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Original Article

Application of induced pluripotent stem cells for cartilage regeneration in CLAWN miniature pig osteochondral replacement model

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ABSTRACT

Introduction: Pluripotent stem cells have an advantage that they can proliferate without reduction of the quality, while they have risk of tumorigenesis. It is desirable that pluripotent stem cells can be utilized safely with minimal effort in cartilage regenerative medicine. To accomplish this, we examined the potential usefulness of induced pluripotent stem cells (iPS cells) after minimal treatment via cell isolation and hydrogel embedding for cartilage regeneration using a large animal model.
Methods: Porcine iPS-like cells were established from the CLAWN miniature pig. In vitro differentiation was examined for porcine iPS-like cells with minimal treatment. For the osteochondral replacement model, osteochondral defect was made in the quarters of the anteromedial sides of the proximal tibias in pigs. Porcine iPS-like cells and human iPS cells with minimal treatment were seeded on scaffold made of thermo-compression-bonded beta-TCP and poly-L-lactic acid and transplanted to the defect, and cartilage regeneration and tumorigenesis were evaluated.
Results: The in vitro analysis indicated that the minimal treatment was sufficient to weaken the pluripotency of the porcine iPS-like cells, while chondrogenic differentiation did not occur in vitro. When porcine iPS-like cells were transplanted into osteochondral replacement model after minimal treatment in vitro, cartilage regeneration was observed without tumor formation. Additionally, fluorescent in situ hybridization (FISH) indicated that the chondrocytes in the regenerative cartilage originated from transplanted porcine iPS-like cells. Transplantation of human iPS cells also showed the regeneration of cartilage in miniature pigs under immunosuppressive treatment.
Conclusion: Minimally-treated iPS cells will be a useful cell source for cartilage regenerative medicine.

1. Introduction

The possible cell sources for cartilage regenerative medicine include chondrocytes, mesenchymal stem cells (MSCs), and the pluripotent stem cells, namely, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells [1]. Use of the pluripotent stem cells has an advantage in the fabrication of large sized regenerative cartilage, because these cells can avoid the decrease in quality during proliferation which is inevitable for chondrocytes and MSCs [2–4].

Many reports have described the in vitro differentiation of ES cells into chondrocytes [5–7]. Although these reports have shown the upregulation of chondrocyte marker genes, they did not show sufficient increases in the extracellular matrices by either the protein quantification or the histological analysis. On the other hand, transplanted ES cells can differentiate into

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chondrocytes with abundant cartilaginous extracellular matrices in the cartilage defects of rats [8,9]. These results suggest that the in vivo environment plays a crucial role in the chondrogenesis of ES cells by providing soluble factors and the mechanical stimulation.

Considering the ethical problems associated with ES cells, that is, the destruction of a potential life, iPS cells induced from mature somatic cells [1,38] should be the best choice as the cell source for cartilage regenerative medicine. However, iPS cells have the same safety issue as ES cells, i.e. the risk of tumorigenesis [1]. Several studies indicate that cancer cells and iPS cells share the gene expression patterns of oncogenes [28,29]. The insufficient silencing of transgenes would increase the risk of tumorigenesis [30]. To reduce the risk of tumor formation, in vitro preparation of mature tissues prior to transplantation is desirable. In a previous report, iPS cells could be differentiated to form a retinal sheet in vitro [11]. For cartilage, Yamashita et al. reported the in vitro formation of cartilage particles using iPS cells [12,13]. Although they succeeded in the fabrication of mature tissues in vitro, the cost for these procedures may hinder the clinical application of them.

Alternatively, treating cells minimally in vitro to prevent tumor formation and expecting cell differentiation and tissue regeneration after transplantation will be more practical. Our previous study showed that the disconnection of cell–cell contacts and the maintenance of cell isolation with a hydrogel were enough to prevent tumorigenesis [14]. In the same study, transplantation of iPS cells could regenerate cartilage in a small defect in a murine patellar groove. However, the effectiveness of the transplantation of iPS cells minimally treated in vitro for the treatment of much larger joint defects is yet to be determined.

The purpose of this study was to demonstrate that the minimally treated iPS cell is a useful cell source for cartilage regenerative medicine. We examined whether cell separation would suppress the pluripotency (also interpreted as tumorigenicity) of porcine iPS-like cells and evaluated the usefulness of these cells using a partial defect in the tibial side of the knee joint in CLAWN miniature pigs. This animal model is adopted to mimic clinical situations in which replacement of tibial plateau with osteochondral implant is desirable, instead of creating joint defect in patellar groove as studied in animal experiments [8,9,14]. For example, meniscectomy causes cartilage degeneration which appears earlier in the tibial plateau than the femoral condyle [46]. Tibial plateau fractures result in damages in the cartilage of the tibial side, which also causes cartilage degeneration of the femoral condyle [47]. In each situation, early restoration of cartilage in the tibial side will prevent the progression of cartilage degeneration in the femoral condyle. The results were compared to those of MSCs, which have already been clinically applied. We also examined the utility of human iPS cells using the same osteochondral replacement model in CLAWN miniature pigs.

2. Materials and methods

2.1. Animals

CLAWN miniature pigs were purchased from Kagoshima Miniature Swine Research Center (Kagoshima, Japan). To perform the syngeneic transplantations, pigs with a C2 attribute in the MHC were used for the collection of cells and to generate the cartilage defect model. Twelve-days- pregnant C57BL/6 J Jcl mice were purchased from CLEA, Japan (Tokyo, Japan). All in vivo experiments were approved by the ethics committee of the University of Tokyo (P10-009).

2.2. Cells

2.2.1. Porcine MSCs

Porcine MSCs were obtained from 6-month-old CLAWN miniature pigs of C2 in MHC, following the procedures of a previous report [26]. Briefly, the pigs were euthanized with deep general anesthesia and the intravenous administration of 1 M KCl (Wako Pure Chemical Ind. Osaka, Japan). Bone marrow cells were collected from the femurs and tibias. Cells were plated onto non-coated polypropylene dishes with MSCGM (LONZA, Basel, Switzerland). The adherent cells were defined as the MSCs. After approximately 7 days, the cells reached confluency and were collected with TRyPLE Select (Thermo Fisher Scientific, Massachusetts, USA). The MSCs were used on passage 1 (P1).

2.2.2. Porcine iPS-like cells

Feeder cells were prepared from Mitomycin C (Sigma Aldrich)-treated embryonic fibroblasts from C57BL/6 J mice. Fibroblasts isolated from the ear fragments of female newborn CLAWN miniature pigs were induced to become iPS-like cells using the STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Merck Millipore, Darmstadt, Germany). Porcine iPS-like cells were cultured on a polypropylene dish coated with 0.1% porcine gelatin (Sigma Aldrich) with feeder cells. The medium for the porcine iPS-like cells contained 1% of penicillin and streptomycin (Sigma Aldrich, Missouri USA), 20% of knockout serum replacement (Thermo Fisher Scientific), 0.1 mM of non-essential amino acids (Thermo Fisher Scientific), 0.05 mM 2-mercaptoethanol (Thermo Fisher Scientific), and 10 ng/mL bFGF (Fiblast Spray, Kaken Pharmaceutical, Tokyo, Japan), in DMEM/F12 Glutamax (Thermo Fisher Scientific).

To analyze the features of the established cells, colonies were stained with Alkaline Phosphatase Staining Kit II (STEMGENT, Massachusetts USA).

Mesodermal differentiation was performed according to a previous report [48].

RNA was extracted from the porcine iPS-like cells with Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription PCR was performed with the TAKARA RT-PCR Perfect Real-Time Kit (TAKARA BIO, Shiga, Japan). Real-time PCR was performed with Platinum Quantitative PCR SuperMix-UDG w/ROX (Thermo Fisher Scientific). PCR was performed with TaKaRa Ex Taq Hot Start Version (TAKARA BIO). The primers used in this study are indicated in Table 1.

2.2.3. Human iPS cells

Human iPS cells, namely, HPS0002, were purchased from RIKEN BRC (Tsukuba, Japan). Cells were cultured in 0.1% porcine gelatin-coated polypropylene dishes with feeder cells. The media for human iPS contained 1% of penicillin and streptomycin, 15% of

Table 1
Primer sequences used for evaluation of porcine iPS-like cells.

Mouse *c-myc*	5′-TGACCTAACTCGAGGAGGAGCTGGAATC-3′
	5′-AAGTTTGAGGCAGTTAAAATTATGGCT GAAGC-3′
Mouse *oct3/4*	5′-TCTTTCCACCAGGCCCCCGGCTC-3′
	5′-TGCGGGCGGACATGGGGAGATCC-3′
Mouse *klf4*	5′-AGTGTGACAGGGCCTTTTCCAGGT-3′
	5′-AAGCTGACTTGCTGGGAACTTGACC-3′
Mouse *sox2*	5′-TAGAGCTAGACTCCGGGCGATGA-3′
	5′-TTGCCTTAAACAAGACCACGAAA-3′
Pig *GAPDH*	5′-ACTGTGGCGTGATGGCCGAG-3′
	5′-TCCACAACCGACACGTTGGCA-3′
Pig *NANOG*	5′-CTGGAAACCTGCCCGTGTGGG-3′
	5′-GGGCACCAGGTCTGGCTGTT-3′
Pig *OCT3/4*	5′-GATCGGGCCGGGGGTTGG-3′
	5′-TCCTCGGGGTTCGGCTCCAG-3′