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In vitro chondrogenesis and *in vivo* repair of osteochondral defect with human induced pluripotent stem cells

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ABSTRACT

The purpose of this study was to investigate the chondrogenic features of human induced pluripotent stem cells (hiPSCs) and examine the differences in the chondrogenesis between hiPSCs and human bone marrow-derived MSCs (hBMMSCs). Embryoid bodies (EBs) were formed from undifferentiated hiPSCs. After EBs were dissociated into single cells, chondrogenic culture was performed in pellets and alginate hydrogel. Chondro-induced hiPSCs were implanted in osteochondral defects created on the patellar groove of immunosuppressed rats and evaluated after 12 weeks. The ESC markers NANOG, SSEA4 and OCT3/4 disappeared while the mesodermal marker BMP-4 appeared in chondro-induced hiPSCs. After 21 days of culture, greater glycosaminoglycan contents and better chondrocytic features including lacuna and abundant matrix formation were observed from chondro-induced hiPSCs compared to chondro-induced hBMMSCs. The expression of chondrogenic markers including SOX-9, type II collagen, and aggrecan in chondro-induced hiPSCs was comparable to or greater than chondro-induced hBMMSCs. A remarkably low level of hypertrophic and osteogenic markers including type X collagen, type I collagen and Runx-2 was noted in chondro-induced hiPSCs compared to chondro-induced hBMMSCs. hiPSCs had significantly greater methylation of several CpG sites in *COL10A1* promoter than hBMMSCs in either undifferentiated or chondro-induced state, suggesting an epigenetic cause of the difference in hypertrophy. The defects implanted with chondro-induced hiPSCs showed a significantly better quality of cartilage repair than the control defects, and the majority of cells in the regenerated cartilage consisted of implanted hiPSCs.

1. Introduction

Articular cartilage (AC) does not heal spontaneously in adults when damaged, progressing to osteoarthritis (OA). This inability to self-repair after damage has encouraged extensive studies on AC regeneration [1,2], the most commonly investigated strategy being cell-based treatments. A number of cell sources have been explored for AC regeneration. Of these, autologous chondrocytes were first used in clinical practice [3]. However, inherent problems were reported with autologous chondrocytes implantation (ACI). Chondrocytes have limited proliferative potential and quickly lose their functional phenotypes in culture [4]. In addition, an extra injury is done to the joint during the harvesting procedure even though the harvest site is located away from the lesion. An alternative method to ACI is the use of adult mesenchymal stem cells (MSCs). Tissue regeneration from MSCs takes advantage of the natural course of embryonic development [5–8]. One critical shortcoming with MSCs is premature hypertrophy which would result in a tissue incapable of enduring the pressure and shear force that the AC is subjected to [9–11]. In addition, the number of MSCs, their proliferative capacity as well as their differentiation potential is known to decline with age [12].

Embryonic stem cells (ESCs) may provide a potential alternative for cartilage tissue engineering. Human embryonic stem cells (hESCs) have drawn great attention as a cell source for regenerative medicine [13,14]. The most compelling advantage of hESCs for cartilage regeneration is that they can potentially provide an unlimited number of chondrocytes or chondroprogenitors for implantation. However, derivation of hESCs from early embryos raises ethical limitations for their use in clinic practice [15]. Induced pluripotent stem cells (iPSCs), generated from somatic cells by transduction of defined reprogramming transcription factors, typically OCT4, SOX2, KLF4, and c-MYC, offer a new path to avoid the controversy of using hESCs [16,17]. iPSCs express many of the markers associated with pluripotent cells, and are known to possess an epigenetic status similar to that of ESCs [16–20]. iPSCs

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function in a manner indistinguishable from ESCs by differentiating into cell types that are characteristic of the three germ layers *in vitro* and *in vivo*. As iPSCs also have high proliferative and differentiation capabilities similar to those of hESCs, they hold great potential for regenerative medicine [21].

In order to be regarded as a clinically viable alternative to MSCs, iPSCs should demonstrate a property superior or comparable to that of MSCs. iPSC-derived mesenchymal cells were able to attenuate the injury associated with hindlimb ischemia in a rodent model and contributed to tissue regeneration to a greater degree than bone marrow-derived stem cells [22]. Further studies have also demonstrated that iPSCs can be differentiated into skeletal muscle, adipocytes, and vascular lineages *in vitro* [23–26]. Several obstacles still need to be overcome before iPSCs can be considered as a potential therapeutic measure for cartilage regeneration. First, the persistence of differentiated phenotypes *in vivo* must be demonstrated. Second, simple and standardized protocols to generate "easy to grow" cell populations are necessary for the clinical application of iPSCs, so that the cells can survive *in vivo* transplantations and regenerate functional tissue without the risk of tumor formation.

Although significant progress has recently been made with regard to iPSCs in musculoskeletal regenerative medicine [27,28], the chondrogenic features of human iPSCs (hiPSCs) in comparison with MSCs were not reported, particularly regarding the difference in hypertrophy induction. In addition, to the best of our knowledge, there are no published reports that examined whether and how the implantation of hiPSCs promotes the repair of chondral defect. The purpose of this study was to investigate the chondrogenic features of hiPSCs in tandem with bone marrow-derived MSCs (BMMSCs) and examine the underlying cause of differences in the chondrogenesis between two cell types. The *in vivo* capacity of hiPSCs for cartilage regeneration was also assessed using an osteochondral defect model.

2. Materials and methods

2.1. hiPSC culture

To avoid potential safety issues associated with the use of viruses, we used the hiPS cell line (SC802A-1, System Biosciences In. Mountain View, CA) created by direct delivery of four proteins fused to a cell penetrating peptide into human fibroblasts in the present study [29]. Undifferentiated hiPSCs were maintained as described previously [17,20]. To prepare feeder-free hiPSCs for differentiation experiments, hiPSCs were passaged to Matrigel-coated polystyrene plates and cultured in the defined mTeSR1 medium (STEMCELL Technologies Inc., Vancouver, BC, Canada). A combined use of mTeSR1 medium and Matrigel-coated substrates had proven to support the feeder-independent maintenance of hiPSCs [30,31].

2.2. Differentiation into embryoid bodies

In vitro differentiation of hiPSCs was performed using the standard embryoid body (EB) differentiation method [32] with minor modifications. For sphere formation, cells were dissociated with 0.05% trypsin-EDTA and plated onto 6-well ultra-low-attachment plates (Corning, Tewksbury, MA, USA). After 2 days of sphere formation, EBs were cultured in ESC medium in the presence of 10^{-7} M all-trans retinoic acid (ATRA) for 10 days. Medium was changed every other day.

2.3. Induction of chondrogenic differentiation

2.3.1. Chondrogenic pellet culture with hiPSCs

hiPSC-EBs were dissociated to a single cell suspension by trypsinizing and then diluting to a final concentration of 5.0×10^5 cells/ml. Micromass pellets were cultured as non-adherent spheres in 5-ml round tubes for 21 days. Chondrogenic medium consisted of DMEM/F-12 as base medium, and was supplemented with 10% FBS, 10^{-7} M dexamethasone, 50 μM ascorbate-2-phosphate, 50 μM L-proline, 1 mM sodium pyruvate, 1% insulin–transferrin–selenium, and 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA) [33]. The tubes were then placed in an incubator at 37 °C in a humidified 5% CO_2 atmosphere. Chondrogenic differentiation of hiPSCs was also performed in alginate gels using the same chondrogenic medium.

2.3.2. Chondrogenic differentiation of hBMMSCs

The bone marrow samples used to isolate hBMMSCs were obtained from three patients (mean age: 64 years, range: 54–72 years) undergoing total hip replacement due to osteoarthritis. Informed consent was obtained from all donors. hBMMSCs were isolated from fresh bone marrow samples, and then expanded as described previously [34]. Chondrogenic pellet cultures were performed in the same method and culture medium as used in hiPSCs for 21 days.

2.3.3. Chondrogenic differentiation of hiPSCs or hBMMSCs in alginate gel

For constructs with alginate, dissociated hiPSCs or hBMMSCs were suspended at a density of 1.5×10^6 cells per 100 μl in 2% alginate (Sigma). Polymerization of alginate was then achieved by dropping the grafts into 100 mM $CaCl_2$ solution. After instantaneous gelation, the alginate was allowed to polymerize further for a period of 8–10 min in the $CaCl_2$ solution. Thereafter, all beads were thoroughly washed with PBS (Gibco, Carlsbad, CA, USA) and were cultured in a 24-well plate (Nunc, Waltham, MA, USA) under the same chondrogenic medium and conditions as in pellet culture. Media were changed every 3–4 days.

Cell viability was determined with the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA, USA). Alginates were washed in PBS and exposed to 4 μM calcein-AM and 2 μM ethidium homodimer in PBS for 20 min at 37 °C. Dye uptake was detected by using a Leica fluorescence microscope with filter cubes for fluorescein (for calcein in live cells) and Texas red (for ethidium homodimer) in dead cells.

2.4. Biochemical assays for DNA and GAG quantification

After 21 days of *in vitro* culture, the alginate hydrogels were dissolved by incubating the beads for 20 min in dissolution solution (55 mM EDTA, 10 mM HEPES, pH 7.4). The pellets were digested overnight in papain buffer at 60 °C. DNA content was determined using the Quant-iT™ dsDNA assay kit and QubitFluorometer system (Invitrogen). Glycosaminoglycan (GAG) production was determined using a Blyscan kit (Biocolor, Carrickfergus, UK) according to the manufacturer's instructions.

2.5. RT-qPCR

Total RNA preparation, cDNA synthesis, and reverse transcription–quantitative polymerase chain reaction (RT-qPCR) reactions were performed as described previously [34]. Primer information is provided in Supplementary Table 1. The relative normalization ratio of PCR products derived from each target gene was calculated using the software of the LightCycler System (Roche, Indianapolis, IN, USA). All experiments were performed in triplicate.

2.6. Western blot analysis

Proteins were extracted from cultures, electrophoresed and transferred to a nitrocellulose membrane. The blot was probed with anti-rabbit type II collagen (COL2A1; 1:500; Abcam, Cambridge, UK), type X collagen (COL10A1; 1:500; Abcam), type I collagen (COL1A1; 1:500; Abcam), SOX-9 (1:1000; Abcam), Runx-2 (1:500; Abcam), an anti-mouse aggrecan (1:100; Abcam), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (1:2000; Cell Signaling Technology, Beverly, MA, USA). This experiment was repeated in three samples, each from different individuals.

2.7. Immunohistochemistry (IHC)

The cells or sections were blocked with 5% normal donkey serum (NDS) and 0.1% Triton X-100 in PBS at room temperature for 1 h. The following primary antibodies were applied overnight at 4 °C: rabbit polyclonal antibodies including NANOG 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), brachyury (1:200; Abcam), COL2A1 (1:200; Abcam), COL1A1 (1:200; Abcam), SOX-9 (1:200; Abcam), and Runx-2 (1:100; Abcam), and mouse monoclonal antibodies including Oct3/4 (1:200; Santa Cruz Biotechnology), SSEA4 (1:100; R&D systems), COL10A1 (1:200; Sigma), human nuclear antigen (HN; 1:100; Chemicon Temecula, CA, USA), and aggrecan (1:100; Abcam). Appropriate fluorescence-tagged secondary antibodies (R&D Systems) were used for visualization.

2.8. Methylation-Specific polymerase chain reaction (MSP)

Genomic DNA was extracted from hiPSCs (triplicates) and hBMMSCs (3 donors) using a GeneAll® Exgene™ Tissue SV kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. Bisulfite-modified genomic DNA was also prepared using an EpiTect® Bisulfite kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. CpG sites within the upstream sequences approximately −5.0 kb from the transcription start site (TSS) of *COL10A1* gene were analyzed (Supplementary Table 2). From the quantified data, methylation index (MI) was defined as intensity of methylated band divided by the sum of intensity of both methylated and unmethylated band.

2.9. Bisulfite sequencing (BSQ) analysis

Genomic DNA extracted in MSP was also used for bisulfite sequencing (BSQ). Primers were designed, based on Zimmermann et al. [35] (Supplementary Table 3).