Original Article

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

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A B S T R A C T

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′-GATCGGGCCGGGGGGTTGG-3′
	5′-TCCTCGGGGTTCGGCTCCAG-3′

knockout serum replacement, 0.1 mM of non-essential amino acid, 0.05 mdM 2-mercaptoethanol, and 5 ng/mL bFGF in DMEM/F12 Glutamax.

2.3. In vitro 3D culture

Porcine MSCs were embedded with 1% atelocollagen (Koken, Tokyo, Japan) diluted with MEM (Thermo Fisher Scientific) at a density of 107 cells/mL. The porcine iPS-like cells were treated with 10 μg/mL Y-27632 (Wako Pure Chemical Ind.) at 37 °C for 3 h, collected with CTK solution (Reprocell, Kanagawa, Japan), pipetted well, and embedded in 1% atelocollagen at a density of 107 cells/mL. The cells and atelocollagen mixture were dispensed in 20 μL aliquots and gelatinized by incubation at 37 °C for 2 h. The resultant pellets were cultured with the chondrogenic medium [26]. Half of the volume of the media was changed twice a week. The experiments were performed in triplicate.

2.4. RT-PCR analysis

RNA was extracted from the porcine iPS-like cells and MSCs cultured on plates or from pellets for the indicated times as described above. Reverse transcription PCR and Real-time PCR was also performed in same manner. The primers used in this study are indicated in Table 2. The experiments were performed in triplicate, and the average values were determined.

2.5. Animal model & transplantation

Cells were collected according to the same procedure used for the in vitro 3D culture. The porcine MSCs and iPS-like cells were mixed in 1% atelocollagen diluted with MEM at densities of 108 cells/mL and 107 cells/mL, respectively. The mixture was soaked in a poly-L-lactic acid (PLLA) construct which was pre-formed to the shape of the defect and was thermo-compression-bonded with beta-TCP, which was also pre-formed to the same shape (Fig. 1 A, B, C, D). The mixture was gelatinized for 2 h with an incubation at 37 °C in moist conditions (Fig. 1E, F). Transplant with human iPS cells was prepared by same procedure as porcine iPS-like cells.

The male CLAWN miniature pigs were given general anesthesia. Approximately one-quarter of the anteromedial side of the proximal tibia was exposed (Fig. 1G). The osteochondral defect was made with a bone chisel (Fig. 1H). The prepared transplant (beta-TCP scaffold, MSCs with the beta-TCP/PLLA scaffold, or iPS-like cells with the beta-TCP/PLLA scaffold) was then placed in the defect and fixed with a mesh plate made of titanium (Okada Medical Supply, Tokyo, Japan) (Fig. 1I). Following transplant fixation, the wound was closed. For pigs that were transplanted with human iPS cells, tacrolimus was orally given 5 days prior to surgery and was maintained at the concentration of 35—50 ng/mL in peripheral blood. The experiments were performed in duplicate.

2.6. Collection of physiological findings

Clinical findings were collected at the indicated times, namely, 2 weeks, 4 weeks, and 8 weeks post-surgery. The collected findings were scored at each time point. The items and references of the scoring analysis are described in Table 3.

2.7. Collection of tissue samples

Tissue samples were collected from pigs transplanted with porcine cells and human cells at 8 weeks and 4 weeks after the surgery, respectively. Pigs were euthanized with deep general anesthesia and the intravenous administration of 1 M KCl. Epiphyses of the distal femurs and proximal tibias were collected and fixed in 4% paraformaldehyde (Wako Pure Chemical Ind.) at 4 °C overnight. Then the samples were sliced in the sagittal direction using diamond band saws and additionally fixed with 4% paraformaldehyde at 4 °C for 1 week. Blood samples were collected on the day of transplantation and on the day of sample collection.

2.8. Histological and histochemical analysis

Samples were decalcified with Morse solution [10% trisodium citrate (Wako Pure Chemical Ind.), 22.5% formic acid (Wako Pure Chemical Ind.) and 67.5% distilled water], sectioned, and paraffin embedded. The sections were stained with hematoxylin & eosin. The samples from the cartilage tissues transplanted with porcine iPS-like cells and the unaffected cartilage tissues (from tibias away from the site of implantation) were also analyzed by fluorescent in situ hybridization (FISH) with a porcine Y chromosome probe.

2.9. Statistical analysis

Statistical analysis was performed with Excel Statistics 2015 (Social Survey Research Information Co., Ltd.). Data were analyzed with Dunnett's test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of porcine iPS-like cells

The characteristics of porcine iPS-like cells were confirmed. Colonies similar to human ES cells which were positive for alkaline phosphatase staining (Fig. 2 A) were obtained. The pluripotency markers NANOG and OCT3/4, and the transgenes oct3/4, sox2, klf4, and c-myc were upregulated (Fig. 2B and C). The upregulation of BRACHYURY was observed with samples with or without mesodermal differentiation supplements (Fig. 2 D). We did not succeed in either epidermal or ectodermal differentiation (data not shown), thus defined the established cells as iPS-like cells.

3.2. Differentiation of porcine stem cells is induced by atelocollagen embedding

In porcine MSCs that were embedded within atelocollagen, the early stage chondrogenic marker SOX9 was upregulated during week 0 compared to the plate culture from the undifferentiated stage (p = 0.0078) (Fig. 3 A). Additionally, the chondrogenic marker AGGRECAN was upregulated during weeks 1 and 2 (p = 0.0036 and p = 0.0001, respectively) (Fig. 3 A). However, the upregulation of COL2 was insufficient (Fig. 3 A).

Regarding the iPS-like cells, RT-PCR analysis of atelocollagen-embedded porcine iPS-like cells showed that the expression of the pluripotency marker NANOG declined immediately after the
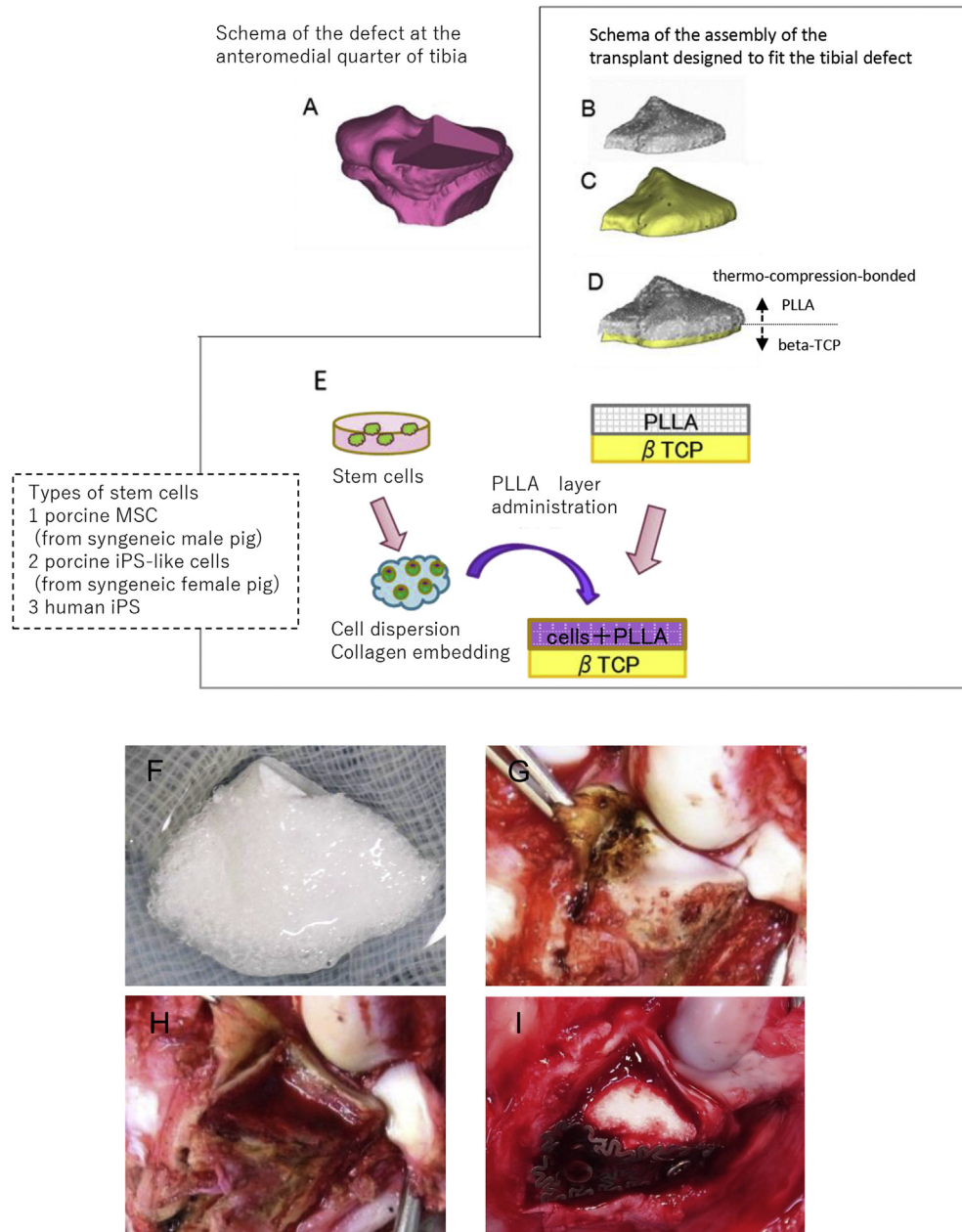
Table 2
Primer sequences used for evaluation of in vitro differentiation of porcine cells.

Pig BETA ACTIN	5′-AAGCCAACCGTGAGAAGATG-3′
	5′-GTACATGGCTGGGGTGTTG-3′
Pig COL1A1	5′-CCAAGAGGAGGGCCAAGAAGAAGG-3′
	5′-GGGGCAGACGGGGCAGCACTC-3′
Pig COL2A1	5′-GCACGGATGGTCCCAAAG-3′
	5′-CAGCAGCTCCCCTCTCAC-3′
Pig SOX9	5′-GCTCACCAGACCTTGAGGAG-3′
	5′-CTTGGAATGTGGGTTCGAGT-3′
Pig AGGRECAN	5′-TCCGCTCCTTGTCGCTTT-3′
	5′-TTTGCTTTGGCGTTTGAGG-3′
Pig NANOG	5′-CCGAAGCATCCATTTCCAGCG-3′
	5′-GGTATTCTGTACTGGCTGAGCC-3′

Fig. 1. Experimental design of in vivo study. (A) Three-dimensional image of the bone and cartilage defect. (B, C) Three-dimensional images of the PLLA (B) and the beta-TCP (C) scaffolds formed to fit the defect in the knee joint. (D) Three-dimensional image of thermo-compression-bonded beta-TCP and PLLA. (E) A Scheme of the preparation of the transplant. Cells were singly isolated and embedded in collagen. The mixture of cells and collagen was administered to PLLA layer. Following administration, collagen was gelatinized. (F) Gross appearance of the transplant. (G) The exposed surgical site. (H) The defect formed at the anteromedial quarter of the tibial side of the knee joint. (I) The surgical site after transplantation. The transplant was placed at the defect and fixed with thin titan mesh plates.

beginning of the embedding culture (p = 0.00004, P = 0.000001, P = 0.000001, and P = 0.000001 each compared to the plate culture) (Fig. 3 B). However, the expression levels of the chondrogenic markers SOX9 or COL2 or AGGRECAN were not upregulated during the embedding culture (Fig. 3 B).

3.3. Syngeneic transplantation of tissue-engineered cartilage using porcine stem cells

Based on the physiological findings, no apparent difference was found among the pigs transplanted with any of the 3 types of transplants (Fig. 4). Weight gain in the pigs was still within the average range of the animals in good health (Kagoshima Miniature Swine Research Center, http://kmsrc.org/index.html). Severe reddening was observed during week 0, which declined by week 2. Throughout the observation period, no abnormal finding was evident at the wound. The avoidance of weight-bearing became very slight by the end of the observation period.

Based on the macroscopic findings, the tissue defect was obvious at the site of the beta-TCP transplant (Fig. 5 A, A', G, G'). Eburnation of the joint cartilage of the opposing femur was also observed (Fig. 5 D, D', J, J'). For the porcine MSCs-transplanted sample, granulation was observed at the transplantation site (Fig. 5 B, B', H, H'). Slight eburnation of the joint cartilage of the pairing femur was observed (Fig. 5 E, E', K, K'). For the porcine iPS-like cells-transplanted samples, granulation was observed at the

Table 3

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Body weight		
Joint swelling	Grade 0	Not observed
	Grade 1	Slight
	Grade 2	Moderate
	Grade 3	Severe
	Grade 4	Extremely severe
Reddening of the skin surrounding a joint	Grade 0	Not observed
	Grade 1	Locally slight
	Grade 2	Locally severe
	Grade 3	Slight in broad area
	Grade 4	Severe in broad area
Weight-bearing on feet	Grade 0	No laterality on standing nor walking
	Grade 1	Slight laterality on standing or walking
	Grade 2	Severe laterality on standing or walking
	Grade 3	Able to stand but disable or reject to walk
	Grade 4	Disable or reject to stand
Wound	Grade 0	No opening, oozing, necrosis or pus
	Grade 1	Either opening or oozing
	Grade 2	Necrosis with or without opening or oozing
	Grade 3	Pus with or without opening, oozing, or necrosis
	Grade 4	Observe all opening, oozing, necrosis and pus
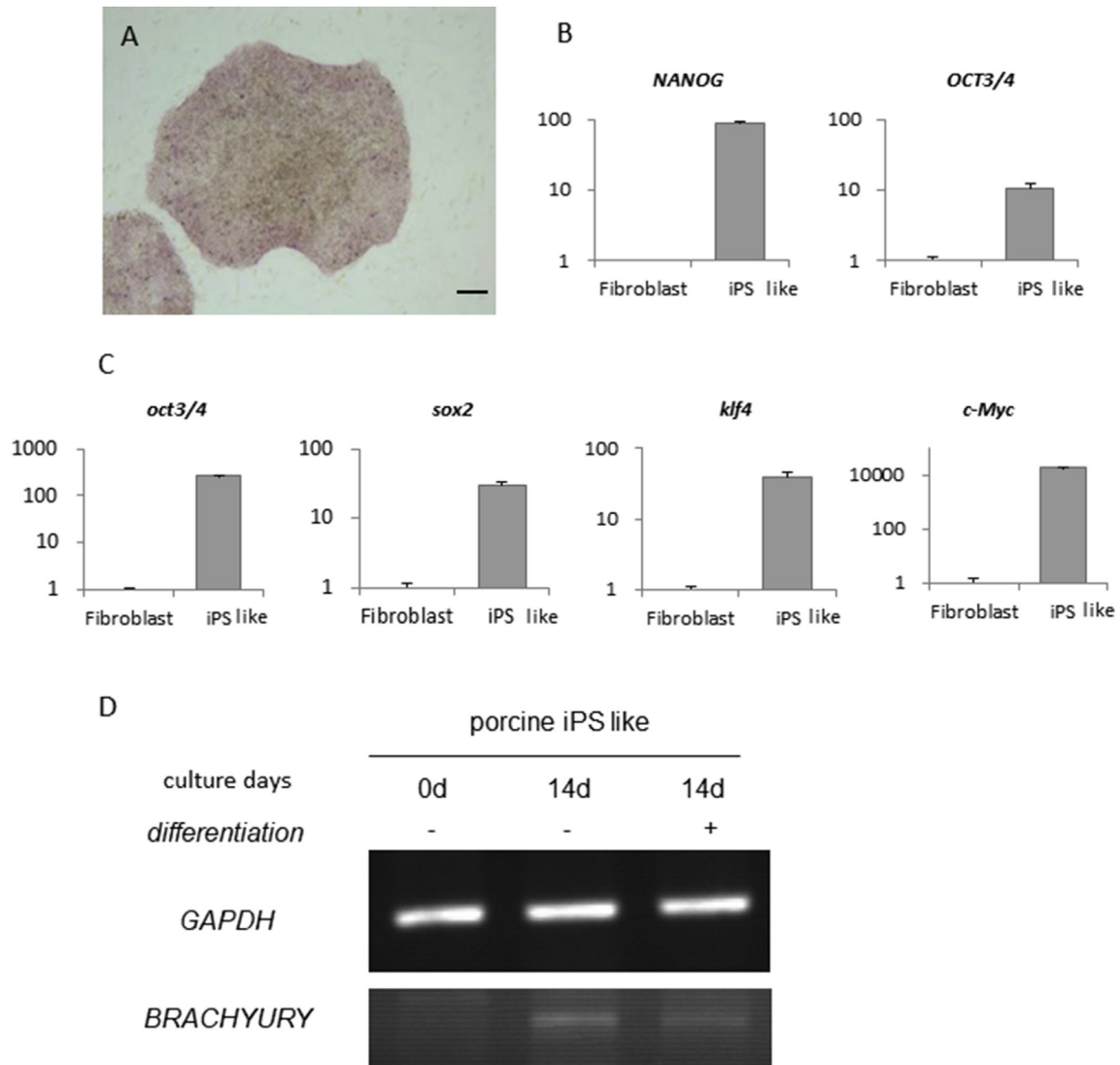
Fig. 2. Characterization of porcine iPS-like cells. (A) Alkaline phosphatase staining of colonies of porcine iPS-like cells. (B, C) Real-time RT-PCR analyses of pluripotent markers *NANOG* and *OCT3/4* (B) and transgenes *oct3/4*, *sox2*, *klf4*, and *c-myc* (C) in porcine iPS-like cells and fibroblasts. (D) RT-PCR analyses of early mesodermal marker *BRACHYURY* in porcine iPS-like cells with or without induction of mesodermal differentiation (D).
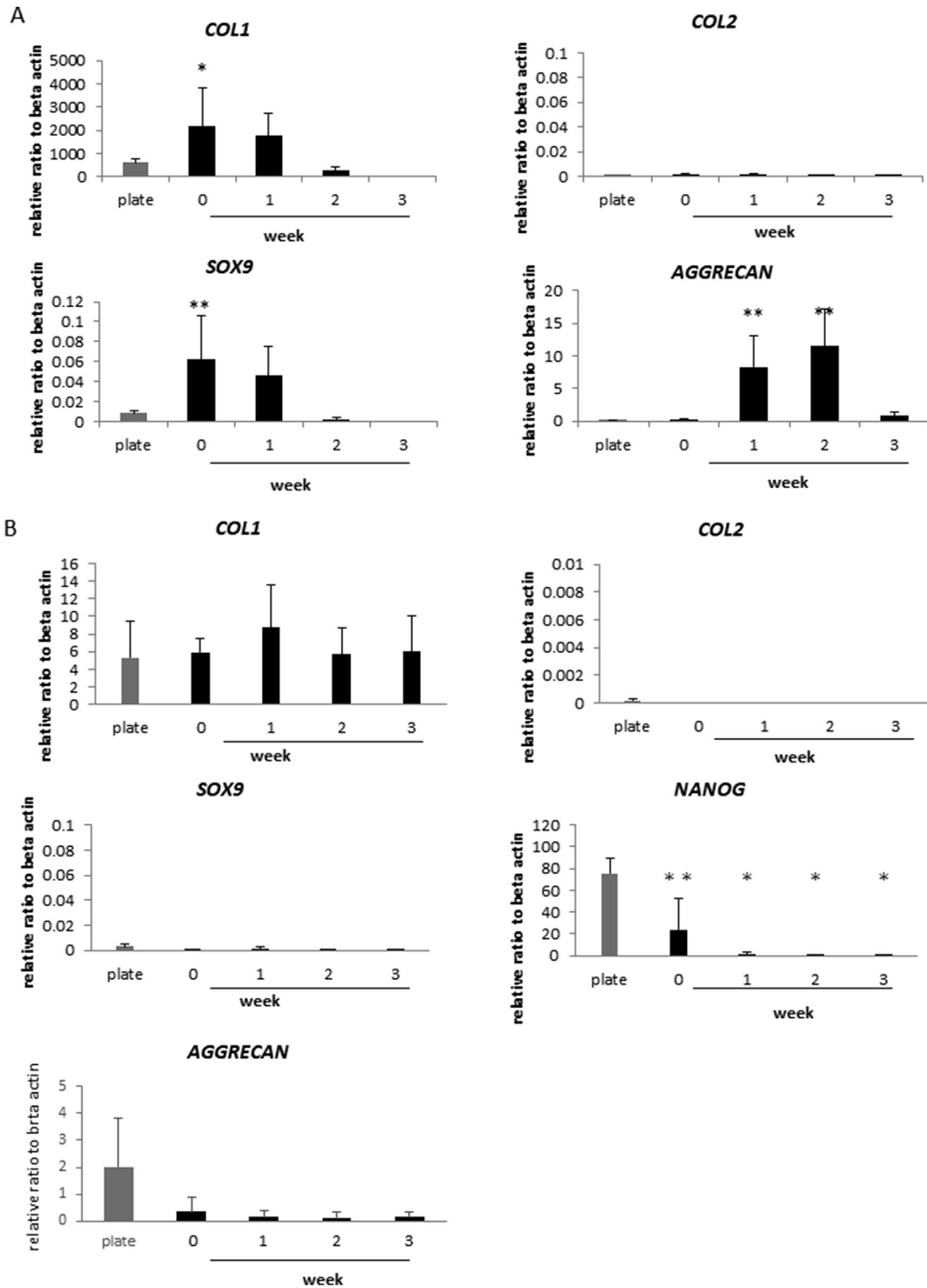
Fig. 3. Gene expression of the porcine cells cultured in 3D chondrogenic condition determined by real-time RT-PCR. MSCs (A) and porcine iPS-like cells (B) were three-dimensionally cultured in chondrogenic differentiation medium and mRNA from each cells was subjected to real time RT-PCR. All values are presented as mean plus standard deviation of 3 samples per group. Statistical analysis was done by Dunnett's test ($^*p < 0.05$, $^{**}p < 0.01$ versus plate).

transplantation site (Fig. 5C, C', I, I'). Eburnation of the joint cartilage of the pairing femur was hardly observed (Fig. 5 F, F', L, L').

As shown in Fig. 6 A, the histology of the unaffected joint showed a bilayer of joint cartilage and subchondral bone (a, e). However, the transplantation site was filled with fibrous tissue connected to the bone in the beta-TCP-transplanted sample. No regenerative cartilage was observed (b, f, i). On the other hand, the

porcine MSC-transplanted samples showed partial regenerative cartilage at the transplantation site (c, g, j). For the porcine iPS-like cell-transplanted samples, regenerative cartilage was also partially observed at the transplantation sites (d, h, k). In all samples, no tumor formation was observed.

As shown in Fig. 6B, the porcine Y chromosome probe was detected in the chondrocytes of the unaffected cartilage of the male
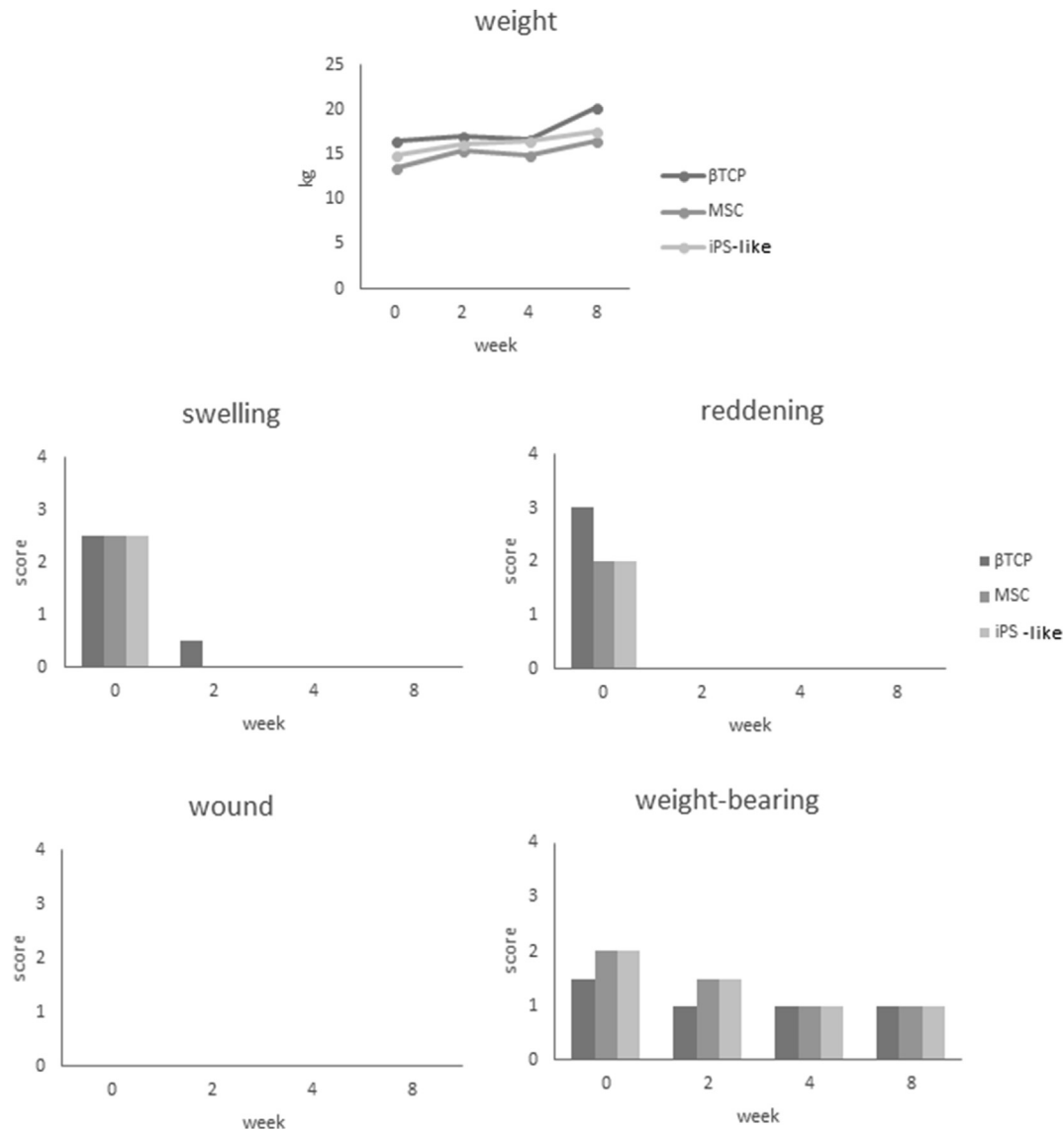
Fig. 4. Physiological findings of the pigs after transplantation. β-TCP: β-TCP scaffold only, MSC: scaffolds (β-TCP + PLLA) with porcine MSCs, iPS-like: scaffolds (β-TCP + PLLA) with porcine iPS-like cells. Values are means of the scores from 2 animals for each group.

pig (j, m). On the other hand, chondrocytes in the regenerative cartilage of the porcine iPS-like cell-transplanted sample were not positive for the Y chromosome (n, o).

3.4. Xenogeneic transplantation of tissue-engineered cartilage using human iPS cells

The blood concentration of tacrolimus showed that the immunosuppressant reached and maintained the desired concentration, suggesting that the risk of xenograft rejection was lowered. The general hematological findings showed that there was no significant difference from the average range of the animals in good health, suggesting there was no sign of severe organ failure due to the intake of the immunosuppressant (data not shown).

Based on the physiological findings, weight gain in the pigs was still within the average range of the animals in good health. Severe swelling and reddening was observed during week 0, which declined by week 2. Throughout the observation period, no abnormal finding was found at the wound. The avoidance of weight-bearing became very slight by the end of the observation

period (Fig. 7). Based on the macroscopic findings, granulation was evident at the transplantation sites (Fig. 8 A). Additionally, eburnation was hardly observed at the joint cartilage of the opposing femur (Fig. 8 B). As is the case with the syngeneic transplantation, no tumor formation was observed.

In the histological findings of the samples with transplanted human iPS cells, regenerative cartilage was formed at the transplantation site (Fig. 8C and D). To determine whether the regenerated tissue originated from the donor human iPS cells, we performed FISH to detect the Y chromosome of the host pig. The chondrocytes in the regenerative cartilage of the human iPS cell-transplanted sample were not positive for the Y chromosome (Fig. 8E).

4. Discussion

In this study, syngeneic and xenogeneic transplantation systems were adopted to examine the safety and the efficacy of minimally-treated iPS cells for the cartilage regenerative medicine. We utilized porcine iPS-like cells established in our lab, which have the
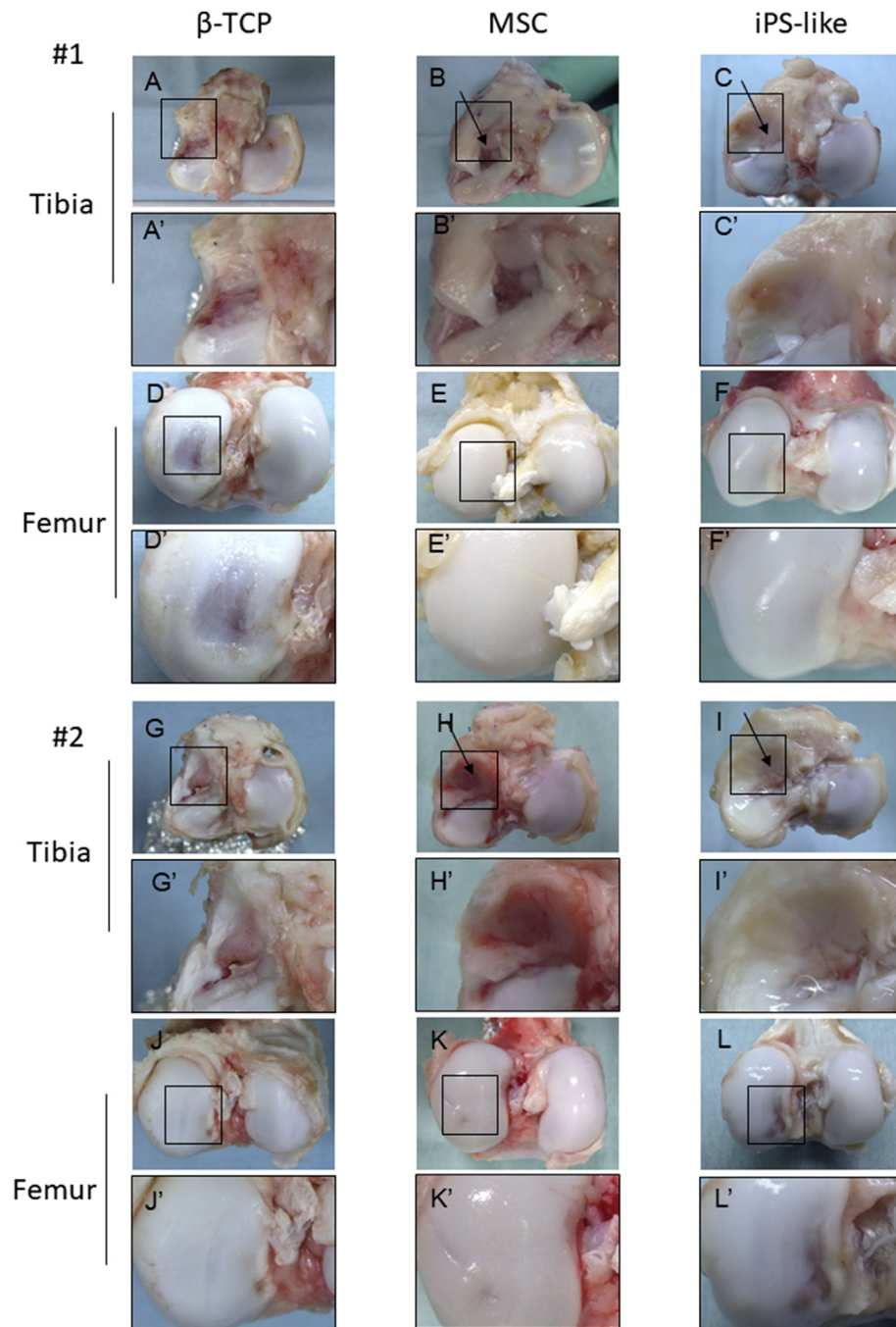
Fig. 5. Macroscopic findings of knee joints 8 weeks after transplantation with porcine cell-based transplants. Tibial (A, B, C, G, H, I) and femoral (D, E, F, J, K, L) sides of knee joints transplanted with the beta-TCP scaffold only (A, D, G, J), the beta-TCP/PLLA scaffold with MSCs (B, E, H, K) and the beta-TCP/PLLA scaffold with porcine iPS-like cells (C, F, I, L) and higher magnifications of each samples indicated by squares (A', B', C', D', E', F', G', H', I', J', K', L'). Granulations are indicated by arrows.

characteristics of iPS cells, except epidermal or ectodermal differentiation (Fig. 2 and data not shown). Considering the difficulty to obtain porcine iPS cells which are yet to be established [42], we utilized human iPS cells to reinforce the data.

The purpose of the minimal treatment is to prevent the tumorigenesis by reducing the pluripotency of the iPS cells. Pluripotency and cell–cell contacts are reported to be closely related. When ES cells differentiate, the protein level of E-cadherin is suppressed, and the gene expression levels of N-cadherin and E cadherin repressors are upregulated, resulting in the loss of cell–cell contact [17,18]. When human iPS cells and human ES cells were

experimentally sandwiched between Matrigel sheets, these cells showed the down-regulation of E-cadherin and upregulation of N-cadherin and Snail, an E-cadherin repressor [19]. Another study showed that human pluripotent stem cells could be switched from the self-renewal stage to the differentiation stage by changing the ECM from non-cell-adhesive hydrogel alginate-dominant ECM to cell-adhesive hydrogel collagen dominant ECM [20]. In this study, porcine iPS-like cells cultured with chondrogenic differentiation medium via single cell dissociation and embedding within atelocollagen showed down regulation of NANOG, suggesting that 3D embedding can weaken the pluripotency of iPS cells (Fig. 3 B). This
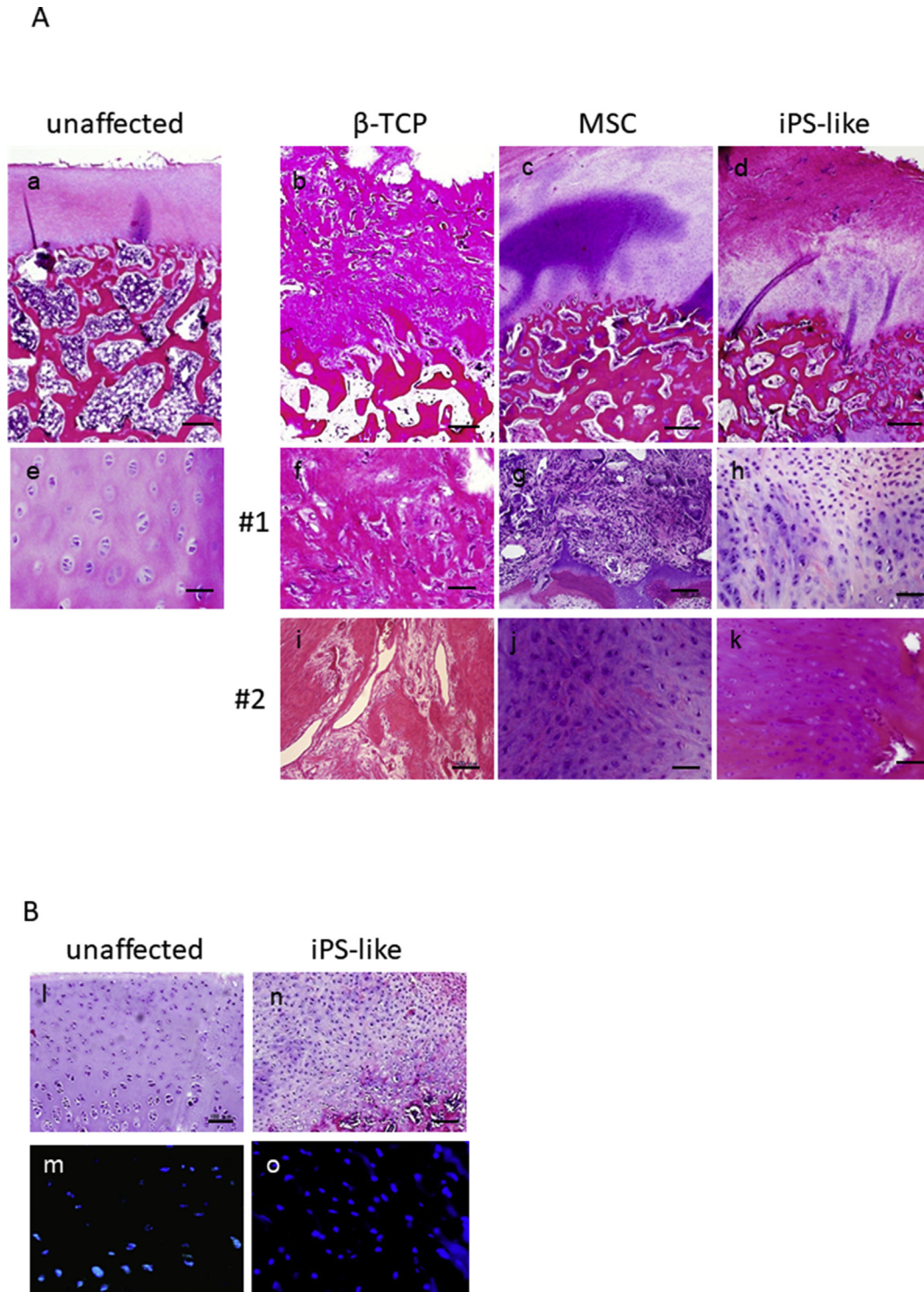
Fig. 6. Histological findings of the transplantation sites. (A) Hematoxylin-eosin staining of the unaffected cartilage (a, e), site with the beta-TCP scaffold only (b, f, i), the beta-TCP/PLLA scaffold with MSCs (c, g, j) and the beta-TCP/PLLA scaffold with porcine iPS-like cells (d, h, k). Scale bars = 1 mm for upper panels and 100 μm for lower panels. (B) FISH analysis. HE staining (upper panels) and FISH (lower panels) for unaffected cartilage (l, m) and regenerated cartilages with of the transplant of the beta-TCP/PLLA scaffold with porcine iPS-like cells (n, o).

result was consistent with those of previous studies showing that the pluripotency of pluripotent stem cells is suppressed by collagen embedding following cell isolation and that the cells are directed toward a certain direction of differentiation [8,16–19]. In the meanwhile, upregulation of chondrogenic markers SOX9 and COL2 were not apparent, indicating that cartilage regeneration mainly depends on the stimuli from in vivo environment after transplantation. On the other hand, porcine MSCs that were cultured with chondrogenic differentiation medium via embedding within atelocollagen showed upregulation of SOX9 and AGGRECAN, although the expression of COL2 was unchanged (Fig. 3 A). These results suggest that the 3D embedding and chondrogenic
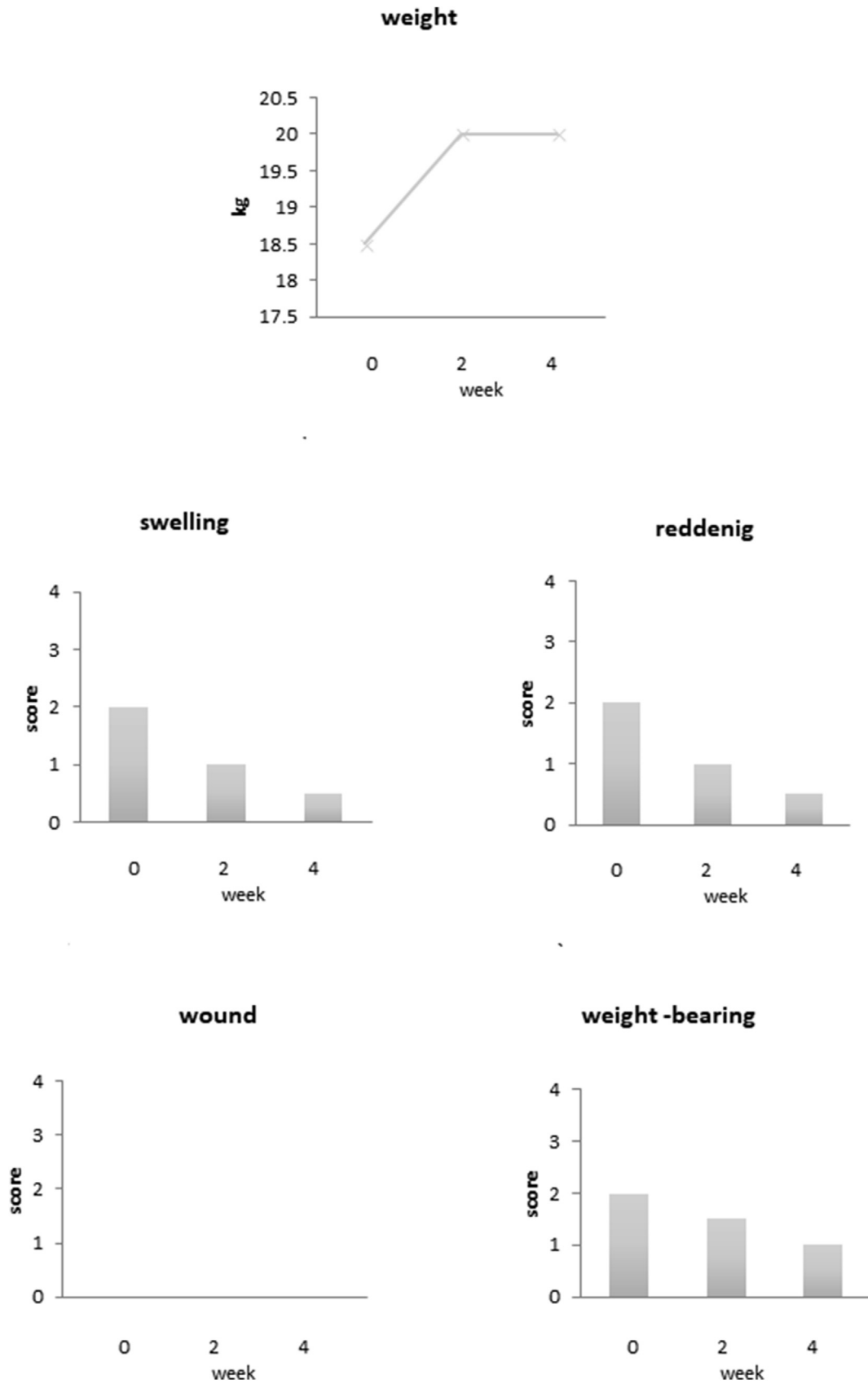
Fig. 7. Physiological findings of the pigs after transplantation of human iPS cells. Scaffolds: (beta-TCP + PLLA). Values are means of the scores from 2 animals for each group.
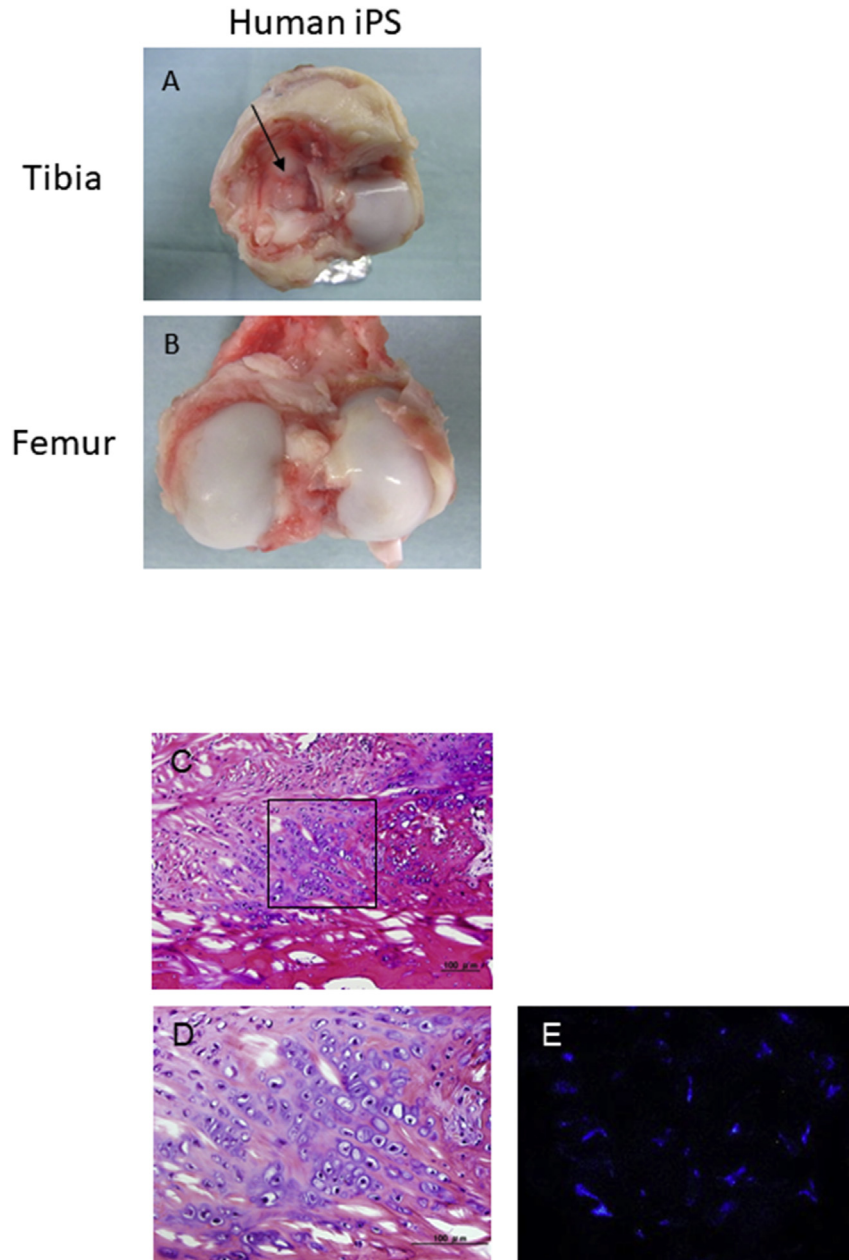
Fig. 8. Macroscopic and histological findings of knee joints 4 weeks after transplantation with human iPS cell-based transplants. Tibial (A) and femoral (B) sides of knee joint transplanted with beta-TCP/PLLA scaffold with human iPS cells. Granulation is indicated by an arrow. (C) Hematoxylin-Eosin staining of regenerative cartilage. (D) Magnification of squared are in figure D. (E) FISH analysis of the same area as figure E. Scale bars = 100 μm.

differentiation culture were not sufficient, but effective for committing MSCs to the chondrocyte lineage.

Using pigs as the animal model, we aimed to determine whether the iPS-like cells precultured under the 3D hydrogel embedding condition could contribute to cartilage regeneration without tumor formation. Transplantation of 3 types of transplants that mimicked joint structures: the beta-TCP scaffold, beta-TCP/PLLA scaffold with MSCs, and beta-TCP/PLLA scaffold with iPS cells into a joint defect at the anteromedial quarter of the knee joint causes no apparent effect on physical status of the animals except the acute reaction in the wounds (Fig. 4).

The macroscopic observation of the knee joints 8 weeks after the transplantation showed that the defects were filled with regenerated tissues, except the animals transplanted with scaffold only (Fig. 5). The shape of the tibial plateau was retained better in the animals transplanted with iPS cells than those with MSCs. The difference in the smoothness of the tibial surface may have affected the extent of the eburnation in the femoral surface, although the effectiveness of each treatment must be evaluated in much longer period.

Histological analysis indicated cartilage formation in the animals transplanted with MSCs or iPS-like cells (Fig. 6A). FISH analysis showed that the regenerative cartilage was negative for Y chromosome, indicating that the transplanted porcine iPS-like cells from female pigs differentiated to chondrocytes in vivo (Fig. 6B). Because in vitro 3D culture failed to induce sufficient chondrogenic differentiation of both cell types, it is obvious that the in vivo environment exerts positive effects on chondrogenesis. The

appropriate mechanical stimulus seems to be a key factor for the chondrogenic differentiation of isolated pluripotent cells. A previous study showed that rats with a knee fixation suffered from teratoma formation, while those without fixation had no teratomas and instead had the formation of regenerative cartilage originating from the transplanted ES cells [9]. Other key factors include the soluble factors from the synovial fluid and surrounding tissues. Recent studies have shown that the intra-articular injection of FGF18, a growth factor that exerts a significant anabolic effect on cartilage, in a surgically induced osteoarthritis rat model promotes the repair of joint cartilage lesions by inducing chondrogenesis [21,22]. IGF-1 is also an anabolic growth factor of articular cartilage, which is provided through the synovial fluid and enhances cartilage repair in articular cartilage defect models [23]. PDGF is a potent mitogenic and chemotactic factor for mesenchymal cells, including chondrocytes, and is expected to enhance tissue repair [23,24]. In the present study, the roles of these factors in cartilage regeneration in vivo were not addressed. To improve the in vivo partial cartilage regeneration that was observed in this study and to induce the in vitro maturation of the cartilage, elucidating the mechanisms of action of these factors during the regeneration of cartilage will be important.

As a cell source of cartilage regeneration, MSCs have been intensively studied [2,25–27]. In this study, porcine MSCs were analyzed together with porcine iPS-like cells for a comparison in terms of chondrogenic ability. The porcine iPS-like cells showed slightly better results than the porcine MSCs, as indicated by the macroscopic view and histological evaluation (Figs. 5 and 6).

Human iPS cells resemble mouse epiblast cells, showing flattened colony figures and being maintained via the FGF2 pathway. Thus, human iPS cells are understood to be primed stem cells. The primed stem cells have functional distinctions, such as the inability to contribute to blastocyst chimeras, from naïve stem cells [39–41]. These facts suggest that human iPS cells may have difficulties in differentiation [49,50]. Lastly, we examined whether human iPS cells could regenerate cartilage only via the stimulation of atelocollagen embedding and transplantation. As the result, regenerative cartilage was observed within transplanted site histologically (Fig. 8). Y-chromosome probe was not detected at the regenerative cartilage by FISH, suggesting that the regenerative cartilage originates from the transplanted human iPS cells. The amount of cartilage regeneration was less than that by porcine iPS cells judged from macroscopic view and histological analysis. One explanation for this is that immunosuppressant treatment with tacrolimus may not have been strong enough, and some of the transplanted human iPS cells may have been rejected by the porcine immune system [43–45]. In addition, the 4-week transplantation time is shorter than that of porcine iPS cells. Anyway, our results show that human iPS cells can differentiate into regenerative cartilage in vivo without tumor formation. However, a larger sample size and longer observation period are needed to confirm the safety of this treatment.

Immunologic mismatch may be critical for the survival of transplanted iPS cells. In the field of clinical organ transplantation, matching HLA haplotypes, especially HLA-A, HLA-B and HLA-DR, between the host and donor is critical to minimizing the risk of rejection. In this study, a syngeneic CLAWN pig was used to avoid any immunoreaction [31–33]. In clinical settings, autologous transplantation is preferred to allogenic transplantation, even though cartilage is immuno priviledged [34,35]. However, the duration and cost of transplantation are problematic for the establishment of iPS cells from each patient. Alternatively, the use of HLA haplotype homozygous iPS cells is being considered for clinical application. The banking of such cells is ongoing, and more than 90% of all Japanese people are estimated to be covered [36,37].

5. Conclusion

In conclusion, this study showed the possibility of using minimally treated iPS cells in combination with beta-TCP/PLLA scaffolds for the regeneration of cartilage in miniature pig osteochondral replacement model. Safety, effectiveness and superiority of iPS cells should be further confirmed by increasing the number of studies for longer period.

Conflicts of interest

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