Video Article

Chondrogenic Differentiation Induction of Adipose-derived Stem Cells by Centrifugal Gravity

Yeonsue Jang[1], Hyerin Jung[1], Ji Hyeon Ju[1]

[1]CiSTEM Laboratory, Convergent Research Consortium for Immunologic Disease, Division of Rheumatology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea

Correspondence to: Ji Hyeon Ju at juji@catholic.ac.kr

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Abstract

Impaired cartilage cannot heal naturally. Currently, the most advanced therapy for defects in cartilage is the transplantation of chondrocytes differentiated from stem cells using cytokines. Unfortunately, cytokine-induced chondrogenic differentiation is costly, time-consuming, and associated with a high risk of contamination during *in vitro* differentiation. However, biomechanical stimuli also serve as crucial regulatory factors for chondrogenesis. For example, mechanical stress can induce chondrogenic differentiation of stem cells, suggesting a potential therapeutic approach for the repair of impaired cartilage. In this study, we demonstrated that centrifugal gravity (CG, 2,400 × g), a mechanical stress easily applied by centrifugation, induced the upregulation of sex determining region Y (SRY)-box 9 (SOX9) in adipose-derived stem cells (ASCs), causing them to express chondrogenic phenotypes. The centrifuged ASCs expressed higher levels of chondrogenic differentiation markers, such as aggrecan (ACAN), collagen type 2 alpha 1 (COL2A1), and collagen type 1 (COL1), but lower levels of collagen type 10 (COL10), a marker of hypertrophic chondrocytes. In addition, chondrogenic aggregate formation, a prerequisite for chondrogenesis, was observed in centrifuged ASCs.

Video Link

The video component of this article can be found at https://www.jove.com/video/54934/

Introduction

Defects in articular cartilage do not heal naturally. Consequently, stem cell transplantation has been proposed as a promising approach for the repair of impaired cartilage. However, this method requires both the acquisition of a sufficient number of stem cells and the induction of these cells to undergo chondrogenic differentiation. Bone marrow (BM) has been widely used as a source of stem cells, but cell isolation from BM has two major disadvantages: invasiveness and insufficient yield. Because of its ease of acquisition, adipose tissue is a preferable source of stem cells. Previous studies demonstrated the feasibility of isolating stem cells from adipose tissue and inducing chondrogenic differentiation in these cells using cytokines, such as TGF-β1[1,2]. These methods are effective but expensive.

As a lower-cost alternative to cytokines, mechanical stress can be used to induce chondrogenic differentiation. Mechanical loading plays a critical role in maintaining the health of articular cartilage[3], and it can induce chondrogenic phenotypes in various cells. For example, hydrostatic pressure induces chondrogenic phenotypes in synovium-derived progenitor cells via the MAP kinase/JNK pathway[4], and mechanical compression induces chondrogenesis in human mesenchymal stem cells (MSCs) by upregulating chondrocytic genes[5]. In addition, shear stress contributes to the expression of chondrogenesis-related extracellular matrix (ECM) in human MSCs[6]. Centrifugal gravity (CG), an easily applied and controlled mechanical stress generated by centrifugation, can induce differential gene expression in cells[7]. For example, in lung epithelial carcinoma cells, the expression of interleukin (IL)-1b is upregulated by centrifugation[8]. Therefore, as an experimentally inducible mechanical stress, CG can be used to induce chondrocytic gene expression in stem cells. However, it remains unclear whether CG can induce the chondrogenic differentiation of stem cells.

In this study, we found that CG induced the upregulation of SOX9, a master regulator of chondrogenesis, in human ASCs, resulting in the overexpression of chondrocytic genes. In addition, we compared the effects of CG on chondrogenesis with those of TGF-β1, the growth factor most commonly used to induce *in vitro* chondrogenesis in stem cells.

Protocol

This study protocol was approved by the institutional review board of The Catholic University of Korea (KC16EAME0162) and performed according to NIH guidelines. All tissues were obtained with written informed consent.

1. Centrifugal Gravity Loading and Pellet Culture

1. Cell culture and harvest
    1. Culture ASCs (P2-P3; see List of Materials) in Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C in a humidified incubator containing 5% CO_2.
    2. When the cells reach 80% confluence, discard the medium and wash the cells with 5 mL of 1x phosphate-buffered saline (PBS).
    3. Add 1 mL of PBS containing 1 mM EDTA and incubate for 2 min at 37 °C in a humidified incubator containing 5% CO_2.
    4. Tap the culture plate gently, add 4 mL of fresh medium, transfer the cells to a 15-mL conical tube, and centrifuge the cells at 250 × g for 2 min at room temperature (RT).

2. Centrifugal gravity loading
    1. After centrifugation (step 1.1.4), remove the supernatant without disturbing the pellet and resuspend the pellet in 10 mL of DMEM-LG with 10% FBS. Count the cells using a hemocytometer.
    2. For CG loading, transfer 2.5 × 10^5 cells to a new 15-mL conical tube and centrifuge at 2,400 × g for 15 min.

3. Pellet culture
    1. Immediately after centrifugation, aspirate the supernatant and add 500 µL of defined chondrogenic differentiation medium (CDM, high-glucose DMEM supplemented with 1% FBS, 1% ITS+Premix, 100 nM dexamethasone, 1x MEM non-essential amino acid solution, 50 µg/mL L-proline, and 1% penicillin/streptomycin). Add 50 µg/mL of freshly prepared L-ascorbic acid 2-phosphate at every medium change. As a positive control, induce chondrogenic differentiation in uncentrifuged cells by adding CDM containing 10 ng/mL TGF-β1.
    2. Place the loosely capped tubes containing the pellets in a standing position and incubate at 37 °C in a humidified incubator containing 5% CO_2.
    3. Change the medium every other day for 3 weeks.

4. Micromass culture
    1. Immediately after centrifugation (step 1.2.2; 2,400 × g for 15 min), aspirate the supernatant and resuspend the pellet in 10 µL of CDM.
    2. To form a micromass, place the resuspended cells in the center of a well of a 24-well plate.
    3. After 2 h, carefully add 1 mL of CDM to the well, pipetting against the wall of the plate to avoid disrupting the micromass. As a positive control, perform a micromass culture with uncentrifuged cells by adding CDM containing 10 ng/mL TGF-β1.
    4. Incubate the micromass at 37 °C in a humidified incubator containing 5% CO_2.
    5. Change the medium every other day for 3 weeks.

2. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) to Detect Transcriptional Upregulation of Chondrogenic Differentiation Markers

1. On day 14, use a pipette to transfer the spheroid pellets to a new 1.5-mL tube and wash them in 1x PBS.
2. Extract the total RNA from the pellets using the guanidinium thiocyanate-phenol-chloroform extraction method[9].
3. Synthesize cDNA from 2 µg of total RNA using reverse transcriptase according to the manufacturer's protocol (incubate at 42 °C for 1 h and then inactivate the enzyme at 70 °C for 5 min).
4. Perform PCR with primers specific for chondrogenic differentiation markers[10].

3. Staining to Detect the Overexpression of Chondrogenic Differentiation Marker Proteins

1. Paraffin-embedding cell pellets
    1. On day 21, use a pipette to harvest the spheroid pellets and wash them with 1x PBS.
    2. Fix the spheroid pellets by immersion in 4% paraformaldehyde for 24 h.
    Caution: Paraformaldehyde is highly toxic. Avoid contact with eyes, skin, or mucous membranes. Minimize exposure and avoid inhalation during preparation. Wear appropriate personal protective equipment.
    3. Discard the fixative and rinse the cell pellets with deionized water (DW).
    4. Place one layer of gauze onto the cassette and transfer the fixed pellets using a pipette. Cover the pellet by folding the gauze and close the cassette lid.
    5. Dehydrate the pellets.
        1. Immerse the pellets in 70% ethanol (EtOH) for 5 min at RT.
        2. Immerse the pellets in 80% EtOH for 5 min at RT.
        3. Immerse the pellets in 95% EtOH for 5 min at RT.
        4. Immerse the pellets in 100% EtOH for 5 min at RT. Repeat twice.
        5. Immerse the pellets in 100% xylene for 15 min at RT. Repeat twice.
    6. Embed the fixed cell pellets in paraffin at 56 °C in a mold; the pellets can be embedded into paraffin using specialized automated tissue processing systems.
    7. Cut 5 µm-thick sections from the paraffin-embedded cell pellet using a rotary microtome.
    8. Float the sections in 50% EtOH and then transfer them to a 50 °C water bath using a slide.
    9. Place the floating sections onto slides.

2. Safranin O and Alcian blue staining
    1. Rehydrate the paraffin sections.
        1. Immerse the slides in xylene for 15 min. Repeat twice.

2. Immerse the slides in 100% EtOH for 5 min. Repeat twice.
3. Immerse the slides in 90% EtOH for 5 min.
4. Immerse the slides in 80% EtOH for 5 min.
5. Immerse the slides in 70% EtOH for 5 min, and then wash them in 1x PBS.

2. Stain the rehydrated sections with 1% Safranin O solution and 3% Alcian blue for 30 min.
3. Discard the staining solution and rinse the sections with DW.
4. Stain the sections with Hematoxylin/Eosin solution (counterstain) for 1 min.
5. Discard the staining solution and rinse the sections with DW.
6. Place a drop of mounting medium on a coverslip after removing any residual water. Carefully place the slide (cell-side down) on the coverslip containing the mounting medium.
7. Allow the slides to dry for 2-3 h at RT.
8. Capture images using a bright-field microscope (automated upright microscope, 50X and 200X).

3. Immunofluorescence assay
   1. Rehydrate the sections as described in section 3.2.1.
   2. Immerse the slides in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), and then maintain them at a sub-boiling temperature for 10 min using a microwave.
   3. Cool the slides for 20 min at RT and then wash them with tap water.
   4. Incubate the slides in 3% H_2O_2 (in 1x PBS) for 15 min, and then wash them with tap water for 15 min[11].
   5. Block the slides with blocking buffer (10% normal goat serum in PBS) for 1 h.
   6. Aspirate the blocking solution, and then apply a primary antibody diluted with 5% normal goat serum onto the slides.
   7. Incubate the slides overnight at 4 °C.
   8. Wash the slides three times in 1x PBS for 5 min each.
   9. Incubate the slides in fluorochrome-conjugated secondary antibody diluted with 5% normal goat serum for 1 h at RT in the dark.
   10. Wash the slides three times in 1x PBS for 5 min each in the dark.
   11. Incubate the slides with DAPI (1 µg/mL) for 10 min, and then wash them two times in 1x PBS for 5 min each.
   12. Place a drop of mounting medium on a coverslip after removing any residual water. Carefully place the slide (cell-side down) on the mounting medium.
   13. Allow the slides to dry for 2-3 h in the dark at RT.
   14. Visualize the slides using fluorescence microscopy (Rhod: 594 nm, DAPI: 340 nm; 60X and 200X magnification)[12].

Representative Results

Centrifugal gravity induces the overexpression of chondrogenic differentiation markers in adipose-derived stem cells.

To determine the degree of centrifugal gravity force that is suitable to induce chondrogenic differentiation, ASCs were stimulated with different degrees of CG (0, 300, 600, 1,200, and 2,400 x g) for 15 min. After stimulation, the ASCs were re-seeded onto culture plates and cultured for 24 h. As shown in **Figure 1A**, SOX9 mRNA expression was significantly increased at 2,400 x g; it was approximately 1.5-fold higher at 2,400 x g than in the control (ASCs not loaded with CG). To confirm whether CG induces chondrogenic differentiation, we stimulated ASCs by CG loading or by TGF-β1 treatment. As a negative control, ASCs were cultured without any stimulation. Samples were harvested on day 14 and qRT-PCR was performed. As shown in **Figure 1**, *COL2A1* mRNA, a representative chondrogenic differentiation marker, was upregulated in ASCs stimulated by CG relative to control cells (the unstimulated ASCs). The upregulation level of *COL2A1* mRNA by CG stimulation was similar to that induced by the TGF-β1 treatment. On the other hand, *COL10*, a marker of hypertrophic chondrocytes, was downregulated by CG stimulation. *ACAN* mRNA, which encodes a major structural component of articular cartilage, was upregulated approximately 2-fold by CG stimulation, whereas *COL1* was not detectably elevated relative to controls. Neither *ACAN* nor *COL1* were upregulated in ASCs treated with TGF-β1.

Centrifugal gravity induces the expression of a chondrogenesis-related extracellular matrix in the pellet culture of adipose-derived stem cells.

Deposition of ECM, such as glycosaminoglycan, is a hallmark phenotype of chondrogenic differentiation. To detect this material, we stained pellet-cultured ASCs with Safranin O and Alcian blue on day 21. As shown in **Figure 2A**, ASCs stimulated with CG deposited more glycosaminoglycan (red for Safranin O and blue for Alcian blue) than controls, at a level similar to that deposited by ASCs treated with TGF-β1. In these experiments, positive staining indicated the formation of a cartilage matrix. For further confirmation, we monitored the expression of COL2A1 protein using the PE-conjugated anti-COL2A1 antibody. COL2A1 was overexpressed in ASCs stimulated with CG to a slightly greater extent than in ASCs treated with TGF-β1 (**Figure 2B**).

Chondrogenic aggregate formation is induced by centrifugal gravity in a micromass culture of adipose-derived stem cells.

The formation of chondrogenic aggregation (or condensation) is a prerequisite for chondrogenic differentiation[13]. Therefore, we compared condensation in the micromass between cultures of control ASCs and ASCs stimulated with CG or TGF-β1. As shown in **Figure 3**, larger and denser aggregations of ASCs (white-colored in the dotted square) were detected in micromass cultures made of ASCs stimulated with CG or TGF-β1 than in control cultures.

SOX9 is upregulated by centrifugal gravity in adipose-derived stem cells.

Sox9 is a master regulator of chondrogenic differentiation[14,15]. To determine whether CG induces the upregulation of Sox9 in ASCs, we monitored the expression of *SOX9* mRNA in ASCs exposed to various durations of CG stimulation (2,400 × g). *SOX9* expression was investigated at various degrees of CG, but it did not differ significantly among CG conditions with forces greater than 2,400 × g (data not shown). Expression of *SOX9* mRNA began to increase after 15 min of CG stimulation, and it was saturated after 30 min (**Figure 4A**). Next, to determine how long CG-induced Sox9 overexpression can be maintained, we harvested CG-stimulated ASCs at the indicated time points and monitored the expression of Sox9. As shown in **Figure 4B**, the expression of Sox9 protein increased until 3 h after CG stimulation and remained at that level for at least 12 h.

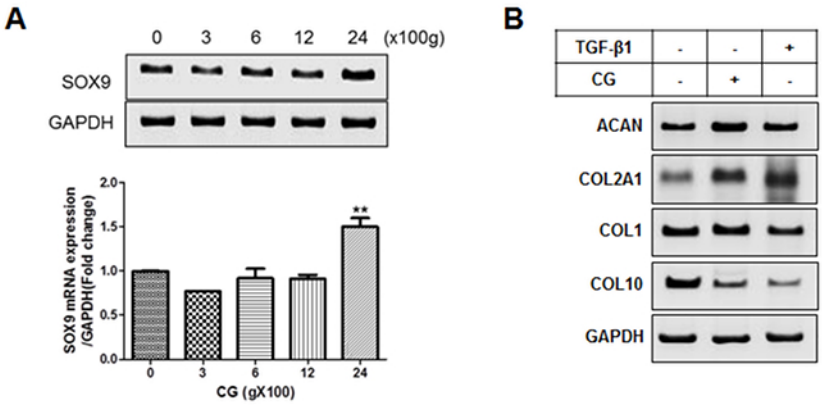

Figure 1. Centrifugal gravity induces the upregulation of chondrogenic differentiation markers in adipose-derived stem cells. A) To determine a suitable centrifugal gravity force, ASCs were centrifuged at different CG (0, 300, 600, 1,200, and 2,400 x g) for 15 min. SOX9 mRNA expression was evaluated at 24 h after CG stimulation. **B**) To evaluate the expression of chondrogenesis-related genes, total RNA was extracted from ASCs that had been centrifuged, treated with TGF-β1 (10 ng/mL), or unstimulated (controls). The RNA was subjected to reverse transcription to synthesize cDNA, which was then amplified by RT-PCR. All experiments were performed at least three times. Error bars represent standard deviation. **p<0.01 for ASCs stimulated with CG vs. the control (non-stimulated ASCs).

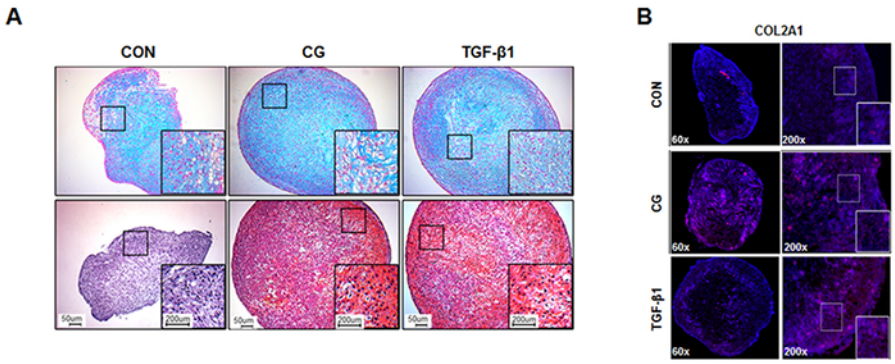

Figure 2. Centrifugal gravity induces a chondrogenesis-related extracellular matrix in pellet cultures of adipose-derived stem cells. **A**) Proteoglycan matrix formation by CG loading. **B**) CG-induced overexpression of collagen type 2. For CG loading, ASCs were centrifuged at 2,400 × g for 15 min. The overexpression of chondrogenesis-related ECM was evaluated in spheroid pellets consisting of centrifuged ASCs through the use of Safranin O and Alcian blue staining. Overexpression of collagen type 2 was confirmed by immunofluorescence using an antibody against collagen type 2 and an Alexa Fluor 594-conjugated secondary antibody (Rhod: 594 nm, DAPI: 340 nm). To determine the effect of CG on chondrogenesis-related ECM overexpression, ASCs were treated with TGF-β1 (10 ng/mL) as a positive control. The negative control was a pellet culture not subjected to CG loading or TGF-β1 treatment. Please click here to view a larger version of this figure.

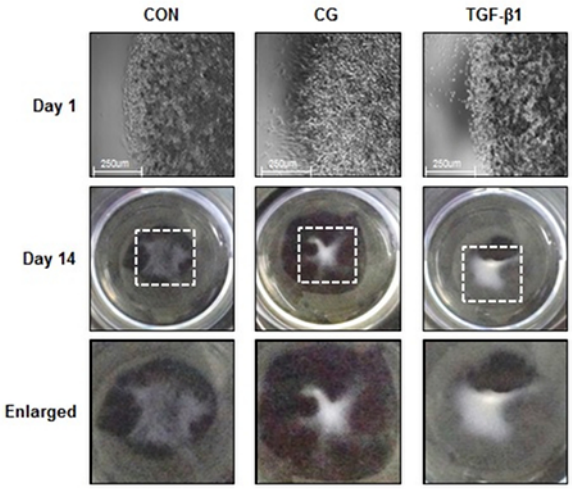

Figure 3. Chondrogenic aggregate formation is induced by centrifugal gravity in micromass cultures of adipose-derived stem cells. To induce chondrogenic differentiation, ASCs (2.5×10^5) were stimulated by centrifugation at 2,400 × g or by treatment with TGF-β1 (10 ng/mL). Unstimulated ASCs were used as controls. To form micromasses, cells (2.5×10^5/10 µL) were placed at the center of wells in a 24-well plate. After 2 h of incubation, fresh medium-CDM for controls and centrifuged ASCs and CDM containing TGF-β1 (10 ng/mL) for ASCs treated with TGF-β1-was added to the wells, and the samples were incubated for 14 days. Chondrocyte condensation was evaluated by the size of the aggregates.

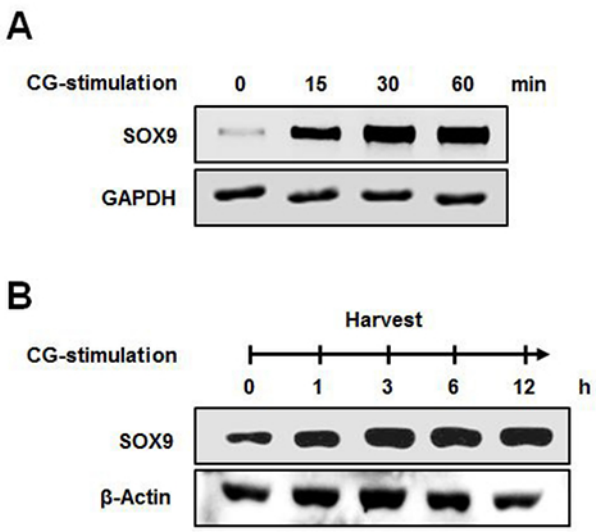

Figure 4. SOX9 is upregulated by centrifugal gravity in adipose-derived stem cells. A) Upregulation of *SOX9* mRNA by CG stimulation for various intervals of time. **B**) Sox9 protein expression in CG-stimulated ASCs at different time points. ASCs were allowed to grow as a monolayer for 24 h after CG loading. mRNA upregulation and the protein expression of Sox9 were confirmed by RT-PCR and Western blotting, respectively.

Discussion

The stemness state of cells is very important for CG-induced overexpression of SOX9. In our study, SOX9 expression could be induced by CG in early-passage ASCs (2-3), but not in later-passage ASCs. It has been reported that, during cultivation, ASCs contain CD34+ cells until 3 passages[16]. ASCs tend to lose the expression of CD34 as the cells are passaged, resulting in a low response to CG.

With centrifugal gravity force, hydrostatic pressure can be loaded onto cells during CG stimulation. Therefore, the volume of medium may be one of the factors that affect the induction of SOX9. To maintain an even hydrostatic pressure environment for each experiment, 1 mL of medium was used per 1×10^5 cells in a 15-mL tube. In addition, a swing-bucket rotor was used to evenly apply CG to the cells (by forming a right angle).

Compared to TGF-β1, CG showed a lower capability to induce SOX9 and chondrogenic marker expression in ASCs. This may be a limitation of this technique. For a clinical application, it should be further investigated whether CG-stimulated ASCs regenerate impaired cartilage up to the level of normal function in an *in vivo* model.

In vitro culture with growth factors, especially TGF-β1, is the most effective method to induce chondrogenic differentiation of stem cells[17]. This method is known to be useful for cell enrichment and chondrogenic differentiation induction. However, early senescence and unexpected lineage differentiation often occur during *in vitro* culture[18,19]. More serious is the high risk of contamination from *in vitro* cultures. On the other hand, our

method does not require *in vitro* chondrogenic differentiation with growth factors. Stem cells can be transplanted into a patient immediately after isolation and following centrifugation, which may guarantee a low contamination risk and a shortened processing time. The microenvironment around stem cells is critical for chondrogenic differentiation induction. Unexpected lineage differentiation may result from an improper *in vitro* environment. As mentioned in our method, because centrifuged stem cells can be transplanted into the cartilage of a patient (the proper environment for chondrogenic differentiation) without an additional differentiation process, unexpected lineage differentiation may be decreased.

Here, we present a protocol that uses CG to induce the chondrogenic differentiation of ASCs. Our results show that CG induces Sox9 upregulation and chondrogenic differentiation phenotypes in ASCs, including COL2A1 overexpression, more extensive ECM deposition, and chondrogenic aggregate formation. Based on our results, the ASCs exposed to CG are a good substitute for the MSCs treated with TGF-β1 and may therefore be used in transplantations for cartilage regeneration.

Disclosures

We declare that we have no conflicts of interest associated with this work.

Acknowledgements

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