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Hyaline cartilage formation and tumorigenesis of implanted tissues derived from human induced pluripotent stem cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are a promising cell source for cartilage regenerative medicine. Meanwhile, the risk of tumorigenesis should be considered in the clinical application of human iPSCs (hiPSCs). Here, we report *in vitro* chondrogenic differentiation of hiPSCs and maturation of the differentiated hiPSCs through transplantation into mouse knee joints. Three hiPSC clones showed efficient chondrogenic differentiation using an established protocol for human embryonic stem cells. The differentiated hiPSCs formed hyaline cartilage tissues at 8 weeks after transplantation into the articular cartilage of NOD/SCID mouse knee joints. Although tumors were not observed during the 8 weeks after transplantation, an immature teratoma had developed in one mouse at 16 weeks. In conclusion, hiPSCs are a potent cell source for regeneration of hyaline articular cartilage. However, the risk of tumorigenesis should be managed for clinical application in the future.

Osteoarthritis (OA) is the most common joint disease characterized by cartilage degradation (8). Because articular cartilage has a poor regenerative capacity, a regenerative method is one of the possible medical treatments for OA (1). In previous studies, articular chondrocytes or somatic stem cells, including bone marrow mesenchymal stem cells, have been used as potent cell sources (6). These cells are useful for repairing focal defects in articular cartilage, but some invasive procedures are required to collect them, such as arthroscopic surgery or bone marrow aspiration. To apply these cells for extensive cartilage repair in OA joints, their limited proliferative potential *in vitro* should be addressed (2, 3). Alternatively, induced pluripotent stem cells (iPSCs) have been considered as one of the promising cell sources for regenerative medicine because of their pluripotency and proliferative activity. In addition, iPSCs are free from ethical concerns that are inevitable when using embryonic stem cells (ESCs). Several studies have shown the potential of iPSCs in cartilage regenerative medicine (9, 12–14). On the other hand, the risk of tumorigenesis should be considered in the clinical application of human iPSCs (hiPSCs) (13).

Recently, Oldershaw *et al.* reported a novel method for differentiation from human ESCs (hESCs) toward chondrocytes (7). Their protocol, which is based on the known developmental progression, uses seven cytokines to direct stepwise differentiation of hESCs during 14-days *in vitro* culture (7). Although the protocol has excellent efficiency and specificity, further *in vivo* maturation and tumorigenesis of the

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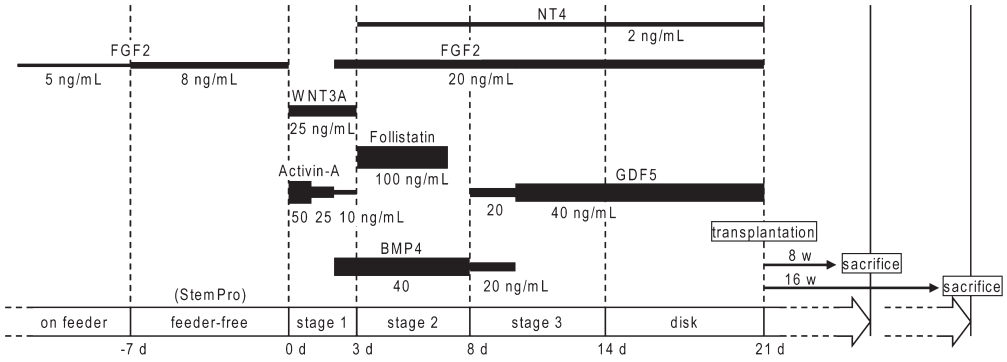

Fig. 1 Schema of hiPSC differentiation. After 1 week of culture under feeder-free conditions in STEMPRO medium, hiPSCs were differentiated with cytokines at the stated concentrations. At day 14, the differentiated hiPSCs were seeded in the cylinder for disk formation. At day 21, the cartilage disks were transplanted into the knee joints of NOD/SCID mice.

differentiated cells have not yet been examined.

In the present study, we subjected three hiPSC clones to the aforementioned chondrogenic differentiation protocol. Cartilage disks were then formed from the differentiated cells and transplanted into mouse knee joints to examine cartilage maturation *in vivo* and the risk of tumorigenesis.

MATERIALS AND METHODS

Cell culture. We isolated mouse embryonic fibroblasts (MEFs) as described previously (11). All hiPSC lines were established from human neonatal dermal fibroblasts (Lonza, Basal, Switzerland) as described previously (4). hiPSCs were cultured on mitomycin C-inactivated MEFs as feeder cells in ES medium (Knockout DMEM (Gibco) supplemented with 15% knockout serum replacement (Gibco), 2 mM L-glutamine (Gibco), 1% (vol/vol) nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), 50 U/mL penicillin (Sigma), 50 μg/mL streptomycin (Sigma)) with 5 ng/mL basic fibroblast growth factor (bFGF; PeproTech). For differentiation, hiPSCs were first cultured under feeder-free conditions using STEMPRO medium (Invitrogen) on a dish coated with Matrigel (BD Biosciences) for 1 week. To induce chondrogenic differentiation, hiPSCs were cultured in a basal medium (DMEM:F12, 2 mM L-glutamine, 1% (vol/vol) insulin-transferrin-selenium (ITS), 1% (vol/vol) nonessential amino acids, and 2% (vol/vol) B-27, 90 μM 2-mercaptoethanol) containing appropriate growth factors as previously described (7). In stage 1, 3×10^5 hiPSCs were differentiated to a primitive streak-mesendoderm state by Wingless-type MMTV integration site family member 3A (WNT3A) and activin A for 3 days in a Matrigel-coated 35-mm dish. At day 3, fibroblast growth factor 2 (bFGF) and bone morphogenetic protein 4 (BMP4) were added to the cells. In stage 2, the cells were passaged onto a Matrigel-coated 60-mm dish and cultured with bFGF, BMP4, follistatin, and neurotrophin 4 (NT4) for differentiation to mesoderm; follistatin was removed at day 8. In stage 3, the cells were passaged onto a Matrigel-coated 100-mm dish and differentiated toward chondrocytes. bFGF and NT4 treatments were continued, and BMP4 was replaced with growth differentiation factor 5 (GDF5). The specific concentrations of the growth factors are shown in Fig. 1.

Real-time RT-PCR. Total RNA was isolated with an RNeasy Mini kit (Qiagen), and 1 μg total RNA was reverse transcribed with a QuantiTect RT kit (Qiagen) according to the manufacturer's protocols. Real-time RT-PCR was performed on a Thermal Cycler Dice (Takara, Otsu, Japan) using FastStart Universal SYBR Green Master (Roche). The mRNA copy number of each specific gene was calculated using a standard curve generated from serially diluted plasmids containing PCR amplicon sequences. The copy number was normalized to human total RNA (Applied Biosystems, Foster City, CA, USA) with human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal control. All reactions were performed in triplicate. Primer sequence information is shown in Table 1.

Disk formation and transplantation. To form a cartilage disk for transplantation, after 2 weeks of differentiation 2×10^6 cells were seeded into a 5 mm-diameter cylinder (internal diameter: 3.4 mm) set on a permeable membrane insert with a pore size of 0.4 μm (Corning). After 1 week of culture, the cylindrical cartilage tissue was cut off by a biopsy

Table 1 *List of primers used for real-time RT-PCR*

Gene	Accession No.	Sequence
NANOG	NM_024865	ttccttcctccatggatctg tctgctggaggctgaggtat
OCT4	NM_002701	gaaggatgtggtccgagtgt gtgaagtgagggctcccata
GSC	NM_173849	cggtcctcatcagaggagtc tcgtctgtctgtgcaagtcc
MIXL1	NM_001282402	cactgtgctcctggaactga tgagtccagctttgaaccaa
T	NM_003181	acccagttcatagcggtgac atgaggatttgcaggtggac
KDR	NM_002253	gtgaccaacatggagtcgtg tgcttcacagaagaccatgc
PDGFRB	NM_002609	gcacttttatccacccagga gtacttggctcagcctccag
SOX9	NM_000346	tacgactacaccgaccacca tcaaggtcgagtgagctgtg
SOX6	NM_017508	gagtcgggaccgtgagataa agctgggtaatcatggtgga
COL2A1	NM_001844	ggtggcttccatttcagcta taccggtatgtttcgtgcag
ACAN	NM_001135	acagctggggacattagtgg gtggaatgcagaggtggttt
GAPDH	NM_002046	gaaggtgaaggtcggagtca gaagatggtgatgggatttc

punch (1 mm in diameter) and transplanted into a full thickness cartilage defect (1 mm in diameter) created in the medial femoral condyles of 12-week-old NOD/SCID mice (CLEA Japan, Tokyo, Japan). Transplantations were performed using 36 mice (12 mice for each hiPSC clone), and their femurs were collected at 8 (n = 21) or 16 weeks (n = 15) after transplantation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. The protocol was approved by the Animal Care and Use Committee of The University of Tokyo (Permit Number: M-P12-131). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Histological analyses. The prepared cartilage disk was fixed with 4% paraformaldehyde phosphate buffer solution (PFA/PBS), embedded in paraffin, sectioned (5 μm in thickness), and stained with toluidine blue solution (Wako, Osaka, Japan). Femurs were fixed with 4% PFA/PBS, decalcified in 20% EDTA/PBS for 2 weeks, embedded in paraffin, and sectioned (5 μm). Safranin O staining, and hematoxylin and eosin (H&E) staining were performed according to a standard protocol. For immunofluorescence, de-

Table 2 *Scoring for histological evaluation*

Cellular morphology	Score
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0

Safranin-O staining of the matrix	Score
Normal or nearly normal	3
Moderate	2
Slight	1
None	0

paraffinized sections were boiled in citrate buffer (10 mM, pH 6.0) for 15 min, blocked with blocking reagent (Roche) for 1 h, and then incubated with an antibody against human vimentin (Abcam). Signals were detected with a TSA Plus Fluorescence System (PerkinElmer Life Sciences). Histological scores for cellular morphology and safranin O staining were determined according to a previous study (Table 2) (10).

RESULTS

Chondrogenic differentiation of hiPSCs

hiPSCs were initially cultured under feeder-free conditions using STEMPRO medium for 1 week, and differentiated toward chondrocytes (Fig. 1). We then analyzed the expression of marker genes during differentiation of clone #1 by real-time RT-PCR. *NANOG* and *OCT4* expression was decreased in earlier stages, and expression of primitive streak marker genes (*GSC*, *MIXL1*, and *T*) was decreased in stage 2 (Fig. 2A). In terms of mesoderm marker genes, *KDR* was gradually decreased while *PDGFRB* was increased during the middle and late stages (Fig. 2A). Expression of chondrocyte marker genes, including *SOX9*, *SOX6*, *COL2A1*, and *ACAN*, was increased in the late stage (Fig. 2A). Clones #2 and #3 showed similar capacities for chondrogenic differentiation at the end of stage 3 (Fig. 2B). These results indicated that the previously established protocol for chondrogenic differentiation was applicable to both hiPSCs.

Preparation of cartilage disks using differentiated hiPSCs

To examine further maturation and safety of the differentiated cells *in vivo*, we planned to transplant them into articular cartilage of immunodeficient mice. We then seeded differentiated hiPSCs (2×10^6) into a 5-mm-diameter cylinder in a transwell dish with a permeable membrane to prepare a cartilage disk (Fig. 3A). After 1 week of culture, a 1-mm cy-

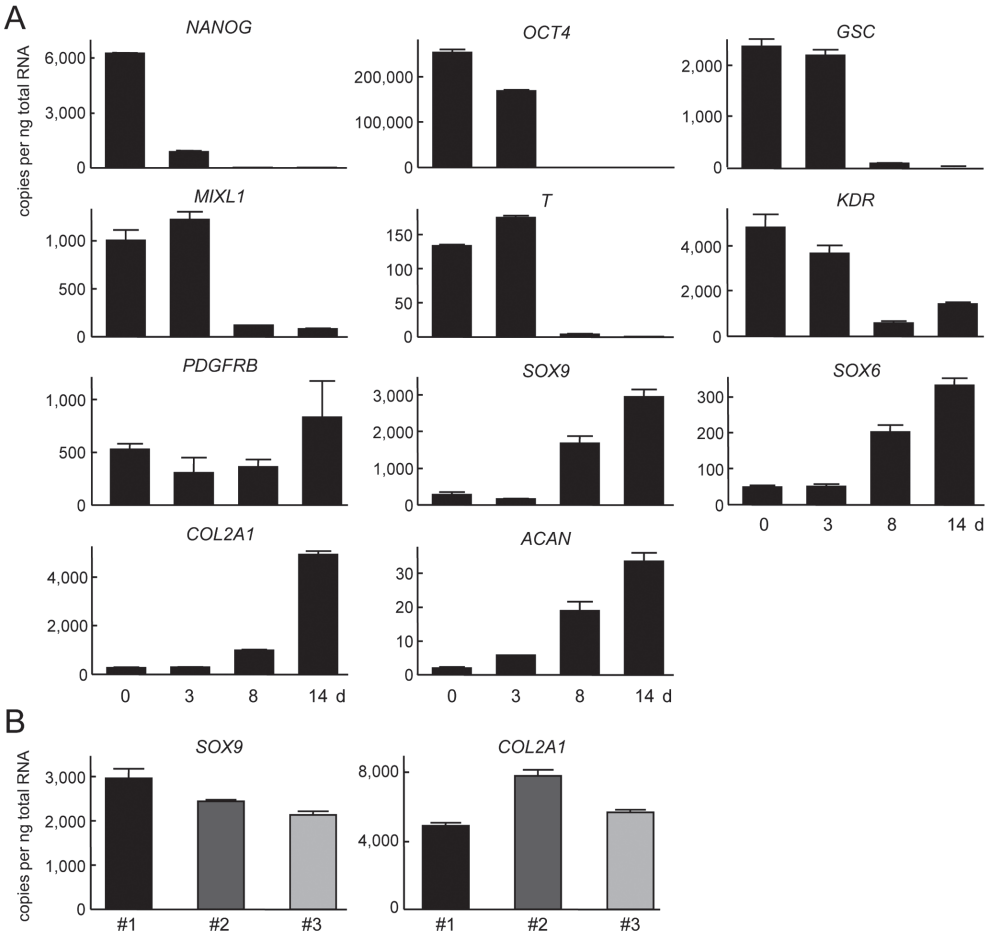

Fig. 2 Chondrogenic differentiation of hiPSCs. (**A**) mRNA levels of marker genes during the 2 weeks of differentiation of hiPSC clone #1. Data are expressed as the means ± SD of three wells/group. (**B**) mRNA levels of *SOX9* and *COL2A1* in the three hiPSC clones (#1–3) at the end of stage 3. Data are expressed as the means ± SD of three wells/group.

lindrical cartilage disk was cut off by a biopsy punch and transplanted into a 1-mm cartilage defect created in the medial femoral condyles of 8-week-old NOD/SCID mice (Fig. 3A). The cell density in the cartilage disk was relatively uniform without large defects or abnormal proliferation after 1-week-culture (Fig. 3B). The expression levels of chondrocyte marker genes were upregulated in all hiPSC clones after disk formation (Fig. 3C).

Transplantation of cartilage disks prepared from differentiated hiPSCs

At 8 weeks after transplantation, we examined *in vivo* cartilage tissue maturation of the chondrocytes derived from hiPSCs. Cartilage tissue formation or regeneration was not observed in cartilage defects without disk transplantation (Fig. 4A). In contrast, a large amount of hyaline cartilage was observed in defects with disk transplantation (Fig. 4A). Immunofluorescence using a specific antibody against human vimentin showed that the hyaline cartilage tissue was derived from human cells (Fig. 4A). There were no significant differences in histological scores of cellular morphology and safranin O staining among the hiPSC clones (Fig. 4B).

To examine the further course of the transplanted tissues, the observation period was extended to 16 weeks. Similar to the results at 8 weeks, a large amount of hyaline cartilage formation was observed in defects transplanted with disks, whereas cartilage tissue formation or regeneration was not observed in defects without disk transplantation (Fig. 4C). Immunofluorescence of human vimentin confirmed that the origin of the hyaline cartilage tissue was human cells (Fig. 4C). There were no significant differences in the histological scores of cellular morphology or safranin O staining among the hiPSC clones (Fig. 4D).

Although tumorigenesis or abnormal tissue forma-

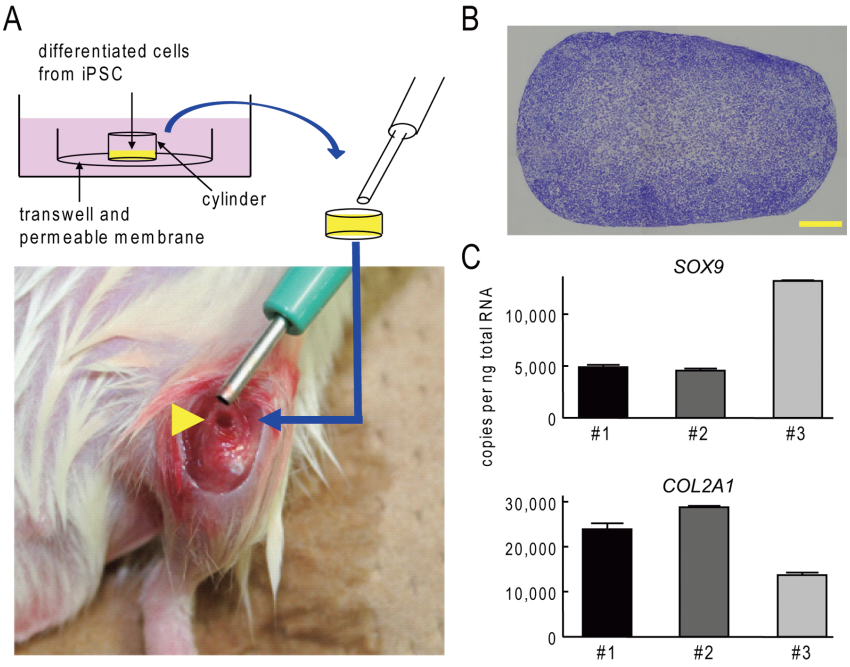

Fig. 3 Transplantation of cartilage disks derived from hiPSCs. (**A**) Differentiated cells were seeded into a 5-mm-diameter cylinder set on a permeable membrane insert. After 1 week of culture, cylindrical cartilage tissue was cut off by a biopsy punch (1 mm in diameter) and transplanted into full thickness cartilage defects (arrowhead, 1 mm in diameter) created in the medial femoral condyles of NOD/SCID mice. (**B**) Histology of a cartilage disk prepared for transplantation after 1-week-culture. Scale bars, 200 μm. (**C**) mRNA levels of *SOX9* and *COL2A1* in cartilage disks derived from the three hiPSC clones (#1–3). Data are expressed as the means ± SD of three wells/group.

tion was not observed during the 8 weeks after transplantation, a large tumor had developed in one mouse transplanted with a disk derived from clone #2 at 16 weeks (Fig. 5A). We observed some safranin O-positive cartilage tissues (Fig. 5B); however, the tumor consisted of non-cartilaginous tissues including various kinds of tissues and cells (Fig. 5C), indicating that the tumor was probably generated from immature hiPSCs.

DISCUSSION

In the present study, we showed that hiPSCs differentiated by a previously established protocol for hESCs could form matured cartilage tissue *in vivo*. The hiPSCs underwent *in vitro* chondrocyte differentiation by the growth factors similarly to the hESC. The differentiated hiPSCs formed cartilage disks at 1 week in high-density three-dimensional culture and developed further into hyaline cartilage tissues in mouse knee joints. The expression patterns of marker genes during the *in vitro* differentiation as well as the histological findings of the transplanted tissues were similar among the three hiPSC clones. These results indicate that the protocol may be useful as a basic method for cartilage regeneration using hiPSCs.

To apply hiPSCs to regenerative medicine for OA, cartilage tissues should be prepared from differentiated hiPSCs. There have been reports of various kinds of tissue engineering techniques with or without matrix scaffolds. Among them, we prepared a cartilage disk using a cylindrical mold, because a simple scaffold-free method appeared to be suitable to analyze the characters of hiPSCs. Cartilage disks were formed from all hiPSC clones, and 1 week of culture for the disk formation enhanced the mRNA levels of chondrocyte marker genes in all hiPSC clones. These results indicated that cartilage tissue engineering by three-dimensional culture may be applicable to properly differentiated hiPSCs. The maturation of transplanted hiPSCs further supports the efficacy of hiPSCs in cartilage regenerative medicine. However, we did not optimize the culture duration for disk formation or test other methods. In contrast to the maturation, bonding of the transplanted tissue to the adjacent cartilage was observed less frequently, indicating that improvements should be made to the tissue preparation protocol.

Transplantation of iPSC-derived tissues involves

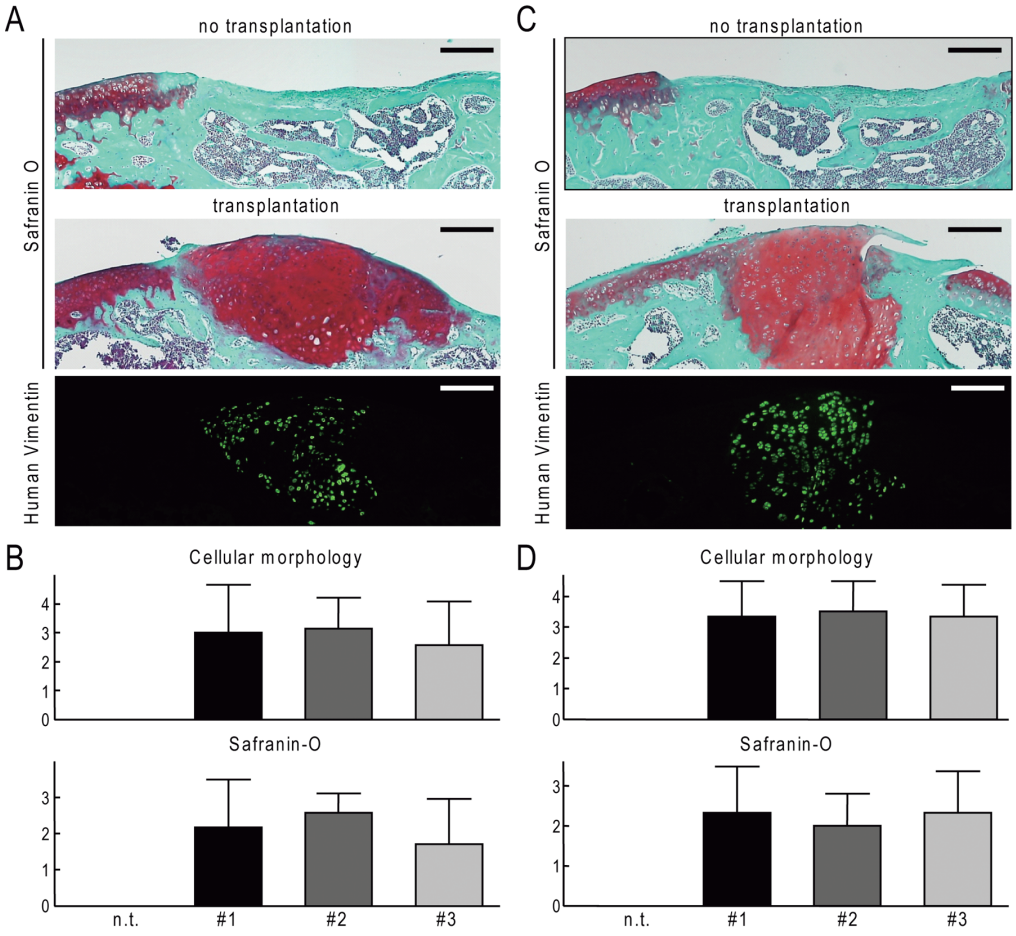

Fig. 4 Maturation of transplanted cartilage disks derived from hiPSCs. (**A**) Representative images of safranin O staining and immunofluorescence of human vimentin in cartilage tissues (hiPSC clone #1) at 8 weeks after transplantation. Scale bars, 200 μm. (**B**) Histological scores of cellular morphology and safranin O staining of transplanted cartilage tissues (hiPSC clone #1–3) and defects without transplantation (n.t.) after 8 weeks. Data are expressed as means ± SD. (**C**) Representative images of safranin O staining and immunofluorescence of human vimentin in cartilage tissues (hiPSC clone #3) at 16 weeks after transplantation. Scale bars, 200 μm. (**D**) Histological scores of cellular morphology and safranin O staining in transplanted cartilage tissue (hiPSC clone #1–3) defects without transplantation (n.t.) after 16 weeks. Data are expressed as means ± SD.

the risk of tumorigenesis that has been observed in a previous study using mouse iPSCs (13). In the present study, a tumor was found in one mouse at 16 weeks after transplantation, indicating an incidence of 6.7%. Considering that no tumors were observed at 8 weeks, the incidence of tumorigenesis may increase when the follow-up period is extended. Because the tumor was an immature teratoma, undifferentiated hiPSCs remaining in the disk had probably developed into the tumor. To prevent tumorigenesis, undifferentiated hiPSCs must be removed after *in vitro* differentiation of a safe hiPSC clone without genomic abnormalities. Recently, Yamashita *et al.* reported a protocol for chondrocyte differentiation from hiPSC in chondrogenic medium supplemented with 1% FBS, ITS, WNT3A, Activin A, bFGF, BMP2, TGFb, and GDF5 (14). They performed three-dimensional culture for 28–70 days to mature the differentiated hiPSCs, and observed no tumor formation in the following transplantation analyses (14). Considering the high nutritional requirement of immature hiPSCs, long-term culture with low FBS may be essential to omit immature hiPSCs, as well as to prepare a matured cartilage tissue *in vitro*. Meanwhile, it is still possible that differentiated hiPSCs form a differentiated tumor such as chondrosarcoma during the longer term using any methods. Novel rescue systems may be necessary to prevent formation of any kind of tumor from transplanted hiPSCs in future.

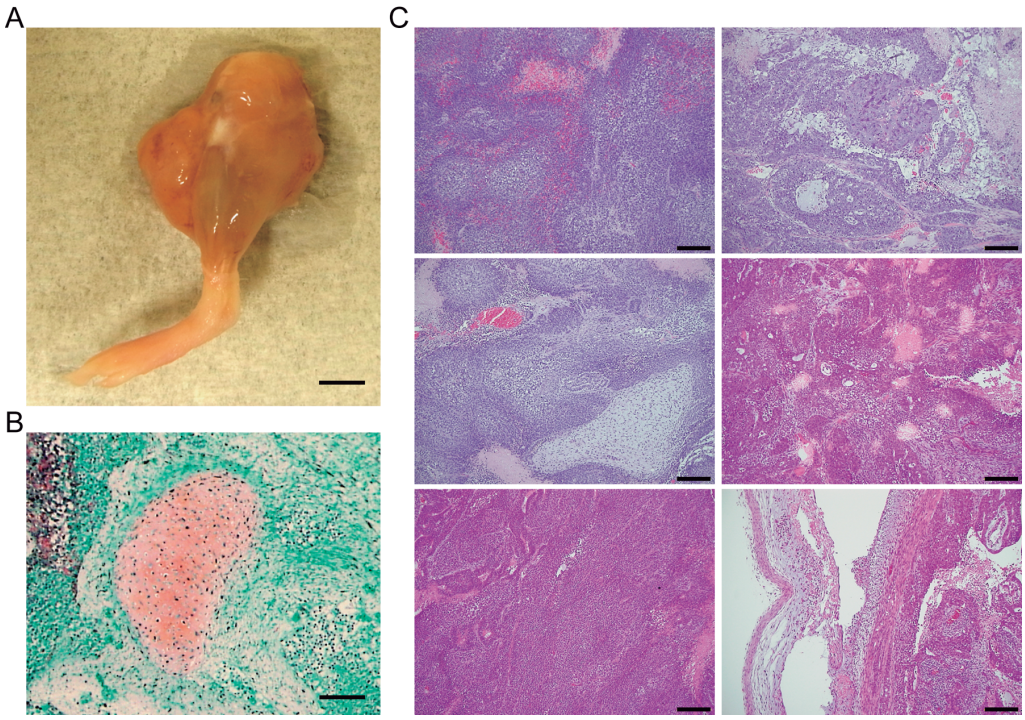

Fig. 5 Teratoma formation in a NOD/SCID mouse transplanted with a cartilage disk derived from clone #3. (**A**) Gross appearance of the knee joint occupied by the tumor. Scale bar, 5 mm. (**B**) Safranin O staining of the tumor. A cartilaginous tissue was stained red in the center. Scale bar, 100 μm. (**C**) H&E staining of the tumor showing various kinds of tissues. Scale bars, 200 μm.

In conclusion, hiPSCs can form hyaline cartilage through *in vitro* differentiation by cartilage disk formation and transplantation into the articular cartilage of mouse knee joints. On the other hand, a risk of tumorigenesis from undifferentiated hiPSCs was found using the present method. For clinical application, we should develop methods to remove undifferentiated or abnormal cells as well as more efficient differentiation and tissue engineering techniques.

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