sites. The size of BMP2-encoding minicircles (mcBMP2) was shown at ~7.3 kb (Figure 1B). TGFβ3-encoding minicircles (mcTGFβ3) had the size of, approximately 7.5 kb. Successful cloning was confirmed by double digestion of the generated minicircles with BamHI and XbaI. A reduced size (~5 kb) of the growth factor-encoding minicircles was confirmed. The resulting fragment of BMP2 and TGFβ3 inserts (arrow) was observed as the size of ~1.1 kb.

The working activity of mcBMP2 and mcTGFβ3 was confirmed by transfecting HEK293T cells. The expression of red fluorescence protein (RFP) was observed (Figure 1C). The cells transfected with the mock vector (mcMock) had the highest transfection rate. The expression of mcBMP2 was relatively lower than that of mcTGFβ3. The transfection rate of mcTGFβ3 was similar to that of mcMock (Figure 1D). The growth factor proteins secreted from the minicircles were detected from the supernatant of the transfected HEK293T cells. The

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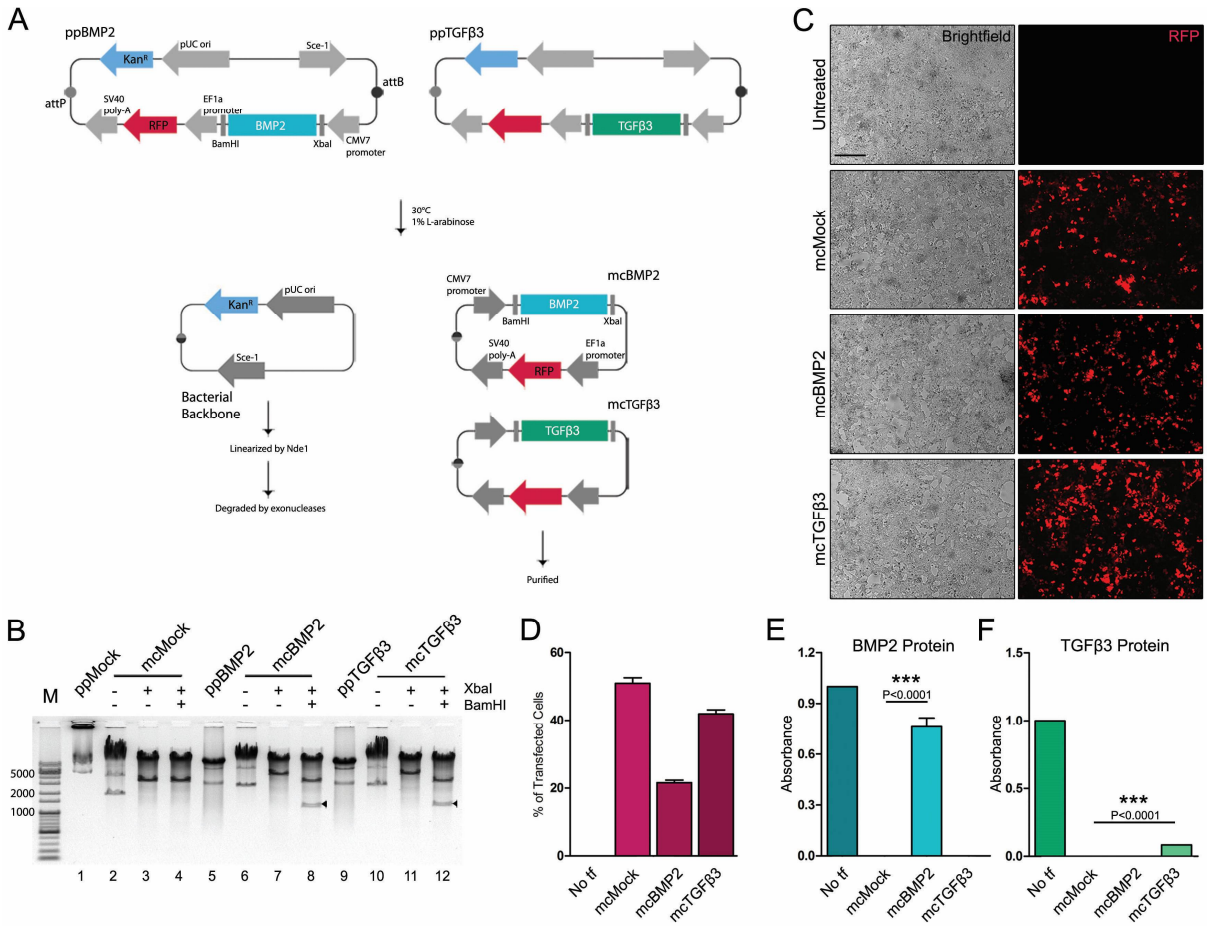

absorbance of recombinant protein BMP2 (conc. 0.1 ng/mL) (Figure 1E). The protein expression of mcTGFβ3 was relatively low (Figure 1F). However, compared to the supernatant of mcMock, the absorbance was detected in the supernatant of mcTGFβ3-transfected HEK293T cells. Based on these results, we confirmed the successful cloning of growth factor-encoding minicircles.

mcBMP2-transfected HEK293T cell supernatant was high as much as the commercial

Figure 1. Generation of BMP2- and TGFβ3-encoding minicircles.

(A) Scheme of minicircle generation process. **(B)** Gel electrophoresis of parental plasmid vectors (pp) and minicircle vectors (mc). **(C)** Fluorescence microscopy of transfected HEK293T cells. **(D)** Percentage of transfected HEK293T cells. **(E)** BMP2 protein expression in mcBMP2-transfected HEK293T sup. **(F)** TGFβ3 protein expression in mcTGFβ3-transfected HEK293T sup. Scale bar represents 200 μm. (**: $p<0.01$; ***: $p<0.001$)

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2.2 Chondrogenesis with minicircles using human iPSC-derived OG cells

hiPSCs were generated into EBs for OG induction. Mesenchymal-like OG cells were induced from the EBs for transfection. Minicircle vectors were transfected in OG cells one day before chondrogenic differentiation using pellet culture system. The chondrogenic pellets were maintained for 30 days without recombinant growth factor addition to observe the differentiation efficacy of mcBMP2 and mcTGFβ3 (Figure 2A). Stable hiPSCs with a compact colony were used for differentiation (Figure 2B). Cells were aggregated to generate EBs (Figure 2C). After attaching the EBs onto a gelatin-coated dish, fibroblast-like OG cells were induced (Figure 2D). After maintaining the OG cells for a week, cells showed a fibrotic morphology that is similar to mesenchymal stem cells. Cells showed a stable morphology after several days before differentiation (Figure 2E). Transfection with mcMock in OG cells showed high expression of RFP (Figure 2F). Similar to the results of HEK293T cells, mcBMP2 showed relatively low expression of RFP in OG cells (Figure 2G). Transfection with mcTGFβ3 showed similar results to that of HEK293T cells (Figure 2H). We have confirmed that the overall transfection tendency of OG cells were similar to the tendency that was shown from HEK293T cells (Figure 2I). The gene expression of MSCs markers were examined in OG cells. Bone marrow-derived MSCs were used as a positive control. CD44 was increased in OG cells, compared to hiPSCs (Figure 2J). CD73 was increased in OG cells as well, however, it was significantly lower than that of MSCs (Figure 2K). Interestingly, the expression of CD90 was higher than that of MSCs (Figure 2L). CD105 was observed in OG cells (Figure 2M). MSC markers were all increased in OG cells. Yet most of the markers were lower than that of MSCs. MSCs are usually characterized by three-lineage differentiation (i.e. apipocyte, chondrocyte and osteoblast). OG cells successfully differentiated to osteogenic lineage (Figure 2N). OG cells were also able to go through adipogenic differentiation (Figure 2O). OG cells were able to generate chondrogenic pellets through differentiation with recombinant growth factors (Figure 2P).

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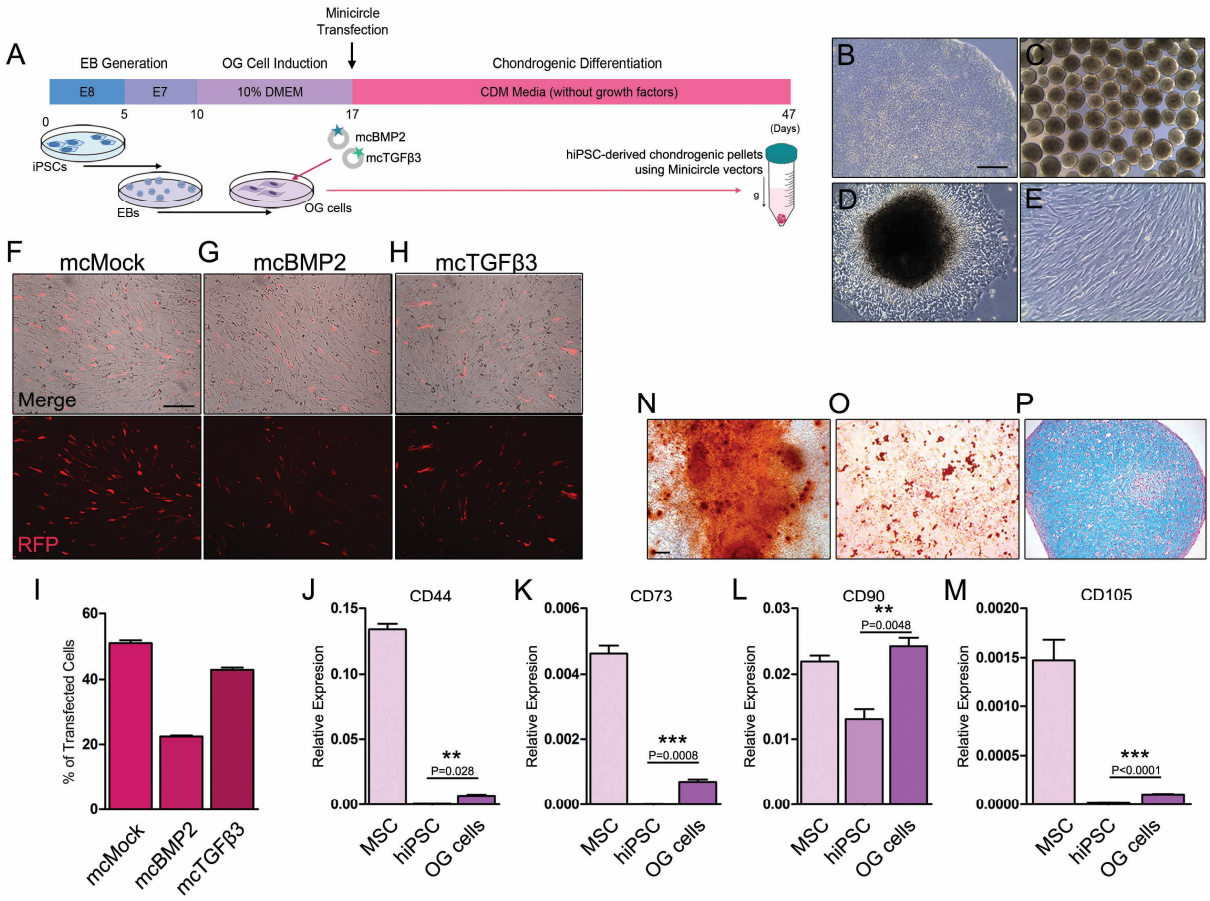

Figure 2. Chondrogenesis using minicircle-transfected hiPSC-derived OG cells.

(A) Scheme of chondrogenic differentiation process from hiPSCs. Minicircles were transfected after OG cells were induced. **(B)** Morphology of hiPSC colony. **(C)** Morphology of generated EBs. **(D)** Image of outgrowth cells derived from EBs attached to a gelatin-coated culture dish. **(E)** Morphology of OG cells before transfection. **(F)** Fluorescence microscopy of mcMock-transfected OG cells. **(G)** Fluorescence microscopy of mcBMP2-transfected OG cells. **(H)** Fluorescence microscopy of mcTGFβ3-transfected OG cells. **(I)** Percentage of transfected OG cells transfected with each minicircle vectors. **(J)** Relative gene expression of CD44 in OG cells. **(K)** Relative gene expression of CD73 in OG cells. **(L)** Relative gene expression of CD90 in OG cells. **(M)** Relative gene expression of CD105 in OG cells. **(N)** Alizarin red-stained osteogenic cells differentiated from OG cells. **(o)** Oil red O staining image of adipogenic cells differentiated from OG cells. **(p)** Chondrogenic pellet generated from OG cells stained with alcian blue. Scale bar represents 200 μm. (**: p<0.01; ***: p<0.001)

2.3 Characterization of minicircle-based chondrogenic pellets

OG cells were transfected with mcMock, mcBMP2 and mcTGFβ. After transfection, cells were aggregated into pellets for differentiation. Cells transfected with mcBMP2 and mcTGFβ3 were mixed with the same portion,

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and generated into pellets by centrifugation as well. Transfected cells formed a pellet after 3 days (Figure 3A). On day 10, pellets maintained its morphology and RFP expression was confirmed in the three-dimensional pellets (Figure 3B). The condensation of mcTGFβ3 pellets was late than others. Pellets were observed under a fluorescence microscope for 30 days. On day 20, mcBMP2 and mcBOTH pellets stayed in a condensed form, however, mcTGFβ3 pellets increased in size (Figure 3C). The RFP expression of mcBMP2 and mcTGFβ3 was maintained up to 20 days but decreased when observed on day 30 (Figure 3D). The RFP expression of mcMock was maintained until day 30 of differentiation.

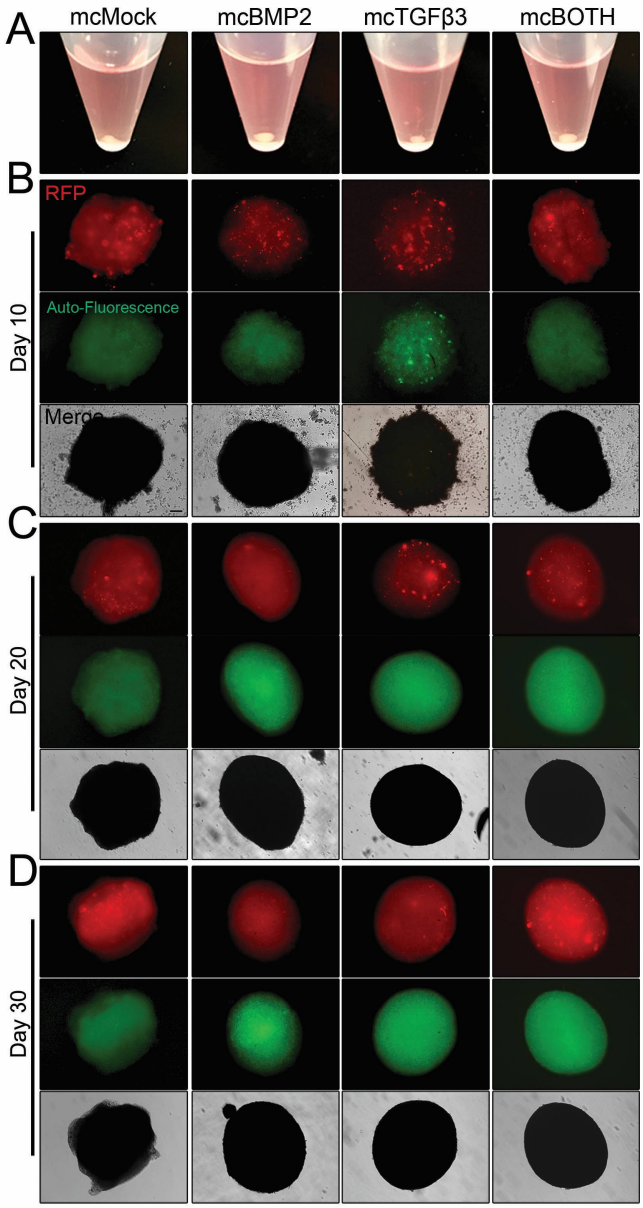

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Figure 3. Minicircle expression in chondrogenic pellets.

(A) Morphology of pellets generated from hiPSC-derived OG cells on day 3. Fluorescence image of chondrogenic pellets on day 10 **(B)**, 20 **(C)**, and 30 **(D)** of differentiation. Minicircle vectors were detected with RFP expression. GFP was detected to consider the auto-fluorescence of the three dimensional pellet. Scale bar represents 200 μm.

The characteristics of the generated chondrogenic pellets were analyzed. The gene expression of chondrogenic markers was evaluated. Chondrogenic pellets generated with human recombinant BMP2 and TGFβ3 were used as a control (Both rhGF). The early chondrogenic marker, SOX9 was increased in the chondrogenic pellets differentiated with the minicircle-transfected cells (Figure 4A). The expression of ACAN was increased, but as SOX9, it was lower than that of the chondrogenic pellet differentiation with recombinant proteins (Figure 4B). SOX9 and ACAN were both significantly increased in mcBMP2, mcTGFβ3, and mcBOTH compared to mcMock. COL2A1 is the gene responsible for the expression of collagen type II. The gene expression of COL2A1 was detected the highest in the minicircle-transfected chondrogenic pellets (Figure 4C). The expression was much higher than the pellets generated using recombinant growth factors. The fibrotic marker COL1A1 were higher in the minicircle-transfected chondrogenic pellets, however, the expression of COL10A1 was lower than other groups (Figure 4D and 4E). Interestingly, mcBMP2 had the highest expression of COL1A1, and mcTGFβ3 had the highest expression of COL10A1. Yet, mcBOTH had the most moderate expression of all markers. The osteogenic marker, RUNX2 was measured in pellets to confirm the osteogenesity of chondrogenic pellets (Figure 4F). All three conditions showed lower expression of RUNX2 expression compared to the pellet differentiated by recombinant growth factors. Pellets generated with mcBOTH had the lowest expression of RUNX2. The ECM accumulation was detected by toluidine blue staining (Figure 4G). Taken all together, we have confirmed the expression of minicircles in the iPSC-derived chondrogenic pellets, and the pellets had the characteristics of the chondrogenic lineage.

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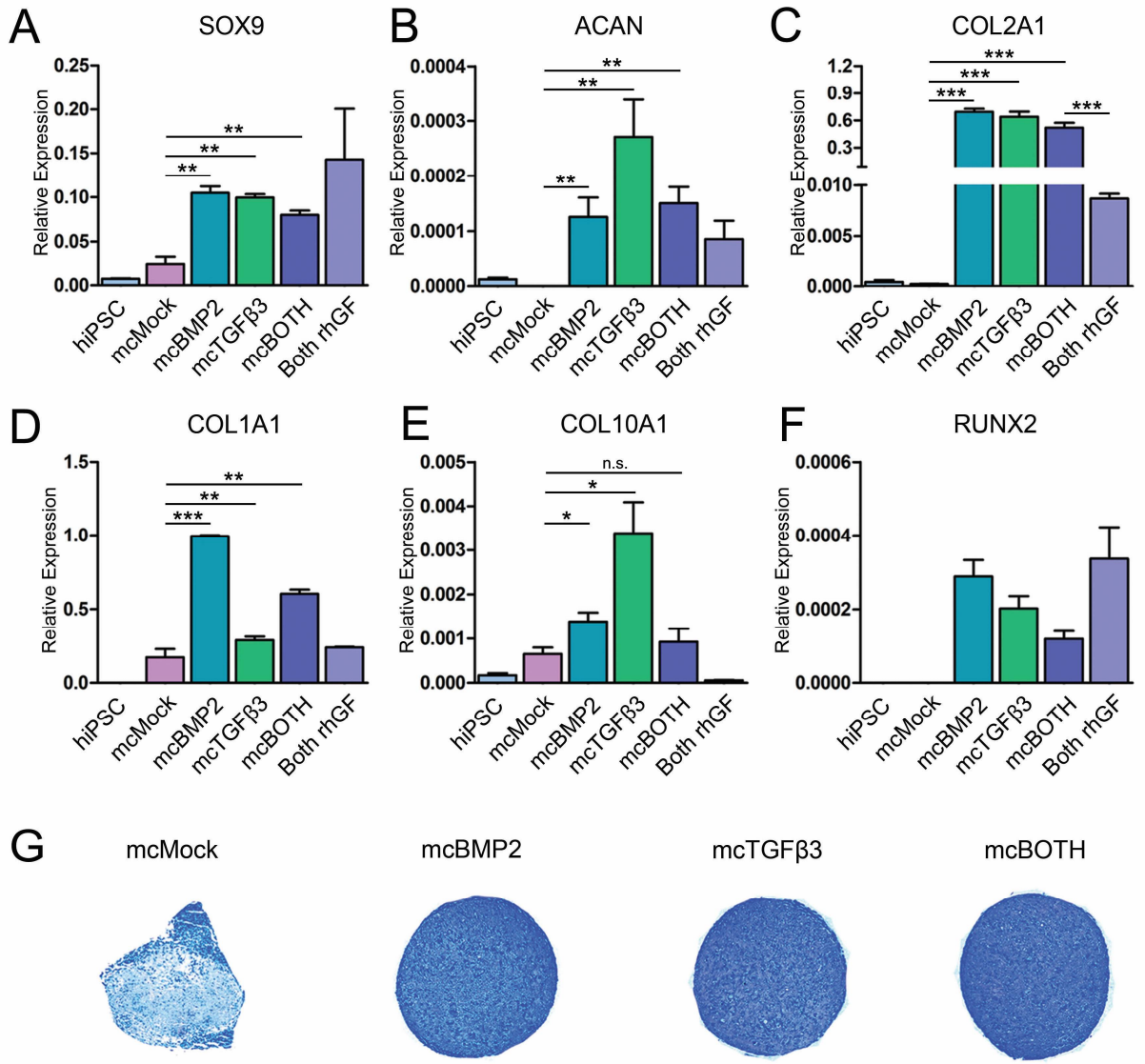

Figure 4. Chondrogenic gene expression of chondrogenic pellets generated with minicircles.

(A) Relative gene expression of SOX9 in chondrogenic pellets. **(B)** Relative gene expression of ACAN. **(C)** Relative gene expression of COL2A1. **(D)** Relative gene expression of COL1A1. **(E)** Relative gene expression of COL10A1. **(F)** Relative gene expression of osteogenic marker, RUNX2. **(G)** Toluidine blue staining image of chondrogenic pellets. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

2.4 Further analysis of minicircle-based chondrogenic pellets

ECM production was detected in all pellets generated with minicircle vectors. For further analyzation, the collagen type that consist the produced ECM was analyzed. Collagen type II represents the hyaline cartilage, and collagen type I represents the fibrotic cartilage *in vivo*. Negative control is shown in Figure 5A. The

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Expression of collagen type II was highly detected even in the cells (blue arrow) of pellets treated with mcBOTH (Figure 5B). Collagen type II was also detected in mcMock pellets as well. The expression of collagen type I was also confirmed in pellets (Figure 5C). Collagen type 1 was relatively high in mcBMP2 and also in mcMock. Collagen type X is usually expressed in hypertrophic cells. The expression of collagen type X was also the highest in mcBMP2 pellets (Figure 5D).

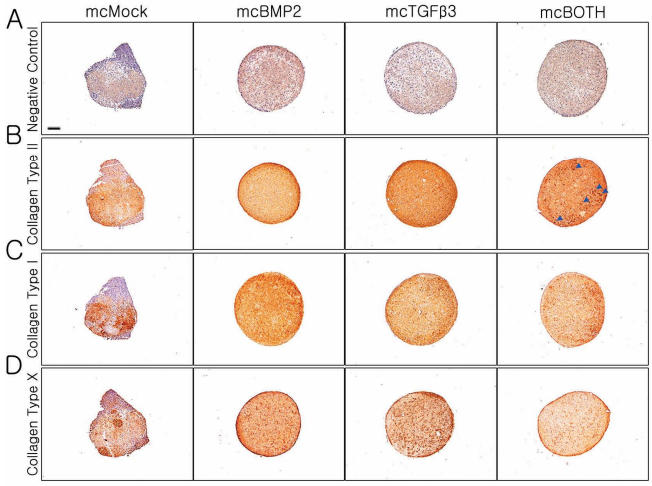

Figure 5. Further characterization of chondrogenic pellets generated with minicircles.

(A) Image of negative control pellets used in immunohistology. **(B)** Image of pellets stained with collagen type II. **(C)** Image of pellets stained with collagen type I. **(D)** Image of pellets stained with collagen type X. Scale bar represents 200 µm.

2.5 In vivo transplantation of mcBOTH chondrogenic pellets in osteochondral rat model

To confirm the recovery ability of minicircle-transfected chondrogenic pellet, we transplanted the pellets in the defect of an osteochondral rat model (Figure 6A). Defects were induced using a microdrill and chondrogenic pellets (day 30) were placed in the defect. Rats were maintained for 8 weeks and sacrificed for further analysis. The recovery was evaluated by several staining methods. Defects treated with minicircle-based chondrogenic pellet were healed, yet, mcBMP2 treated joints showed less accumulation of ECM (Figure 6B). The defect in joints implant with mcMock pellets, however, had almost no accumulation of ECM proteins. We examined the protein type that was accumulated in the healed ECM matrix. Negative control is shown in Figure 6C. Collagen type II was highly expressed in joints implanted with mcTGFβ3 and mcBOTH (Figure 6D). The two groups had

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higher expression of collagen type II than that of mcMock treated with both growth factors (Both rhGF). The expression of collagen type I was detected in mcBMP2 implanted joints (Figure 6E). Collagen I was barely detected in joints with mcTGFβ3, mcBOTH and Both rhGF. Collagen type X was thought to be highly expressed in mcTGFβ3-implanted joints, however, it seemed similar to that of mcBMP2-implanted joints (Figure 6F). Through these results, we confirmed that minicircle-transfected chondrogenic pellets efficiently healed under in vivo conditions.

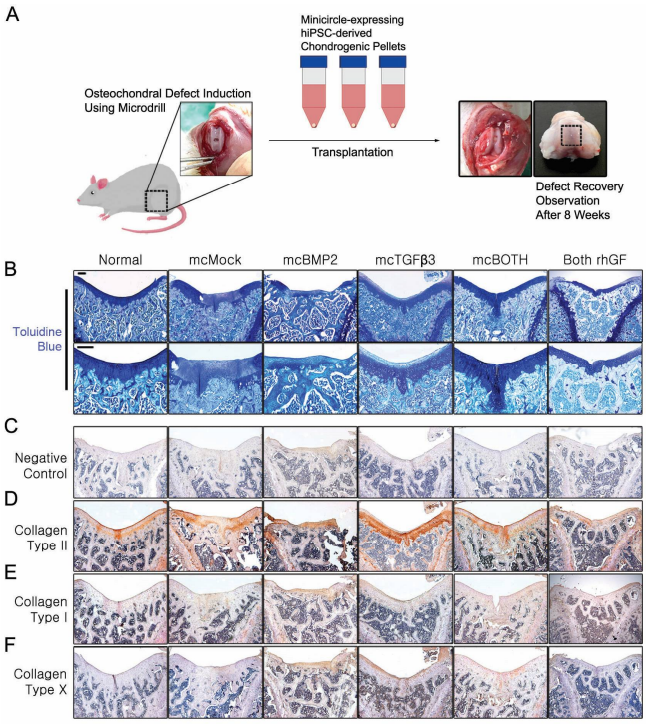

Figure 6. *In vivo* transplantation of minicircle-based chondrogenic pellets in osteochondral defect rat model.

(A) Scheme of defect induction and transplantation. **(B)** Image of pellet-transplanted joints stained with toluidine blue. **(C)** Image of negative control joint sections. **(D)** Image of joints stained with collagen type II. **(E)** Image of joints stained with collagen type I. **(F)** Image of joints stained with collagen type X. Scale bar represents 200 μm.

3. Discussion

The low cellularity and avascularity limits the ability of cartilage recovery. The current option for cartilage

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recovery using cell-based therapy is the use of chondrocytes or MSCs. However, autologous chondrocytes and MSCs lose their original characteristics after several passages under *in vitro* conditions [29, 30]. Dedifferentiated cells result in fibrocartilage after differentiation or transplantation. This challenge must be overcome for better cartilage recovery using cell-based therapy.

Human iPSCs are stem cells generated from the adult somatic cells obtained from the donor. The identical immunity with the donor makes hiPSCs able to avoid immune rejection. hiPSCs can be expanded limitlessly *in vitro*. Therefore it is a possible candidate as a for the next generation material for cell-based therapy. The use of iPSCs in cartilage regeneration and recovery can suggest options for cell-based therapy to treat cartilage defects.

Non-viral gene delivery is a subject that should be solved for future application. Minicircles are supercoiled DNA vectors lacking the bacterial backbone sequence (i.e. origin of replication, selection marker gene and CpG motifs) [25, 31]. The bacterial backbone in the commercial plasmid vectors can induce immune reactions in cells [32]. The spreading of the antibiotic resistance gene can be unsafe to be used in vivo. With its robust construct and improved safety, minicircle vectors were considered as a possible agent for DNA vaccination that can actually be used in clinic [33].

Here, we have shown the combination of minicircle technology and hiPSCs. Minicircle vectors encoding human BMP2 and TGFβ3 were successfully generated by cloning (Figure 1). OG cells induced from iPSC-derived EBs were transfected and showed similar transfection efficacy to that of HEK293T cells. Chondrogenic pellets differentiated using growth factor proteins secreted from minicircles went through chondrogenesis *in vitro* (Figure 3). The mixture of OG cells transfected with each minicircles had the most adequate quality. Minicircle-based chondrogenic pellets were transplanted in osteochondral defect rat joints. Minicircle transfected chondrogenic pellets showed increase ECM in the defected cartilage (Figure 6).

The delivery of mcBMP2 and mcTGFβ3 in hiPSC-dervied OG cells successfully led to chondrogenesis. BMP2 and TGFβ3 are reported to induce chondrogenic differentiation. BMP2 restored and enhanced the chondrogenic potential of expanded chondrocytes under *in vitro* conditions [34]. TGFβ3 delivery was essential for neocartilage formation and increased the proliferation in MSCs [35]. Co-stimulation with both BMP2 and TGFβ3 resulted in improved chondrogenesis compared to the standard one growth factor-based method. BMP2

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synergistically enhance the effect of TGFβ3 [17]. We induced chondrogenesis by transfecting mcBMP2 and mcTGFβ3 into EB-derived outgrowth cells. Separately transfected outgrowth cells were mixed to confirm the effect on co-stimulation of BMP2 and TGFβ3. Co-stimulation with both growth factors showed similar expression of SOX9, COL2A1 compared to that of the other two conditions (Figure 4A and 4B). However, mcBOTH-transfected outgrowth cells showed low expression levels of COL1A1 and COL10A1. Pellets generated with mcBOTH-transfected cells had the lowest expression of RUNX2 (Figure 4F). Previous reports have shown that co-stimulation with both growth factors had lower bone gamma-carboxyglutamate protein (BGLAP, Osteocalcin) compared to either MSCs treated with TGFβ3 or undifferentiated MSCs [17]. These results correspond with the previous reports, suggesting that co-stimulation with both BMP2 and TGFβ3 has the lowest possibilities to develop into bone.

ECM was slightly accumulated in mcMock-transfected pellets (Figure 4-5). This might be caused by the pellet formation itself, as it is known that a high density of cells can still lead to accumulation of ECM proteins. However, the genetic levels were much higher in growth factor-encoding minicircle transfected pellets, especially aggrecan and collagen type II (Figure 4B and 4C). Most importantly, the expression of COL2A1 explosively increased compared to the pellets treated with recombinant growth factors. This might be caused by the secretion of growth factor proteins by the cell itself. And it may be more feasible to affect the cells in the center of the pellet for differentiation. The remaining of minicircle vectors could be a problem for actual use in clinic, however, the short term secretion of growth factor proteins by the controlled use of minicircle vectors may lead to the formation of cartilage with better quality and the maintenance of implanted regenerated cartilage tissue.

OG cells generated from hiPSC-derived EBs are reported to have similar qualities to that of MSCs [5, 36, 37]. The relative expression of MSC markers (i.e. CD44, CD73, CD90, CD105) was evaluated (Figure 2J-2M). OG cells had increased levels of CD44, CD73 and CD105 compared to that of hiPSCs. CD90 was even higher than that of MSCs. OG cells were able to differentiate into all three lineages that are required for MSC characterization (i.e. osteogenic, adipogenic, and chondrogenic differentiation) (Figure 2N-2P). The potential to adipogenic lineage seemed to be the weakest in hiPSC-derived OG cells. The characterization of MSCs-derived from hiPSCs was reported by several researchers. It was previously reported by Kang and colleagues that MSCs

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generated from hiPSCs had adequate osteogenicity and chondrogenicity, however, relatively less adipogenicity [38]. There is still less evidence about the low adipogenecity, however there are several other reports that reports the low adipogenesis from hiPSC-derived MSCs and even ESC-derived MSCs [39-41].

MSCs are infamous for having low transfection efficiency with non-viral delivery. Lipid-based non-viral transfection systems showed low efficiency in bone marrow-derived MSCs [42]. These results also agree with the report that shown transfection efficacy lower than 5% in adipose-derived MSCs using lipofectants [43]. Even with minicircles, we previously reported low transfection in bone marrow-derived MSCs using chemical transfection reagents [26]. The transfection efficacy in MSCs was increased when using electroporation along with minicircle vectors. However, MSC-like outgrowth cells derived from hiPSCs showed high transfection efficacy even with lipofectants. This can suggest hiPSCs as a solution for non-viral gene modified cell source for gene and cell therapy. In our results, mcBMP2 showed relatively low transfection rate compared to mcMock or mcTGFβ3. However, the protein secreted from the minicircle did not correlate with the transfection efficacy. Even with low transfection rate, mcBMP2 secreted relatively high levels of growth factor protein than mcTGFβ3. It is thought to be caused by the working structure of the protein. TGFβ3 is reported to have low solubility at physiological pH and form aggregates more easily than the other TGF isoforms [44].

The healing ability of chondrogenic pellets were confirmed in osteochondral defected rat models (Figure 6). However, unlike mcTGFβ3 and mcBOTH, mcBMP2 had a relatively small amount of accumulated ECMs. Also, the ECM proteins were expressing collagen type I and X. BMP2 was used as an agent for chondrogenesis, however, it's ultimate function is related with bone generation. The high expression of fibrotic and hypertrophic marker is thought to be related to the calcification of cartilage that can lead to bone formation. Therefore, mcTGFβ3 and mcBOTH are thought to be relatively more applicable for cartilage regeneration for future use.

4. Materials and Methods

4.1 Minicircle production

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The minicircle parental plasmid (CMV-MCS-EF1-RFP-SV40-PolyA) was purchased from SBI (System Biosciences, Mountain View, CA, USA). The cDNA sequence of a codon optimized human BMP2 and TGFβ3 was subcloned into the mock parental plasmid. The growth factor cDNA was inserted at the BamHI and XbaI restriction sites in the multiple cloning sites downstream to the CMV promoter. The sequence of BMP2 and TGFβ3 is shown in Additional file 1. The minicircle vectors were produced following the manufacturer's instructions. ZYCY10P3S2T E.coli cells were transformed with the parental vectors containing each growth factor. A single colony was obtained and grown for 2 hours in 2 mL of Luria-Bertani broth (LB) with 500 µg/mL kanamycin at 30°C. In a 1 litre flask, 100 µL of starter culture was inoculated into 200 mL terrific broth (TB) and incubated at 30°C with shaking at 200 rpm for 15 hours. Induction medium consisting of 200 mL LB, 4% 1N NaOH, and 200 µL of 20% L-arabinose was added to the TB bacterial culture. The mixture was incubated for 5 hours at 30°C with shaking at 200 rpm. Bacterial cells were harvested and plasmid DNA was extracted using NucleoBond Xtra plasmid purification kits (Macherey-Nagel, Duren, Germany). The inserts encoded in the minicircles were confirmed by double digestion in XbaI and BamHI.

4.2 hiPSC culture

All iPS cell lines used in this experiment (n=3) were generated using cord blood mononuclear cells. Reprogramming and characterization was performed as previously described [4]. Cells were maintained in a vitronectin-coated dish (Thermo Fisher Scientific, Waltham, MA, USA) and media were changed daily with fresh E8 medium (STEMCELL Technologies).

4.3 Embryoid body generation and outgrowth cell induction

Maintained iPSCs were detached and 2 x 10^6 cells were prepared. A 1:1 mixture of TeSR-E8 and Aggrewell media (STEMCELL Technologies) was used to generate EBs. Cells were incubated in the media mixture for 24 hours at 5% CO_2, 37°C. Media were changed daily with fresh E8 media for 3 days. On day 4, EBs were transferred to E7 media. EBs were maintained for additional 3 days. Gelatin-coated dished were prepared for OG cell induction. Culture dishes were coated with 0.1% gelatin for 30 minutes and completely dried. EBs were

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harvested and resuspended in OG induction media consisted of Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific), 20% Fetal bovine serum (FBS, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific). EBs were counted and 50-70 EBs per cm^2 were seeded onto a gelatin-coated dish. OG cells were induced from the attached EBs for 3 days at 5% CO_2, 37°C. Next, cells were detached and remaining EB clumps were removed using a 40 µm cell strainer (BD Technologies, Franklin Lakes, NJ, USA). Single OG cells were harvested and plated onto a new gelatin-coated dish (1-5 x 10^4 cell per cm^2). Cells were used up to passage 5.

4.4 Minicircle transfection

OG cells were seeded onto a gelatin-coated plate. On the day before transfection, culture media were changed to DMEM without serum and antibiotics. Cells were transfected with the minicircle plasmids using Lipofectamine 2000 reagent (Thermo Fisher Scientific) following the manufacturer's instruction. Briefly, plamid DNA and lipofectamine were mixed in Opti-MEM (Thermo Fisher Scientific) for 20 minutes. The DNA-lipid mixture was added to the culture media and incubated for 6 hours at 5% CO_2, 37°C. Media were changed into OG induction media and incubated overnight. On the next day, the expression of RFP in transfected OG cells was measured using fluorescence microscopy. The total cells and transfected cells were counted using the Image J program and the percentage of transfected cells was evaluated.

4.5 Enzyme-linked immunosorbent assay

The amount of BMP2 and TGFβ3 was detected in the cultured media. The cultured media of transfected 293T cells were harvested 72 hours after transfection. Wells of a 96-well plate was coated with the cultured media and incubated overnight in 4°C. Recombinant human BMP2 and TGFβ3 (R&D Systems, Minneapolis, MN, USA) were used as standards. Plates were washed and BMP2 and TGFβ3 antibodies (Abcam, Cambridge, MA, USA) were added. The plate was incubated in room temperature for 2 hours. Plates were washed and applied with the HRP-conjugated secondary antibodies for 1 hour at room temperature. 1x TMB solution (eBioscience, San Diego, CA, USA) was added and incubated for 10 minutes. After stopping the reaction, plates were read at 450

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nm.

4.6 Osteogenic differentiation

OG cells were seeded onto a gelatin-coated plate at $5x10^4$ cells/cm^2. Cells were maintained in osteogenic differentiation media (DMEM supplemented with 15% FBS, 50 µg/ml ascorbate-2-phosphate, 10 nmol/l dexamethasone, and 10 mmol/l β-glycerophosphate) for 21 days with media changed every other day. Osteogenesis was confirmed by alizarin red staining using a commercial kit (Sigma Aldrich, St. Louis, MO, USA).

4.7 Adipogenic differentiation

OG cells were seeded onto a gelatin-coated plate at $5x10^4$ cells/cm^2. Cells were maintained in adipogenesis differentiation media (Thermo Fisher Scientific) for 21 days with media changed every other day. Adipogenesis was confirmed by staining with oil red O (Sigma Aldrich).

4.8 Chondrogenic differentiation using pellet culture

Minicircle-transfected OG cells were counted and 3 x 10^5 cells per pellet were prepared. Cells were harvested in a 15 mL conical tube and media were changed into chondrogenic differentiation media (CDM; DMEM supplemented with 20% knockout serum replacement, 1x non-essential amino acids, 1 mM L-glutamine, 1% sodium pyruvate, 1% ITS+ Premix, 10^{-7} M dexamethasone, 50 mM ascorbic acid, 40 µg/mL L-proline) without additional recombinant growth factors. Cells resuspended in CDM were centrifuged at 750 xg for 5 minutes. Generated pellets were maintained for 30 days and media were changed every 3 days. As a positive control, pellets were supplemented with 10 ng/mL recombinant human TGFβ3 and 50 ng/mL recombinant human BMP2 as described in our previous work [5].

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4.9 Polymerase chain reaction

Harvested chondrogenic pellets were stored at -80°C before use. Samples were snap-frozen with liquid nitrogen and ground using a pestle. Ground chondrogenic pellet samples were incubated with Trizol (Thermo Fisher Scientific) and mRNA was extracted. RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used to synthesize cDNAs from the extracted RNAs. Mean cycle threshold values from triplicate experiments were used to calculate the gene expression normalized to GAPDH as an internal control. Real-time PCR was carried out using LightCycler® 480 Instrument II (Roche, Basel, Switzerland).

4.10 Ethics

All procedures involving animals were 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 Experimentation provided by the Institutional Animal Care and Use Committee of the College of Medicine of the Catholic University of Korea. This study protocol was approved by the Institutional Review Board of The Catholic University of Korea (CUMC-2016-0226-01).

4.11 Osteochondral defect model

Sprague-Dawley rats were anaesthetized. An osteochondral defect (1.5 mm x 1.5 mm x 1.5 mm) was created using a microdrill on the articular cartilage of the trochlear groove of the distal femur. After 10 days of *in vitro* differentiation, chondrogenic pellets were placed in the defect. The arthrotomy and skin were closed with interrupted nylon sutures. After 4 weeks, rats were sacrificed for gross and histological analysis. The recovery was examined using the International Cartilage Repair Society (ICRS) scoring system.

4.12 Histological analysis

Chondrogenic pellet samples or rat joint samples were washed with phosphate-buffered saline (PBS). Samples

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were fixed in 4% paraformaldehyde for 2 hours at room temperature (RT). Dehydration was performed with increasing sequential ethanol solutions. Additional clearing was done with sequential ethanol-zylene mixtures and samples were infilterated with paraffin overnight. Paraffin blocks were fixed and 7 µm sections were obtained using a microtome. Before staining the sections, slides were placed in a 60°C oven for at least 10 minutes. Slides were immediately deparaffinized using zylene. Slides were rehydrated with decreasing sequential ethanol series and were rinsed with running tap water for 1 minute each. For alcian blue staining, slides were incubated in 1% alcian blue solution (Sigma Aldrich) for 30 minutes at RT. Slides were washed with running tap water and counterstained with nuclear fast red solution. For safranin O staining, slides were stained with Weigert's hematoxylin (Sigma Aldrich) for 10 minutes at RT. Slides were washed in running tap water for 10 minutes. Slides were stained with 0.001% fast green solution (Sigma Aldrich) and 0.1% safranin O (Sigma Aldrich) solution for 5 minutes each. Toluidine blue staining was done by incubating the hydrated slides in 0.04% toluidine blue (Sigma Aldrich) solution for 10 minutes. Slides were washed in running tap water and dried for 10 minutes until complete dryness. After the staining process, slides were dehydrated with an increasing sequential ethanol series. Ethanol was cleared with 2 cycles of 100% zylene and slides were mounted with VectaMount™ Permanent Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

4.13 Immunohistochemistry

Slides were placed in a 60°C oven for 10 minutes and deparafinized with 2 cycles of zylene. Slides were rehydrated and incubated in boiling citrate buffer (Sigma Aldrich) for antigen unmasking. After cooling the unmasked slides, endogenous peroxidase activity was blocked by treating the slides with 3% hydrogen peroxide (Sigma Aldrich). Slides were washed and blocked with tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA). Primary antibodies were diluted in blocking solution in the following ratios: collagen type I (1/200; Abcam), collagen type II (1/100; Abcam). Slides were incubated with diluted primary antibody at 4°C overnight. The next day, slides were washed with TBS containing 0.1% tween-20. Secondary antibodies (1/200; Vector Laboratories) diluted in blocking buffer was treated for 40 minutes at RT. After washing out the secondary antibody, slides were treated with ABC reagent drops (Vector Laboratories) for 30 minutes. DAB solution (Vector Laboratories) was followed and incubated for 5 minutes. Slides were washed and

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counterstained with Mayer's hematoxylin (Sigma Aldrich) for 1 minute. Slides were dehydrated and cleared. Slides were mounted, and staining was confirmed under a bright field microscope.

4.14 Statistical analysis

The results are shown as mean and standard error of the mean. Error bars represent the standard error of the mean. Statistical analysis was performed and graphs were drawn using GraphPad Prism 5 (GraphPad). T-test was applied to analyze non-parametric quantitive datasets, and the one-tailed p-value was calculated. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ indicated statistical significance.

5. Conclusions

We conclude that the minicircle encoding human BMP2 and TGFβ3 induced chondrogenesis in hiPSC-derived OG cells. Pellets generated with OG cells transfected with both mcBMP2 and mcTGFβ3 had the highest efficacy of differentiation. Differentiation using human growth factors secreted from minicircles suggests a new regeneration process that can offer *in vitro* tissue generation. Especially in combination with hiPSCs, this study suggests a new strategy for future regenerative medicine.

SUPPLEMENTARY MATERIALS

This study

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AUTHOR CONTRIBUTIONS

YAR designed and performed the experiment. NP and YAR generated the growth factor encoding minicircle vectors. YN performed the chondrogenic pellet related experiment and analyzed the data. YAR, NP, YJ analyzed the results. YAR and JHJ wrote the manuscript. JHJ supervised the overall study.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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