Video Article

Differentiating Chondrocytes from Peripheral Blood-derived Human Induced Pluripotent Stem Cells

Yueying Li[2], Yong Hai[1], Jiayu Chen[3], Tie Liu[1]

[1]Department of Orthopedics, Beijing Chao-Yang Hospital, Capital Medical University

[2]Key Laboratory of Genomic and Precision Medicine, Beijing Institute of Genomics, Chinese Academy of Sciences

[3]Clinical and Translational Research Center of Shanghai First Maternity & Infant Hospital, School of Life Sciences and Technology, Tongji University

Correspondence to: Tie Liu at waterkins@163.com

URL: https://www.jove.com/video/55722
DOI: doi:10.3791/55722

Keywords: Developmental Biology, Issue 125, induced pluripotent stem cells, peripheral blood, differentiation, embryoid body, fibroblastic cells, chondrocyte

Date Published: 7/18/2017

Citation: Li, Y., Hai, Y., Chen, J., Liu, T. Differentiating Chondrocytes from Peripheral Blood-derived Human Induced Pluripotent Stem Cells. *J. Vis. Exp.* (125), e55722, doi:10.3791/55722 (2017).

Abstract

In this study, we used peripheral blood cells (PBCs) as seed cells to produce chondrocytes via induced pluripotent stem cells (iPSCs) in an integration-free method. Following embryoid body (EB) formation and fibroblastic cell expansion, the iPSCs are induced for chondrogenic differentiation for 21 days under serum-free and xeno-free conditions. After chondrocyte induction, the phenotypes of the cells are evaluated by morphological, immunohistochemical, and biochemical analyses, as well as by the quantitative real-time PCR examination of chondrogenic differentiation markers. The chondrogenic pellets show positive alcian blue and toluidine blue staining. The immunohistochemistry of collagen II and X staining is also positive. The sulfated glycosaminoglycan (sGAG) content and the chondrogenic differentiation markers *COLLAGEN 2* (*COL2*), *COLLAGEN 10* (*COL10*), *SOX9*, and *AGGRECAN* are significantly upregulated in chondrogenic pellets compared to hiPSCs and fibroblastic cells. These results suggest that PBCs can be used as seed cells to generate iPSCs for cartilage repair, which is patient-specific and cost-effective.

Video Link

The video component of this article can be found at https://www.jove.com/video/55722/

Introduction

Cartilage tissue has a very poor capacity for self-repair and regeneration. Various surgical interventions and biological treatments are used to restore cartilage and joint function, with unsatisfying results. The recent development of stem cell technology may change the entire cartilage repair field[1]. Various stem cells have been studied as seed cells, but human induced pluripotent stem cells (hiPSCs) appear to be the most promising choice, as they can provide many types of patient-specific cells without causing rejection reactions[2]. Furthermore, they can overcome the limited proliferative nature of adult cells and maintain their self-renewal and pluripotent abilities. Moreover, gene targeting can be used to change the genotype to obtain specific types of chondrocytes.

Fibroblasts have been widely used to generate iPSCs because their reprogramming potentials have also been well studied. However, there are still some limitations that must be overcome, such as the painful biopsy from patients and the need for the *in vitro* expansion of the fibroblasts, which may result in gene mutations[3]. Recently, PBCs were found to be advantageous for reprogramming[4]; moreover, they were commonly utilized and abundantly stored. It is possible that they may redirect study focus from the skin. However, to the best of our knowledge, there are few reports on PBC reprogramming followed by differentiation into chondrocytes.

In the current study, we utilize PBCs as an alternative source by reprogramming them into iPSCs and then differentiating the iPSCs into the chondrogenic lineage through a pellet culture system in order to mimic chondrocyte formation.

Protocol

The protocol for the generation of hiPSCs from PBCs can be found in our previous study[5]. The study was approved by the Institutional Review Board of our institution.

1. Embryoid Body (EB) Formation

1. Make 50 mL of hiPSC medium: Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% knockout serum replacement (KSR), 5% fetal bovine serum (FBS), 1× nonessential amino acids, 55 μM 2-mercaptoethanol, 2 mM L-glutamine, and 8 ng/mL basic fibroblast growth factor (bFGF).
2. Make 50 mLof EB formation medium: DMEM supplemented with 15% KSR, 5% FBS, 1x nonessential amino acids, 55 μM 2-mercaptoethanol, and 2 mM L-glutamine.
3. Make 50 mL of basal culture medium: DMEM supplemented with 20% FBS, 1× nonessential amino acids, 55 μM 2-mercaptoethanol, and 2 mM L-glutamine.
4. Prepare 10 mL of dispase solution, 1 mg/mL in knockout DMEM.
5. Culture hiPSCs onto 60-mm tissue culture dishes with feeder cells (*i.e.,* a monolayer of irradiated mouse embryonic fibroblast cells). When the cells are 80-90% confluent, disassociate the cells with dispase and passage the hiPSCs 1:3 every 4-5 days. Place the cells into a 37 °C and 5% CO_2 incubator.
6. **Dissect the undifferentiated hiPSC colonies into smaller pieces (about 50-100 μm in diameter) using a fire-drawn glass needle when the iPSCs are 80-90% confluent. Generally, use hiPSC colonies in a 60-mm dish to generate EBs in a 100 mm Petri dish.**
    1. Culture less than 100 small pieces of colonies in a 100 mm, non-adherent Petri dish containing 10 mL of EB formation medium. Place the dishes into a 37 °C and 5% CO_2 incubator.
7. Replace approximately 25% of the initial medium with an equal amount of the basal culture medium every 2 days. Tilt the dish to let the EBs settle. Carefully remove 3 mL of upper medium and add 4 mL of fresh basal culture medium. Do not disturb the EBs.
   NOTE: The EBs are morphologically characterized by the pieces of colonies, taking on a round appearance with smooth borders under the microscope.
8. After 10 days of culture in the non-adherent Petri dish, coat a new 100-mm tissue culture dish with 4 mL of 0.1% gelatin for 30 min at 37 °C before use.
9. Transfer the medium plus EBs from a 100 mm, non-adherent Petri dish to a 15-mL conical tube. Let the EBs sediment for 4-5 min. Aspirate the supernatant carefully and leave less than 0.5 mL of medium plus EBs
10. Seed less than 100 EBs onto a 100 mm, gelatin-coated tissue culture dish with 10 mL of basal culture medium. Place the dishes into a 37 °C and 5% CO_2 incubator.

2. Cell Pellet Formation and Chondrocyte Differentiation

1. Make 10 mL of 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA). Make 80 mL of basal culture medium: DMEM supplemented with 20% FBS, 1x nonessential amino acids, 55 μM 2-mercaptoethanol, and 2 mM L-glutamine.
2. Make 10 mL of chondrogenic differentiation medium: DMEM (high glucose) supplemented with 10% insulin-transferrin-selenium solution (ITS), 0.1 μM dexamethasone, 1 mM ascorbic acid, 1% sodium pyruvate, and 10 ng/mL transforming growth factor-beta 1(TGF-β1).
3. **Refresh the medium with 10 mL of basal culture medium after 48 h. Thereafter, refresh the medium every three days with 10 mL of basal culture medium.**
   NOTE: After 10 days in culture, fibroblastic cell outgrowths should have expanded from the EBs.
    1. Coat the 100 mm dishes with 4 mL of 0.1% gelatin for 30 min at 37 °C before use. Discard the cell supernatant and wash the cells with Dulbecco's Phosphate-Buffered Saline (DPBS) once.
    2. Digest the cells with 3 mL of 0.25% trypsin/EDTA at 37 °C for 5 min and neutralize with 4 mL of basal culture medium.
4. Dissociate the cells into single cells by pipetting up and down 5-10 times and passing them through a 70 μm nylon mesh. Centrifuge the cell suspension at 200 x g for 5 min. Re-seed the cells on a new 100 mm, gelatin-coated tissue culture dish with 10 mL of basal culture medium.
5. Refresh the medium with 10 mL of basal culture medium after 48 h. Thereafter, refresh the medium every three days with 10 mL of basal culture medium.
   NOTE: The cells acquire a homogenous, fibroblast-like morphology.
6. **When ~90-100% confluence is reached (*i.e.,* about 5-7 days), harvest the cells with 3 mL of 0.25% trypsin/EDTA at 37 °C for 5 min. Neutralize with 4 mL of basal culture medium. Dissociate the cells into single cells by pipetting up and down 5 times. Use a hemocytometer to count the cell number.**
    1. Place 3 x 10^5 cells in a 15 mL polypropylene tube. Centrifuge at 200 x g for 5 min at room temperature (RT). Re-suspend the cells in 1 mL of chondrogenic differentiation medium.
7. Re-centrifuge the cells at 300 x g for 3 min and maintain the cells in small pellet form. Put the tube into the 37 °C and 5% CO_2 incubator for 21 days. Do not screw the lid on tightly and let the gas exchange.
8. Replace 3/4 of the culture medium every three days with fresh chondrogenic differentiation medium.
   NOTE: After 21 days in culture, hiPSC-chondrogenic pellets (hiPSC-Chon) should have formed. Human mesenchymal stem cells (MSCs), as a positive control, are also collected and cultured in the chondrogenic differentiation medium for 21 days to form chondrogenic pellets (hMSC-Chon).

3. Analysis of Chondrogenic Differentiation

1. Prepare 10 mL of 10% neutral-buffered formalin.
2. Prepare 50 mL of 0.1% alcian blue reagent and 50 mL of 1% toluidine blue reagent.
3. Prepare 1 mL of the primary antibodies: rabbit polyclonal antibodies against collagen II (1:50) or mouse monoclonal antibodies against collagen X (1:50). Also, prepare anti-rabbit or mouse secondary antibodies.
4. Make 1 mL of papain solution: 10 U/mL in PBS with 0.1 M sodium acetate, 2.4 mM EDTA, and 5 mM L-cysteine.

5. Make 100 mL dimethylmethylene blue (DMMB) dye solution: 100 µL of 16 mg/L 1,9-dimethylmethylene blue, 40 mM glycine, 40 mM NaCl, and 9.5 mM HCl; pH 3.0.
6. **Assess chondrogenic differentiation by alcian blue and toluidine blue stains of the pellet sections.**
    1. Fix one hiPSC-Chon pellet or hMSC-Chon pellet in 1 mL of 10% neutral-buffered formalin for 24 h.
    2. Transfer the pellet to 1 mL of 70% ethanol in H_2O. Dehydrate the pellet with 1 mL of a graded ethanol series (*i.e.,* 25, 50, 75, 90, 95, 100, and 100%, 3 min each).
    3. Clarify the pellet in 1 mL of 100% xylene three times. Infiltrate the pellet with paraffin for 1 h in a 65 °C oven. Embed the pellet into paraffin blocks with a 7 x 7 x 5 mm^3 base mold, following routine histological procedures[6].
    4. Make adjacent sections by microtome with a thickness of approximately 4 µm[7]. Adhere the sections onto glass slides.
    5. Dry the slides for 2 h at 60 °C in an oven. Deparaffinize the sections in three cycles (3 min each) using 100% xylene.
    6. Use a decreasing alcohol series for rehydration (*i.e.,* 100, 100, 95, 95, 70, 50, and 25% in H_2O, 3 min each) and then perform a final rinse with deionized water for 5 min.
    7. Stain the sections with 0.1% alcian blue reagent or 1% toluidine blue staining for 4-5 h and then rinse them with distilled water.
    8. Dehydrate with a graded ethanol series (*i.e.,* 25, 50, 75, 90, 95, 100, and 100%, 3 min each) followed by three consecutive steps of clarification in 100% xylene. Mount the slides and visualize them under a microscope.

7. **Perform immunohistochemistry.**
    NOTE: Additional pellet sections are further assessed by immunohistochemistry.
    1. After deparaffinization and rehydration, bring the slides to a boil in 1 mM EDTA, pH 8.0 (waterproof in water boiled by autoclave). Let them sit for 8 min at a sub-boiling temperature and then allow the slides to cool at RT.
    2. Rinse the slides with deionized water 3 times. Incubate the sections in 3% H_2O_2 solution in methanol at RT for 15 min to block endogenous peroxidase activity.
    3. Rinse the slides with deionized water and immerse them in DPBS for 5 min. Apply 50-100 µL of appropriately diluted (1:50) primary antibody to the sections on the slides and then incubate them in a humidified chamber at RT for 1 h.
    4. Wash the slides 3 times (5 min each) with DPBS. Incubate the samples with the corresponding secondary antibodies (*i.e.,* anti-rabbit or mouse) for 15 min at RT.
    5. Wash the slides 3 times (for 5 min each) with DPBS. Perform DAB detection under a microscope.
    6. Wash the slides with DPBS 3 times (2 min each). Counterstain the cell nuclei by immersing the slides in hematoxylin for 1-2 min. Dehydrate with a graded ethanol series (25, 50, 75, 90, 95, 100, and 100%; 3 min each) followed by three consecutive steps of clarification with xylene. Finally, mount the slides and visualize them under the microscope.

8. **Detect sGAG content.**
    1. Digest chondrogenic pellets in papain solution at 60 °C for 2 h.
    2. Determine the DNA content using the dsDNA assay kit and fluorometer system. Measure the sGAG content by mixing it with DMMB dye solution under measuring absorbance at 525 nm[8].
    3. Calculate the concentration of sGAG against a standard curve of shark chondroitin sulfate.

9. **Perform the real-time PCR analysis of the chondrogenic differentiation markers in hiPSC-Chon pellets.**
    1. Harvest 3-4 of the same chondrogenic pellets by adding 500 µL of ice-cold extraction reagent to one tube. Vortex thoroughly. Incubate the sample for 5 min at RT.
    2. Add 0.1 mL of chloroform. Vortex the sample for 15 s and incubate it at RT for 3 min. Centrifuge the sample for 15 min at 12,000 x g and 4 °C. Transfer the upper aqueous phase (about 250 µL) into a new 1.5 mL microcentrifuge tube.
    3. Add 25 µL of sodium acetate and 1 µL of glycogen to the sample. Precipitate the RNA by mixing it with 250 µL of isopropyl alcohol. Mix thoroughly. Incubate the sample at RT for 10 min.
    4. Centrifuge for 10 min at 12,000 x g and 4 °C. Remove the supernatant completely. Wash the RNA pellet twice with 500 µL of 75% ethanol.
    5. Centrifuge for 5 min at 7,500 x g and 4 °C. Remove all leftover ethanol and air-dry the RNA pellet for 5-10 min. Dissolve the RNA in 10 µL of nuclease-free water.
    6. Convert the RNA into cDNA using a reverse transcriptase system. Subject the cDNA samples to real-time PCR using qPCR kit master mix (2x) and a real-time PCR system[9].
        NOTE: The primer sequences are:
        h*AGGRECAN*-F:TCGAGGACAGCGAGGCC;
        h*AGGRECAN*-R: TCGAGGGTGTAGCGTGTAGAGA;
        h*β-ACTIN*-F: TTTGAATGATGAGCCTTCGTCCCC;
        h*β-ACTIN*-R: GGTCTCAAGTCAGTGTACAGGTAAGC;
        h*COL2*-F:TGGACGATCAGGCGAAACC;
        h*COL2*-R:GCTGCGGATGCTCTCAATCT;
        h*SOX9*-F:AGCGAACGCACATCAAGAC;
        h*SOX9*-R:CTGTAGGCGATCTGTTGGGG;
        h*COL10*-F: ATGCTGCCACAAATACCCTTT;
        h*COL10*-R: GGTAGTGGGCCTTTTATGCCT.

Representative Results

Chondrogenic Differentiation of hiPSCs:

EB formation medium and basal culture medium were used to differentiate the hiPSCs into the mesenchymal lineage. A multi-step culture method was used (**Figure 1**). First, the hiPSCs were spontaneously differentiated via EB formation for 10 days (D10; **Figure 2A**). Second, cells outgrew from the EBs for another 10 days (D10+10). During these two steps, the iPSCs gradually lost their original morphologies and obtained spindle-shaped morphologies (**Figure 2B**), which then changed to a fibroblastic shape after passage. Third, cells were expanded in monolayer after subculture (**Figure 2C**). Residual undifferentiated cells were excluded during this step. Then, the cells were expanded and committed to fibroblastic-like cells after 5-7 days in monolayer culture (D10+10+7). Fourth, when the hiPSC-fibroblastic-like cells (hiPSC-F) reached about 90% confluence, they were induced to differentiate into chondrocytes via a 3D pellet culture (**Figure 2D**)[10].

Characterization of hiPSC-Chon Pellets:

hiPSC-F were cultured in 15 mL polypropylene tubes in pellet for 21 days. Chondrogenic cells may assemble *in vitro* and produce a characteristic extracellular matrix when in high-density culture. At the end of culture, we could see a dense, cartilage-like aggregate, the hiPSC-Chon pellet, which was up to 2-3 mm long and 3 mm thick (**Figure 2D**). The cells were positive for alcian blue (**Figure 3A**) and toluidine blue (**Figure 3B**) staining, which indicated the successful chondrogenic differentiation of hiPSC pellets. Immunohistochemistry analysis for collagen II (**Figure 3C**) and collagen X (**Figure 3D**) further proved that the hiPSC-Chon pellets had developed a chondrocyte-like phenotype. Negative controls of immunohistochemistry for collagen II and collagen X were performed to better prove the positive staining (data not shown)[5].

sGAG analysis was also performed after chondrogenic differentiation (**Figure 3E**). sGAG contents were detected in hiPSC-Chon pellets, hiPSC-F, EBs, and the undifferentiated hiPSCs. sGAG content was significantly upregulated in hiPSC-Chon pellets than in the other groups (P <0.05). In the positive control of hMSC-Chon, the sGAG content was also significantly upregulated (P < 0.05) compared to hMSCs. However, the sGAG contents between hMSC pellets and hiPSC-Chon pellets demonstrated no difference (P >0.05).

Gene Expression of Chondrogenic Differentiation Markers:

Gene expression of the differentiation markers for the chondro-progenitor lineage (*SOX9* and *COL2*) and fully-differentiated chondrocytes (*AGGRECAN* and *COL10*) were used to characterize the phenotype of the chondrogenic pellets (**Figure 4**). In comparisons between hiPSCs, hiPSC-F, and hiPSC-Chon pellets, expressions of *COL2*, *COL10*, *SOX9*, and *AGGRECAN* were significantly upregulated in hiPSC-Chon than in the other groups (P <0.05). In the positive control of hMSC-Chon, the expression of these markers was also significantly upregulated than in hMSCs (P < 0.05). However, the gene expression between hMSC pellets and hiPSC-Chon pellets demonstrated no differences (P >0.05). In all, these results suggest a successful chondrogenic differentiation process from human iPSCs.

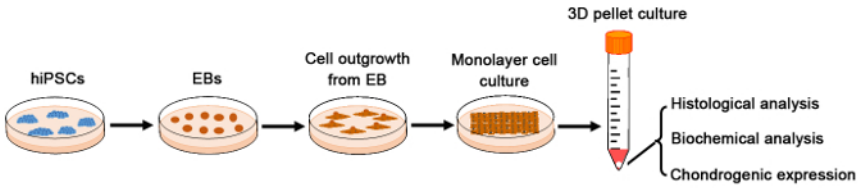

Figure 1: Schematic Overview of the Protocol. A multi-step culture method used to differentiate human iPSCs into chondrocytes, including: 1) spontaneous differentiation *via* EB formation, 2) cell outgrowth from EBs, 3) monolayer cell culture after subculture, and 4) 3D pellet culture. The chondrocyte phenotype is assessed by histological analysis, biochemical analysis, and chondrogenic gene expression. Please click here to view a larger version of this figure.

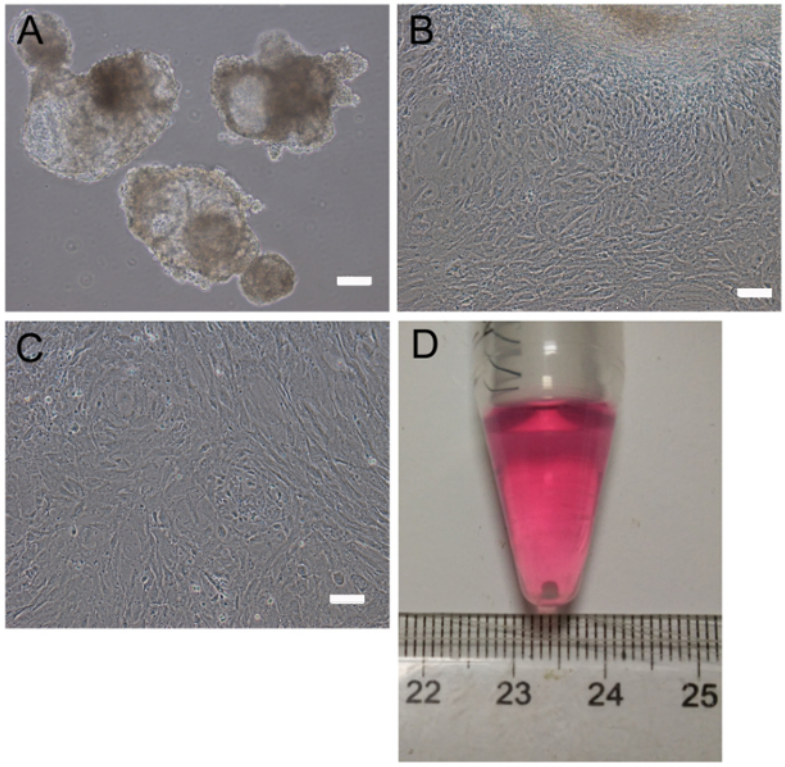

Figure 2: Generation of Chondrocytes from hiPSCs. (**A**) EB formation on D10. Scale bar = 100 µm. (**B**) Cell outgrowth from EBs on D10+10. Scale bar = 100 µm. (**C**) Monolayer cell culture on D10+10+7. Scale bar = 100 µm. (**D**) 3D pellet culture. This figure has been modified from our previous study[5]. Please click here to view a larger version of this figure.

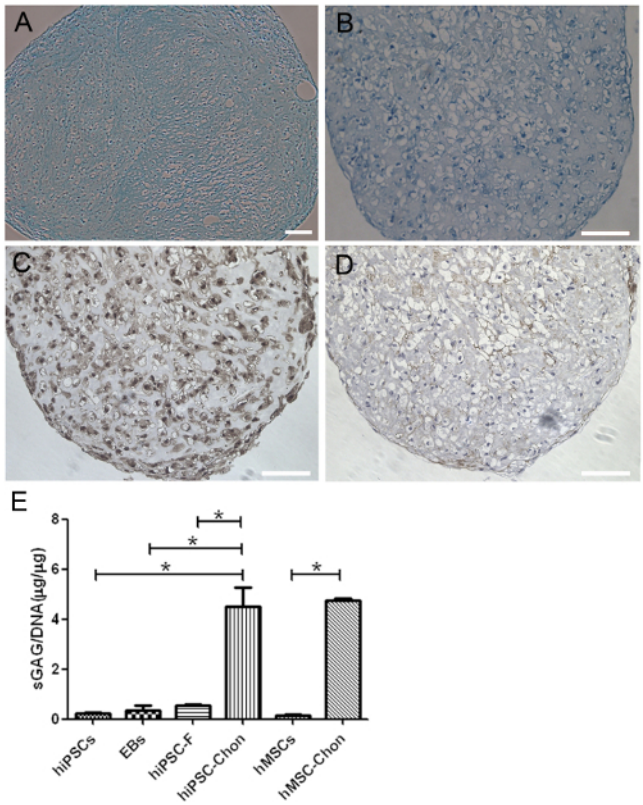

Figure 3: Characterization of hiPSC-Chon Pellets. (**A**) Alcian blue staining and (**B**) toluidine blue staining of glycosaminoglycans and proteoglycans. Scale bar = 100 µm. (**C** and **D**) Immunohistochemistry for collagen II and collagen X. Scale bar = 100 µm. (**E**) Biochemical characterization of hiPSC-Chon pellets versus hiPSCs, EBs, and hiPSC-F compared with hMSC-Chon versus hMSCs. sGAG per DNA. The bar represents the mean ± SEM. N = 3, *P < 0.05. This figure has been modified from our previous study[5]. Please click here to view a larger version of this figure.

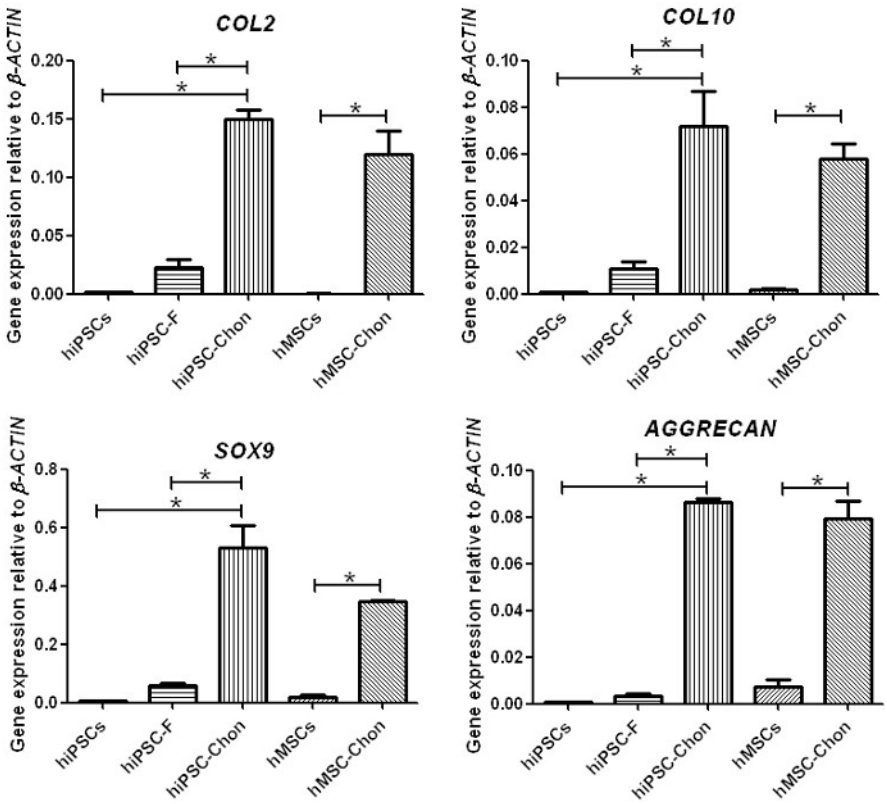

Figure 4: Gene Expression Analysis. RT-qPCR gene expression analysis of chondrogenic differentiation markers (*COL2*, *COL10*, *SOX9*,*and AGGRECAN*) in hiPSC-Chon versus hiPSCs and hiPSC-F compared with hMSC-Chon versus hMSCs. The bar represents the mean ± SEM. N = 3, *P <0.05. This figure has been modified from our previous study[5]. Please click here to view a larger version of this figure.

Discussion

Here, we provide a protocol to generate chondrocytes from PBCs via iPSCs. Because PBCs are more common and widely used in the clinical field, they are presented as a potential alternative for reprogramming. In this study, episomal vectors (EV) were utilized to reprogram PBCs into iPSCs, following the method established by Zhang *et al.*[11]. This integration-free approach does not involve integrating virus-associated genotoxicity, which is believed to have a broad effect in the clinical field[12,13]. The reprogramming efficiency of generating integration-free iPSCs from blood cells in this study was satisfied. More than 30 iPSCs could be produced from 2 mL of peripheral blood. Therefore, PBCs have the potential to be the seed cells used to generate iPSCs for cartilage engineering and other clinical applications.

The main steps of chondrogenic differentiation from hiPSCs included: EB formation, cell outgrowth from EBs, monolayer culture, and 3D pellet culture. Undifferentiated hiPSC colonies are dissected into smaller pieces using a fire-drawn glass needle. The mechanical method, although more technical, is better than enzymatic digestion (such as dispase or collagenase) because of the reduced damage and the specific size (50 - 100 µm in diameter) at acquisition. Furthermore, mechanical digestion can manually dispose of the feeder cells, which will repress the hiPSC differentiation. hiPSCs spontaneously differentiate to form EBs, which are characterized as the three-dimensional, multi-cellular aggregates with smooth borders. Several EBs can cluster together to form irregular shapes. In order to maintain the EBs in good conditions, less than 100 EBs are cultured in a 100 mm, non-adherent Petri dish, with 10 mL of EB formation medium. About 50 EBs in one 100 mm dish is thought to be the best concentration. EBs are then seeded onto 10 cm, gelatin-coated dishes with basal culture medium. The density of the distribution of EBs is important for the sufficient outgrowth of EBs. Less than 100 EBs are cultured onto a 100-mm dish. Within 10 days of culture, fibroblastic cells are gradually outgrown and expanded from the EBs. The monolayer step is performed to exclude residual undifferentiated cells present in the EBs, as well as to expand cells committed to the mesenchymal lineage. 0.5-1 x 10^6 cells are seeded in a 100-mm dish for monolayer cell culture. The expression of cell surface markers on hiPSC-F were analyzed by flow-cytometric analysis in our previous study[5]. The results showed that the majority of hiPSC-F expressed CD73 (81.81 ± 2.05%) and CD105 (endoglin; 81.90 ± 1.61%), which are known to be the positive human mesenchymal markers. Furthermore, six different iPSCs and one human embryonic stem cell (ESC) have been used to reproduce these methods.

The induced chondrogenic differentiation of pluripotent cells is also a complex key process. In view of this, classic chondrogenic medium was utilized for the induction of chondrogenesis from hiPSCs. TGF-beta1 and dexamethasone are supplemented in the pellet culture medium. These factors have been demonstrated to have a significant influence on chondrogenic potential abilities[14]. Another difference from other protocols was the concentration of 10% ITS, which is much higher than the typically reported 1% ITS[15,16]. 1% ITS plus 10% FBS enhanced cartilage formation in other methods[17,18]. ITS as a serum substitute can promote chondrocyte proliferation and formation and retain the chondrogenic phenotypes. In order to replace the animal components of FBS, we upgraded the concentration of ITS to 10%, which has been proved to efficiently promote chondrocyte differentiation[7].

A high-density cell culture is another essential factor for chondrogenic differentiation. There are many other cell culture methods that can be used to induce chondrogenic differentiation, such as micromass culture, co-culture with other cells, biomaterial-based culture, and genetic manipulation[1,19,15]. The 3D pellet culture in our study, which results in a high cell density and high cell-cell interaction, is easier to perform without other cells or materials. Since it is performed in the 15 mL centrifuge tubes, one limitation is that it can only be used in a small-scale chondrogenic differentiation assay. However, 96-well plates with round bottoms could be used as a promising alternative[7]. Therefore, other improvement to the culture methods could promote the efficiency of chondrogenic differentiation *in vitro*. In our study, the chondrogenic induction for as long as 21 days was done under serum-free and xeno-free condition, during which all animal-related components were removed. Therefore, the procedure in our study is adaptable for future clinical applications.

It is believed that autologous stem cells would be the ideal choice for cartilage repair, as they may not only decrease rejection, but also achieve tissue regeneration by taking advantage of the natural course of embryonic development[20,21]. However, they were found to have limited proliferative potential *in vitro*[22]. Therefore, an integration-free method for generating chondrocytes from PBCs via iPSCs may be a more promising approach for cartilage tissue engineering. With our method, 2 mL of blood could be enough to induce the patient-specific chondrocytes needed for cartilage defects. Moreover, we also used hMSCs as a positive control to compare with cells differentiated from iPSCs, which suggested that iPSCs have good chondrogenic differentiation potentials.

In conclusion, this study proved that PBCs can be used as candidates for chondrocyte regeneration. This could further reflect a future direction to generate seed cells for cartilage repair in a patient-specific and cost-effective approach to regenerative medicine.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors wish to thank Xiaobin Zhang for his plasmid. We also thank Shaorong Gao and Qianfei Wang for their kind help during the experiment. This study is supported by the National Natural Science Foundation of China (No.81101346, 81271963, 81100331), the Beijing 215 high-level talent project (No.2014-3-025), and the Beijing Chao-Yang Hospital Fund (No. CYXX-2017-01), and the Youth Innovation Promotion Association of Chinese Academy of Sciences (Y.L.).

References

1. Diekman, B. O. *et al.* Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells. *Proc Natl Acad Sci USA.* **109** (47), 19172-19177 (2012).
2. Park, I. H. *et al.* Disease-specific induced pluripotent stem cells. *Cell.* **134** (5), 877-886 (2008).
3. Loh, Y. H. *et al.* Generation of induced pluripotent stem cells from human blood. *Blood.* **113** (22), 5476-5479 (2009).
4. Zhang, X. B. Cellular reprogramming of human peripheral blood cells. *Genomics Proteomics Bioinformatics.* **11** (5), 264-274 (2013).
5. Li, Y. *et al.* Reprogramming of blood cells into induced pluripotent stem cells as a new cell source for cartilage repair. *Stem Cell Res Ther.* **7** (31) (2016).
6. Canene-Adams, K. Preparation of formalin-fixed paraffin-embedded tissue for immunohistochemistry. *Methods Enzymol.* **533**, 225-233 (2013).
7. Solchaga, L. A., Penick, K. J., Welter, J. F. Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks. *Methods Mol Biol.* **698**, 253-278 (2011).
8. Oldershaw, R. A. *et al.* Directed differentiation of human embryonic stem cells toward chondrocytes. *Nat Biotechnol.* **28** (11), 1187-1194 (2010).
9. Monje, L., Varayoud, J., Luque, E. H., Ramos, J. G. Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor alpha transcripts with alternative 5'-untranslated regions in the female rat preoptic area. *J Endocrinol.* **194** (1), 201-212 (2007).
10. Teramura, T. *et al.* Induction of mesenchymal progenitor cells with chondrogenic property from mouse-induced pluripotent stem cells. *Cell Reprogram.* **12** (3), 249-261 (2010).
11. Su, R. J. *et al.* Efficient generation of integration-free ips cells from human adult peripheral blood using BCL-XL together with Yamanaka factors. *PLoS One.* **8** (5), e64496 (2013).
12. Carey, B. W. *et al.* Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells. *Cell Stem Cell.* **9** (6), 588-598 (2011).
13. Okita, K. *et al.* An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. *Stem Cells.* **31** (3), 458-466 (2013).
14. Staerk, J. *et al.* Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell.* **7** (1), 20-24 (2010).
15. Qu, C. *et al.* Chondrogenic differentiation of human pluripotent stem cells in chondrocyte co-culture. *Int J Biochem Cell Biol.* **45** (8), 1802-1812 (2013).
16. Guzzo, R. M., Gibson, J., Xu, R. H., Lee, F. Y., Drissi, H. Efficient differentiation of human iPSC-derived mesenchymal stem cells to chondroprogenitor cells. *J Cell Biochem.* **114** (2), 480-490 (2013).
17. Koyama, N. *et al.* Human induced pluripotent stem cells differentiated into chondrogenic lineage via generation of mesenchymal progenitor cells. *Stem Cells Dev.* **22** (1), 102-113 (2013).
18. Liu, X. *et al.* Role of insulin-transferrin-selenium in auricular chondrocyte proliferation and engineered cartilage formation in vitro. *Int J Mol Sci.* **15** (1), 1525-1537 (2014).
19. Guzzo, R. M., Scanlon, V., Sanjay, A., Xu, R. H., Drissi, H. Establishment of human cell type-specific iPS cells with enhanced chondrogenic potential. *Stem Cell Rev.* **10** (6), 820-829 (2014).

20. Goepfert, C., Slobodianski, A., Schilling, A. F., Adamietz, P., Portner, R. Cartilage engineering from mesenchymal stem cells. *Adv Biochem Eng Biotechnol.* **123**, 163-200 (2010).
21. Ingber, D. E. *et al.* Tissue engineering and developmental biology: going biomimetic. *Tissue Eng.* **12** (12), 3265-3283 (2006).
22. Yoshida, Y., Yamanaka, S. Recent stem cell advances: induced pluripotent stem cells for disease modeling and stem cell-based regeneration. *Circulation.* **122** (1), 80-87 (2010).

