Biofabrication

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Bio-3D printing iPSC-derived human chondrocytes for articular cartilage regeneration

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Abstract

Osteoarthritis is a leading cause of pain and joint immobility, the incidence of which is increasing worldwide. Currently, total joint replacement is the only treatment for end-stage disease. Scaffold-based tissue engineering is a promising alternative approach for joint repair but is subject to limitations such as poor cytocompatibility and degradation-associated toxicity. To overcome these limitations, a completely scaffold-free Kenzan method for bio-3D printing was used to fabricate cartilage constructs feasible for repairing large chondral defects. Human induced pluripotent stem cell (iPSC)-derived neural crest cells with high potential to undergo chondrogenesis through mesenchymal stem cell differentiation were used to fabricate the cartilage. Unified, self-sufficient, and functional cartilaginous constructs up to 6 cm² in size were assembled by optimizing fabrication time during chondrogenic induction. Maturation for 3 weeks facilitated the self-organisation of the cells, which improved the construct’s mechanical strength (compressive and tensile properties) and induced changes in glycosaminoglycan and type II collagen expression, resulting in improved tissue function. The compressive modulus of the construct reached the native cartilage range of 0.88 MPa in the 5th week of maturation. This paper reports the fabrication of anatomically sized and shaped cartilage constructs, achieved by combining novel iPSCs and bio-3D printers using a Kenzan needle array technology, which may facilitate chondral resurfacing of articular cartilage defects.

1. Introduction

Articular cartilage is a connective tissue covering the ends of bones. It is a load-bearing tissue that lowers friction and protects joints. Owing to its avascularity and aneural nature, articular cartilage has low metabolic activity and self-healing ability [1]. Cartilage damage is a consequence of disease or trauma that can occur at all ages and is a source of pain and loss of function. The progression of focal cartilage defects into degenerative conditions, such as osteoarthritis (OA), can lead to disability. Over 300 million people worldwide are affected by OA, causing a huge clinical and economic burden [2]. Current surgical treatments, including microfractures and autologous and allogenic implantations [3], address only focal defects and do not generate long-lasting functional cartilage. Total joint replacement (TJR) is the only treatment for symptomatic OA. However, TJR has limitations such as bone shortening, significant complications, and lack of long-term durability [4, 5].

Tissue engineering is considered an alternative therapeutic strategy for cartilage repair and joint preservation. Although controlling the shape and positioning of cells is a promising approach for cartilage repair, there is a lack of functional cell types and biomaterials. To date, chondrocytes and mesenchymal stem cells (MSCs) are considered the most promising sources of cells for regeneration therapy. Despite many advantages, both cell types have significant limitations. Donor age, density, activity, and senescence of the cells influence differentiation potential,
immunomodulatory properties, and migration abilities, thus restricting their use for some patients. The application of chondrocytes is limited by phenotype instability and spontaneous dedifferentiation occurring during expansion.

Induced pluripotent stem cells (iPSCs) are an alternative cell source that overcomes the limitations associated with loss of cell phenotype. Their self-renewal potential provides an almost unlimited number of cells with pluripotent capacities. While engineering autologous transplants from patient-specific iPSCs is possible [6], preparation remains very costly. Therefore, an iPSC library composed of iPSC lines derived from human leukocyte antigen (HLA) homozygous individuals has been established [7, 8]. In addition, a recent study demonstrated the successful development of human iPSC-derived HLA-1-deficient platelets [9], which may open the door to the possibility of resolving HLA-mediated donor shortage.

The potential of iPSCs to undergo chondrogenesis has already been established [10, 11]. iPSCs may carry the risk of tumour formation due to the artificial reprogramming process [12]; therefore, it is important to appropriately differentiate iPSCs toward chondrocytes before transplantation. Recent studies have reported the possibility of differentiating iPSC-derived neural crest cells (iNCCs), through MSC induction (iNCMSCs), toward chondrocytes [10, 13]. iNCCs and derived iNCMSCs can be easily expanded and cryopreserved and are a potent, readily available cell source for tissue engineering applications. Repairing focal articular cartilage defects in vivo with iNCMSC aggregates has been reported [11], although building an anatomically sized and shaped construct feasible for cartilage regeneration remains challenging.

Currently, tissue engineering technologies aimed at cartilage regeneration therapy can be generally classified as scaffold-based and scaffold-free [14, 15]. Scaffold-based methods include 3D printing, light-based technologies, bioextrusion, wet-spin extrusion, ink-jet bioprinting, and electrospinning [16]. Utilizing biomaterials allows for the fabrication of desired structures and provides an initial foundation for cells that is beneficial in highly loaded environments such as those undergone by cartilage [17]. However, the use of biomaterials imparts a rigidity that restricts construct flexibility for fitting into defects, thus causing difficulties during surgical procedures, and can lead to unfavourable outcomes for patients. Another important consideration is the degradability of materials used in traditional bioprinting [18]. Non-degradable or slowly degradable bio-ink may cause complications due to infections and chronic foreign body response. Furthermore, prolonged scaffold presence after transplantation may suppress sufficient extracellular matrix (ECM) deposition and cell migration as is necessary for adequate tissue remodelling during regeneration [19].

The objective of the current study was to utilize a bio-3D printer to engineer a scaffold-free cartilage construct of anatomical size and shape. Optimizing the printing and maturation timing to the unique iPSC chondrogenic potential facilitated the fabrication of a cartilage construct that provides a functional and cost-effective product for cartilage regeneration.

2. Material and methods

2.1. Cell culture and differentiation

Human NCCs derived from human iPSC line 414C2 (iNCCs) kindly provided by Dr Toguchida from the Centre for iPSC Cell Research and Application (CiRA, Kyoto University, Japan) were maintained according to a previously described protocol [13]. iNCCs were cultured in Chemically Defined Medium containing Iscove’s Modified Dulbecco’s Medium (Sigma–Aldrich, St Louis, MO, USA)/Ham’s F-12 (Gibco, Grand Island, NY, USA) (1:1 ratio), 1% chemically defined lipid concentrate (Gibco), 15 µg ml⁻¹ apo-transferrin (Sigma–Aldrich), 5 mg ml⁻¹ bovine serum albumin (BSA; Sigma–Aldrich), 7 µg ml⁻¹ insulin (Wako Pure Chemical Industries, Osaka, Japan), 10 µM SB-431 542 inhibitor (Selleck Chemicals, Huston, TX, USA), 20 µg ml⁻¹ epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA), 20 µg ml⁻¹ fibroblast growth factor (FGF; Wako Pure Chemical Industries) and 50 U ml⁻¹ penicillin/50 µg ml⁻¹ streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured on fibronectin (Millipore, Billerica, MA, USA) and passaged using Accutase cell detachment solution (Wako Pure Chemical Industries). Poietics normal human bone marrow-derived MSCs (hBMMSCs) were purchased from Lonza (Lonza, Walkersville, MD, USA). Cells were expanded and maintained in MSC growth medium (Lonza) according to the protocol. hBMMSCs were passaged with 0.25% Trypsin-ethylenediaminetetraacetic (EDTA) (Gibco) and seeded at 5 × 10⁵ cell cm⁻². Cells were used at passage 4 for experiments. Culture medium was replaced every other day.

2.2. MSC induction

iNCMSCs were obtained by continuous passage and culture of iNCCs in iNCMSC medium containing minimal essential medium Eagle-alpha modification (α-MEM; Nacalai Tesque Inc., Kyoto, Japan), 10% foetal bovine serum (Hyclone, Logan, UT, USA), 5 ng ml⁻¹ basic FGF (Wako Pure Chemical Industries) and 50 U ml⁻¹ penicillin/50 µg ml⁻¹ streptomycin (Invitrogen). Cells were passaged with 0.25% Trypsin-EDTA (Gibco) and used at passage 3. The culture medium was replaced every other day.
2.3. Flow cytometry
Flow cytometry was used to characterize the expression of cell surface markers CD271, CD44, CD45, CD73, CD90, and CD105 (BD Pharmingen Inc., San Diego, CA, USA) (supplementary table 1 (available online at stacks.iop.org/BF/13/044103/mmedia)) in iNCCs and iNCMSCs. Flow cytometry was performed using the BD Accuri C6 Plus Flow Cytometer (BD Bioscience, San Jose, CA, USA) according to the manufacturer’s protocol. Cells suspended in 0.1% BSA/Dulbecco’s phosphate-buffered saline (DPBS; Gibco) were incubated with fluorescence-conjugated antibodies for 30 min at room temperature.

2.4. Chondrogenic induction
Chondrogenic induction was achieved by the addition of three growth factors at specific time points to the Chondrogenic Differentiation Basal Medium (Lonza, Walkersville, MID, USA) supplemented with hMSC Chondrogenic SingleQuots (Lonza). A previously described protocol [13] is illustrated in figure 2(A). During chondrogenic initiation, cells were treated with 40 ng ml⁻¹ platelet-derived growth factor-BB (PDGF-BB; R&D System) from day 0 to day 10 and 10 ng ml⁻¹ transforming growth factor beta-3 (TGF-β3; Peprotech Inc., Rocky Hill, NJ, USA) from day 6 through the rest of chondrogenic maturation. From day 10, cells were treated with 50 ng ml⁻¹ bone morphogenetic protein (BMP)-4 (R&D Systems). Cells were seeded at 1.5 × 10⁴ or 4 × 10⁴ cells/well on ultra-low attachment 96 U-well plates (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) to obtain 500 µm or 700 µm diameter spheroids at day 10 of induction. The chondrogenic differentiation medium was changed initially on days 3, 6, and 10. From day 10, the medium was replaced once every 3 and 4 d. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

2.5. Engineering construct using Bio-3D printer
The Regenova Bio-3D printer (Cyfuse Biomedical KK, Tokyo, Japan) was used to assemble the scaffold-free construct, as previously reported [20, 21]. The bioprinting process is illustrated in figure 1. Briefly, spheroids were automatically aspirated using a nozzle from 96 U-well plates and squared into the Kenzan, which is an array of fine needles. Spheroids were placed in the Kenzan based on a pre-designed configuration. An in-house software was used to convert the 3D model format to one that is compatible with the Kenzan technology and to control spheroid deposition. In this study, 9 × 9 square-type, 26 × 26 square-type, and 7 mm diameter round-type Kenzans were used depending on the experiment. The 9 × 9 square Kenzan is an array of 81 needles; the 26 × 26 square Kenzan is an array of 676 needles arranged in 9 × 9 and 26 × 26 rows and columns to create a rectangle. The 7 mm round Kenzan is an array of a single row of 36 needles arranged in a 7 mm diameter circle. The diameter of the spheroids used for printing both the 9 × 9 and 26 × 26 Kenzans was about 550 µm; for the 7 mm round Kenzan, it was 700 µm.

2.6. Optimization of fabrication
In the 1st attempt to create the construct, spheroids from day 26 of chondrogenesis/spheroid formation were used. A total of 24 spheroids were printed in two layers of three rows and two columns each to create a cube-shaped construct. The construct was cultured on the Kenzan for an additional 20 d and then fixed and analysed.

In the 2nd attempt, to optimize fabrication time, spheroids from days 3, 6, or 10 of chondrogenesis/spheroid formation were used to assemble the constructs. In total, 81 spheroids were printed in nine layers of one row and nine columns each to create a sheet-shape construct. Each of the constructs was returned to the culture medium to continue chondrogenic differentiation. After removing from the Kenzan on day 20 of chondrogenic induction, the constructs were cultured in a bioreactor for an additional 36 d, then fixed and analysed.

2.7. Comparison of chondrogenesis in spheroids and constructs
To compare chondrogenesis efficiency in spheroid cultures and the constructs, spheroids from day 10 of chondrogenesis were randomly divided into two groups; the culture of one was continued in 96 U-well plates, and the other group was printed on the 7 mm round Kenzan. Spheroids from individual cultures and the constructs were fixed before and right after printing (day 0), then 3, 6, and 21 d after printing. The culture medium was concurrently changed for individually cultured spheroids and constructs. The same spheroids number (36) were used in the DNA quantity and biochemical evaluations for glycosaminoglycans (GAGs) deposition in both groups.

The amount of DNA was measured using the CyQuant Cell Proliferation Assay (Invitrogen, Eugene, OR USA) according to the manufacturer’s instructions after papain-digestion. After CyQuant GR dye/cell-lysis buffer was added to the samples, fluorescence was measured with ~480 nm to ~520 nm filters using a GloMax Microplate Reader (Promega, Madison, WI, USA).

2.8. Comparison of chondrogenesis in iNCMSC and BMMSC constructs
To compare the chondrogenesis efficiency of the constructs fabricated using iNCMSCs with BMMSCs, 180 spheroids were prepared simultaneously from the same number of cells (4 × 10⁴). Constructs were printed using day 10 spheroids. Five layers of 36 spheroids per layer were printed on 7 mm round Kenzan to create ring constructs. The constructs were cultured on the Kenzan 21 d after printing (31 d of chondrogenesis), then fixed and analysed.
The chondrogenic differentiation protocol described above was used for iNCMSC and BMMSC construct maturation.

2.9. Optimization of the maturation period
To evaluate the optimal time for construct maturation, 81 spheroids were printed in nine layers of one row and nine columns each to create a sheet-shape construct. Constructs fabricated using spheroids from day 10 of chondrogenesis were removed from the Kenzan needle array 7 d later, and then cultured in a chondrogenic differentiation medium for an additional 2–5 weeks. The medium was replaced every 3 d. Constructs were stored at $-80^\circ$C until biochemical evaluation.

To evaluate the potential of the construct to self-remodel after removing the Kenzan, two iNCMSC constructs were printed on 7 mm round Kenzan. Thirty-six spheroids from day 10 of chondrogenesis were used to print 40 layers construct. Twenty days after printing, constructs were removed from the Kenzan and cultured on a custom-made 5 mm diameter polydimethyldsiloxane (PDMS) tube in the bioreactor for an additional 9 or 43 d.

2.10. Histological and immunohistochemical examination
Spheroids or constructs were fixed in 10% formalin neutral buffer solution (Wako Pure Chemical Industries) for 24 h at 4 °C, washed three times with PBS, and then dehydrated using a graded ethanol series. The samples were then embedded in paraffin wax, cut into 5 μm thick sections, and stained with 0.1% SOFG (Nacalai Tesque Inc.), 0.05% fast green solution (Tokyo Chemical Industry, Tokyo, Japan), goat anti-type I collagen-UNLB (Southern Biotech, Birmingham, AL, USA), or goat anti-type II collagen-UNLB (Southern Biotech) antibodies to detect GAGs, type I and type II collagen, respectively. Samples were then treated with DAB solution (Agilent Technologies, Tokyo, Japan), mounted, and imaged using a BZ-X700 microscope (Keyence, Osaka, Japan). Porcine native tissue samples were isolated from the femoral condyle of a miniature pig and processed in the same manner.

The TUNEL assay was performed using an ApopTag Peroxidase in situ Apoptosis Detection Kit S7100 (Millipore Corp., CA USA) following their protocol.

2.11. Mechanical characterization
To measure the compressive modulus, stress was applied to the construct in uniaxial, unconfined compression tests using a ZTS digital force gauge (IMADA Co, Ltd, Toyohashi, Japan), and the amount of deformation was measured. The construct’s area was recorded before and at the highest point of stress application. The recorded area was recognized, measured, and calculated using an in-house software.

The suture pull-out test was performed using a uniaxial tension apparatus (Tissue Puller 560TP; DMT, Aarhus, Denmark). 6-0 nylon sutures (Alfresa Pharma Corporation, Osaka, Japan) were passed through the construct at 2 mm from the edge of the
opposite sides. The uniaxial pull-apart test was performed at a speed rate of 0.015 mm s$^{-1}$ until the suture tore from one side of the construct.

2.12. Biochemical characterization
To examine GAG distribution, constructs were digested for 3 h at 60°C with 125 µg ml$^{-1}$ papain (Sigma-Aldrich) in a mixture of 0.05 M PBS, 5 mM cysteine hydrochloride, and 0.05 M EDTA acid, at pH 6.0, using approximately 1 ml/construct. To quantify type II collagen distribution, constructs were subjected to guanidine chloride (GuCl) extraction for 24 h following three repeated digestions with pepsin (Chondrex Inc., Redmond, VA, USA) and digestion with elastase (Chondrex Inc.) for 24 h. GAG and type II collagen contents were measured using a GAGs Assay Kit (chondrex) and type II collagen detection Kit (chondrex), respectively, according to the manufacturer’s protocols. Absorbance was measured using a GloMax Microplate Reader (Promega). The GAG and type II collagen content were calculated based on the standard curve and normalized to the construct wet weight or µg DNA.

2.13. Fabrication of constructs of different shapes
A 9 × 9, 26 × 26, 7 mm diameter round, or two 26 × 26 type Kenzan needle arrays were used to create cube-, sheet-, rectangular-, L-, tube-, or articu-
lar surface-shape constructs, respectively. The diameter of the spheroids used for printing 9 × 9 and 26 × 26 type Kenzans was about 550 µm and included 1.5 × 10⁴ cells. The diameter of the 7 mm round Kenzan was about 700 µm and included 4 × 10⁴ cells.

All constructs were fabricated using spheroids from day 10 of chondrogenesis. The maturation of all constructs was performed in bioreactors equipped with a flow system. The size of the bioreactor and volume of the maturation medium were adjusted depending on the total cell number used for construct formation.

For L-shape construct that include two square faces joined at 90º angles, we used 544 spheroids printed on 32 needles in two rows of 17 layers for each face. The construct was removed from the Kenzan 18 d after printing; 40 d after printing, it was fixed and stained. The tube-shape construct was built with 1800 spheroids arranged in 50 layers of 36 spheroids each. The construct was cultured on the Kenzan for an additional 36 d before fixing and staining. The articular cartilage surface-shape construct was composed of 1677 spheroids placed on needles according to the design. Design was obtained by translating porcine distal femoral condyle and groove scan data. Mini pig was purchased from the Nippon Institute for Biological Science. Spheroids from day 10 of chondrogenesis were used to print two constructs. The two separately printed constructs were then joined and cultured in the originally 3D-printed foundation built into the bioreactor for 15 d. After being removed from the Kenzan, construct was cultured for the following 24 d.

2.14. Statistical analysis
All values are expressed as means (±SD). The results shown are representative of three independent experiments conducted in triplicate. For comparison of chondrogenesis in spheroid and construct (figure 5(A)) one-way ANOVA followed by Scheffe multiple comparisons post-test was performed. For statistical analysis of construct maturation optimizations (figure 7) Student’s t-test were performed. Differences between experimental groups with a *P*-value < 0.05 and **P*-value < 0.001 were considered statistically significant.

3. Results
3.1. Characterization of induced iNCMSCs
iNCMSCs were collected using a well-defined differentiation protocol (supplementary figure 1(A)). Cell morphology and size changed drastically after the change from iNCC to iNCMSC culture medium (data not shown). Expression of the neural crest marker CD271 was rapidly lost, and the expression pattern of MSC markers CD44, CD73, and CD105 became comparable with that for human bone marrow cells (supplementary figure 1(B)) in a similar manner to that reported in a previous study [13]. Due to progressively decreasing MSC marker CD90 expression through the differentiation process (supplementary figure 1(B)), we used passage three cells in all experiments to maintain a homogeneous population.

3.2. Chondrogenic potential of iNCMSCs
The potential to undergo chondrogenic differentiation was first confirmed in the spheroid culture. Chondrogenic induction could be observed as a gradual and continuous increase of spheroid diameter (figure 2(B)). At day 39 of chondrogenesis, whole spheroid imaging revealed deposition of two cartilage-specific markers, SOFG, detecting proteoglycan and type II collagen (figure 2(C), supplementary figure 2). Type I collagen signal was also present; it was prominent only in the outside area of the spheroids. The spheroids exhibited a homogeneous consistency, and necrotic areas were not observed in the middle of the aggregates.

3.3. Fabrication time optimization
In the 1st attempt to assemble the chondrogenic construct, fully matured spheroids from day 26 of chondrogenesis could not create an integrated structure. After an additional 20 d of maturation, individual spheroids were still observed on microscopic images of the construct (figure 3).
Figure 2. iNCMSCs chondrogenic differentiation. (A) Schematic protocol for chondrogenic induction. Three growth factors: PDGF-BB, TGFβ3 and BMP-4 were added to Chondrogenic Differentiation Basal Medium + Chondrogenic SingleQuots Kit Supplement at specific time of induction. (B) Chondrogenic differentiation in the spheroid culture. Increase of spheroid diameter in time dependent manner over a 31 d period of induction. (C) Histological evaluations of iNCMSCs spheroids. Representative images of spheroids at day 39 of chondrogenesis stained with safranin O (SOFG), types I and II collagen. Images of histological evaluations were taken at ×4, ×10 and ×20 magnification. Scale bars represent 500 µm on all magnification images.

In the 2nd attempt, spheroids formed during the 1st 10 d of differentiation was used to assemble constructs. Constructs printed at three different time points were compared to evaluate the optimal fabrication time (figures 4(A) and (B)). Macroscopic observations showed that at day 26 of chondrogenesis, the constructs were of significantly different sizes (figure 4(A)). Six days after removal from the Kenzan, the day 3 constructs showed a dramatic deformation, while the day 6 constructs maintained the shape and size provided by the Kenzan needles. The day 10 constructs showed no structural deformation and the highest integrity with the most prominent thickness and smoothness (figure 4(A)).

Histological evaluation showed that the constructs fabricated on day 10 of induction exhibited uniform consistency with equally distributed cartilage structure, with no observable boundaries between spheroids. In contrast, individual spheroid positions could be observed in constructs from day 3 and day 6 (figure 4(B)). Day 10 constructs showed the strongest SOFG signal with the highest number of chondrocytes with lacunae (figure 4(B)) which is
3.4. Comparison of chondrogenesis in spheroid and construct

A comparison of spheroids cultured individually and spheroids that existed inside the constructs for a 3 week period after printing revealed no significant differences in DNA quantity between the two groups (figure 5(A)). Histological evaluations showed a slightly elevated level of TUNEL-positive cells present around the holes right after printing (day 0). The TUNEL-positive area observed in printed spheroids did not vary significantly from that observed in individually cultured spheroids at days 3, 6 and 21 (figure 5(B)). Proteoglycans deposition increased in a time-dependent manner was similar in both groups of spheroids (figure 5(B)). Although quantitative biochemical evaluations of GAGs confirmed the histologically observed gradual deposition, they also revealed markedly increased deposition in the individually cultured spheroids compared to the spheroids located inside the construct (figure 5(A)). Type I collagen was strongly expressed in both groups of spheroids over three weeks, which is compatible with the previous notion that the reduction of type I collagen expression occurs in the individually cultured spheroids on day 70 and not on day 40 of chondrogenesis [11]. Deposition pattern was transitioning in both groups. First, homogeneously expressed, 21 d after printing relocated into outside area of individual spheroid. Inside the construct, type I collagen contents were located around needles and close to the outer rims (figure 5(B), supplementary figure 3). Type II collagen deposition increased in both groups of spheroids in time dependent manner.

Data suggest that spheroids from both groups trend toward hyaline cartilage rather than fibrocartilage.

3.5. Comparison of chondrogenesis in iNCMSC and BMMSC constructs

To compare the efficiency of iNCMSCs to that of other cell sources to create a functional construct, simultaneously fabricated iNCMSC and BMMSC constructs were analysed.

Macroscopic observations revealed that the length of the iNCMSC construct did not change during the 1st 3 weeks after printing. In contrast, the BMMSC construct showed a steady decrease in size during maturation. Interestingly, the thickness of the iNCMSC construct gradually increased during the 1st 3 weeks of maturation (figure 6(A)). Histological evaluations confirmed the difference between the thicknesses of the two constructs and revealed functional superiority of iNCMSCs over BMMSC. Homogeneous and intense staining of ECM with SOFG and type II collagen was observed in the iNCMSC construct, while it was sporadic and weak in the BMMSC.
Figure 4. Fabrication time optimization. (A) Gross images of constructs on the Kenzan and 6 d after removal from the Kenzan. (B) Histological evaluations of constructs fabricated using spheroids from day 3, 6 and 10 of chondrogenesis and porcine native articular cartilage. Constructs were removed from the Kenzan at day 20 of chondrogenesis and cultured for additional 36 d. Images of histological evaluations were taken at ×4 and ×20 magnification. Scale bars represent 1000 µm on ×4 and 500 µm on ×20 magnification images.

construct (figure 6(B)). TUNEL evaluations revealed a higher level of damaged cells around the holes after printing in the BMSC construct (figure 6(B)) than in the iNCMSC construct.

3.6. Construct maturation optimization
After validating the printability of iNCMSCs, constructs were placed in long-term culture to investigate the minimum maturation period necessary to increase their functionality.

Printed constructs analysed at five different time points after printing showed increased deposition of GAGs and type II collagen matrix in a time-dependent manner (figure 7). The GAG content of the construct from the 3rd week of maturation was 41.9 µg mg⁻¹ and reached a plateau. The GAG content increased almost three-
Figure 5. Comparison of iNCMSC spheroids cultured individually and inside the construct. DNA quantity and GAG deposition of 36 spheroids cultured in 96 well culture plates and printed on 7 mm round Kenzan. DNA quantity graph indicates a not significant (ns) difference between all samples from both groups. Mean value (±SD) of triplicate well is shown. * indicates $P < 0.05$, ** indicates $P < 0.01$ by one-way analysis of variance (ANOVA) followed by Scheffe post-hoc test. Representative images of spheroids and constructs at day 0, 3, 6 and 21 after printing stained with SOFG, TUNEL, type I and type II collagen. Images of histological evaluations were taken at ×4 magnification. Scale bars represent 500 µm.

fold during the 1st 3 weeks of maturation. The type II collagen content also increased gradually from 0.2 to 2.4 µg mg$^{-1}$ during the 1st 4 weeks of maturation and reached a plateau. Type II collagen content increased four-fold during this time.
The increased GAG and type II collagen content were corroborated by the mechanical analysis results of the constructs. The compressive modulus was 0.59 MPa, 0.77 MPa and 0.88 MPa for the constructs after 3, 4, and 5 weeks of maturation, respectively.

The maximum force necessary to tear the construct increased from 90 mN mm$^{-2}$ to 209 mN mm$^{-2}$ from 3 to 5 weeks of maturation, as observed in the suture retention test (figure 7).

To confirm the high viability of the iNCMSC construct and gain insight into its potential self-renewal characteristics, we compared two constructs that underwent a maturation process after removal from the Kenzan for 9 and 43 d. Histological evaluations of the constructs maintained in the bioreactor to mature 43 d after removal from the Kenzan revealed that almost all the holes formed by removing the Kenzan needles were filled up with new tissue compared to that in the construct matured only for 9 d (figure 8).

Despite the presence of the TUNEL-positive signal in the middle of the construct that had been matured for 43 d, the cells were able to remodel the holes after the needles were removed.
Figure 7. Maturation time optimization. (A) Mechanical and (B) GAG and type II collagen biochemical evaluations of constructs undergo 5 weeks maturation process. The same spheroids number construct samples ($n = 3$) were removed from Kenzan 7 d after printing (week 1) and then cultured for following 4 weeks. Mean value ($\pm$SD) of triplicate well is shown. * indicates $P < 0.05$, ** indicates $P < 0.01$ by unpaired two-sided $t$-test.

Figure 8. Self-remodel potential of iNCMSC construct. Comparison of iNCMSC constructs cultured on PDMS tube for 9 and 43 d after removal from the Kenzan. Both constructs were removed from the Kenzan 20 d after printing. Representative images of constructs stained with SOFG, type I, type II collagen and TUNEL. Images of histological evaluations were taken at $\times 4$, $\times 10$ and $\times 20$ magnification. Scale bars represent 1000 $\mu$m on $\times 4$ and 500 $\mu$m on $\times 10$ and $\times 20$ magnification images.

3.7. Constructs of different shapes
Following optimizations, the L-shape construct was engineered (figure 9) as a composition of two squares joined at a 90° angle. The construct possessed great stiffness and maintained its shape during mechanical manipulations (supplementary movie 1).

Histological analysis revealed an empty space between two layers of spheroids (figure 9). The empty space was not created on purpose but occurred due to placing the two layers of spheroids not close enough to fuse with each other. Consequently, separately maturated layers closed the empty space depriving the inner area of the medium. Microscopic observation of the construct confirmed SOFG-negative and TUNEL-positive tissue inside the empty space (figure 9).
Figure 9. Different shape construct. L-shape construct. (A) Bio-3D-printer design data and gross images of the construct removed from the Kenzan 18 d after printing. (B) Overview images and representative area of the construct stained with SOFG, types I, II collagen and TUNEL 40 d after printing. Histological evaluations revealed empty space build up between two layers of spheroids. Images of histological evaluations were taken at $\times 4$, $\times 10$ and $\times 20$ magnification. Scale bars represent 1000 $\mu$m on the $\times 4$ and 500 $\mu$m on $\times 10$ and $\times 20$ magnification.

To create a construct of a larger area, a tube-shape structure was printed using a 7 mm round Kenzan (figure 10). Tube-shape construct, 3 cm long and 7 mm in diameter, were cut and unfolded along the longer axis to create a rectangular structure of about 6 cm$^2$. The unfolded construct
maintained the half-pipe shape. Histological evaluations of constructs cultured for 36 d on the Kenzan revealed a marginally elevated TUNEL-positive signal present around the holes formed by the needles (figure 10) compared to similar constructs removed 20 d after printing (figure 8), indicating that early removal would be beneficial for construct viability.

Histological analysis of L-shape and tube-shape constructs confirmed high homogeneity of chondrogenic differentiation and integrity of both structures (figures 9 and 10).

In a subsequent experiment, we printed a surface-shaped construct to reproduce the anatomical topography of the porcine femoral groove. Two separately printed constructs were joined and matured together to obtain one integrated larger construct. The construct shape reliably replicated the groove topography (figure 11). Finely adjusted bonding between the two constructs obtained due to the location of two Kenzans in the originally 3D printed foundation resulted in the creation of one highly integrated structure (supplementary movie 2). Macro and microscopic observations, however, revealed areas of unequal maturation and elevated TUNEL signal (figure 11) in the inner part of the construct, similar to that observed in empty space of the L-shape construct. Data suggests deprivation of essential nutrients due to insufficient inflow of the culture medium into these areas.

4. Discussion

Great technological advances have been made in tissue engineering in the past decade, however the technology has translated into limited clinical applications for cartilage regeneration.

While there are many scaffold-based approaches, only two scaffold-free bio-engineered cartilage constructs have been evaluated in clinical trials: chondrosphere (co don AG) and revaflex (ISTO Technologies) [22]. The 1st uses autologous chondrocytes to create aggregates; in the 2nd, a 22 mm circular graft is formed from allogeneic juvenile chondrocytes. Although both constructs enhanced the level of activity and quality of life of patients after
Figure 11. Different shape construct. Articular surface-shape construct. (A) Bio-3D printer design data and gross images of the construct. (B) The miniature pig distal femoral condyle and groove and bio-3D printer converted data. Gross image of two separately printed constructs on two Kenzans after printing and 3 d later. Gross image of the construct removed from the Kenzan at day 15 shows some poorly matured area. (C) Overview images and representative area of the construct stained with SOFG, types I, II collagen and TUNEL. Images of histological evaluations were taken at ×4, ×10 and ×20 magnification. Scale bars represent 1000 μm on the ×4 and 500 μm on the ×10 and ×20 magnifications.
up to 12 months after treatment, both focus only on single cartilage defects. A system that enables the treatment of larger defects has not yet been developed.

In the current study, scaffold-free, self-sustainable cartilage constructs were successfully fabricated, suitably shaped and sized for repairing larger defects. First, highly chondrogenic, potent iNCCMSCs were optimized for the bioprinting process. Next, the minimum maturation period was determined, then various types of Kenzan were employed to fabricate larger cartilage constructs adequately shaped for irregular defects.

Bio-engineered constructs are based on a large number of cells [14]. Due to the need to minimize the harvest of stem cells a readily available and abundant source of chondroprogenitor cells is urgently needed for engineering cartilage constructs. For this reason, iNCCMSCs were used as the source material in this study. Starting with $5 \times 10^5$ iNCC stock cells, we fabricated a 6 cm$^2$ construct composed of $8.6 \times 10^5$ MSC-induced cells. The already well-known chondrogenic differentiation capacity and population homogeneity of iNCCs was confirmed in spheroids culture (figure 2(B), supplement figure 2) [13]. Previous studies have reported the utility of iNCCMSCs in cartilage defect treatment, although only as an implantation of aggregates, using fibril glue to achieve integration between the aggregates and the host tissue [11].

In this study, a bio-3D printer was employed to assemble iNCCMSC aggregates into one large, integral construct. The central approach to obtain that goal was printing time optimization. A previous study showed that similar to MSCs, human pluripotent stem (hPS) cell chondrogenic differentiation mimics early embryogenesis [10], which is an organized process composed of a sequence of multistep processes that involve mesenchymal condensation followed by ECM synthesis and remodelling. Combinations of essential growth factors present in the cell environment promote and maintain each of these steps. On the other hand, construct fabrication process combining small building unit-spheroids into a bigger structure, although coordinated, for cells, is quite sudden and can disrupt cells environment. The placement of individual spheroids inside a complex structure drastically decreased spheroid surface area and lowered levels of growth factors on the cells.

Therefore, combining these two processes, in appropriate timing, is essential to obtain a fully integrated, functional construct. The requirement for assembly time optimization was indicated by our first experiment when the spheroids from day 26 of chondrogenesis, due to prolonged exposure to TGF-β3 and BMP-4 after PDGF-BB induction, fully went through the multistep maturation process to a point that did not allow them to integrate with each other (figure 3), despite following a 20 d maturation period.

For that reason, in the 2nd attempt, we focused on printing optimization at the early phase of chondrogenic induction. Printing constructs using spheroids at three different points of chondrogenic induction allowed us to evaluate optimal time for scaling up without causing spheroid environmental disturbances. Constructs fabricated with day 3 spheroids showed strong type I collagen and a lack of proteoglycan deposition despite 56 d of maturation (figure 4(B)). This corresponded well with findings from hPS studies which stated that PDGF receptor α-positive hPS cells, after pre-treatment with PDGF-BB, were able to condensate and produce cartilage nodules after 16 d and with treatment with TGF-β3 from days 3–6 [10]. PDGFRα-negative hPS cells could not form mesenchymal condensation and, therefore, could not undergo chondrogenesis. Our results suggest that printing constructs at day 3 from induction might interrupt PDGF-BB-regulated iNCCMSC proliferation, which is essential for condensation, causing chondrogenesis termination (figure 4(B)). Interruption of the TGF-β3 treatment due to the fabrication process, in constructs fabricated with day 6 spheroids, results in the deprivation of the maturation process, observed as decreased proteoglycan production and significantly higher type I and type II collagen deposition, characteristic of fibrotic cartilage formation. Constructs printed with day 10 spheroids, abundant in round chondrocytes, showed the highest proteoglycan deposition level among the three constructs, comparable to those of native porcine articular cartilage used as a control (figure 4(B)). Day 10 spheroid constructs, similarly to the day 6 constructs highly expressed types I and II collagen, however showed different deposition pattern. We were not able to obtain an improvement of the chondrogenic maturation process to obtain the ideal type II/type I collagen ratio observed in the native articular cartilage although it might greatly lower considerations of dedifferentiation to fibrotic cartilage leading to hypertrophy.

However, our study on individually cultured spheroid and construct located spheroids allowed us to gain more insight into the dynamics of the iNCCMSC construct maturation process. DNA quantity and TUNEL evaluations revealed continued high viability of spheroids in both groups. Weak TUNEL-positive signals present in both groups indicate its occurrence due to nutrient deprivation-related stress rather than stress from the printing process (figure 5(B)). Regardless, spheroids from both groups were able to gradually mature over a period of 21 d after printing, indicated by their strong proteoglycan and type II collagen deposition. GAG biochemical evaluations revealed that although a gradual increase of deposition persists in both groups, as expected, it was more prominent in individual spheroid culture due to the higher level of the growth factors on the cells. Deposition of two
chondrogenic markers, SOFG and type II collagen, in a time-dependent manner, coincide with the relocation of type I collagen, visible, in individual and construct-located spheroids. Events observed inside the iNCMSC spheroids and constructs suggest the well-known remodelling process occurring during chondrogenic development after mesenchymal condensation, when tissue ECM transformation is activated. In order to mature, cartilage ECM type I collagen gradually disappears and is substituted by type II collagen [23]. The tendency of the iNCMSC spheroids to increase type II collagen and type II/type I collagen expression-ratio indicate their increased potential to remodel when cultured individually and inside constructs. Further, observations of iNCMSC constructs maintained on the PDMS tube after removal from the Kenzan confirmed its high viability and self-remodelling potential. The high viability of the cartilage construct is important not only for development but also maintenance of matrix contents necessary to repair chondral defects, absorb load, and provide lubrication during movement [24].

An iNCMSC construct optimized for printing at day 10 showed highly competitive characteristics compared to a fabricated BMMSC construct (figure 6). The overall integrity of the iNCMSC construct which could maintain the desired shape and size, combined with highly expressed chondrogenic characteristics, indicates its effectiveness for chondral defect repair applications, minimizing gaps and offering improved integration with host tissue, leading to more effective clinical applications.

On the other hand, chondrogenic maturation of engineered constructs with prolonged in vitro culture is not only highly time- and money-consuming but also may result in lower ability of the construct to integrate and build continuity with native tissue.

To achieve a compromise and determine the minimal necessary maturation period for our construct to sustain its pre-design shape, further observations of engineered constructs and evaluations of their mechanical and biochemical properties were conducted.

Cultivation of printed constructs for an additional 5 weeks resulted in significantly increased compressive and tensile properties, correlated with changes in GAGs and type II collagen expression (figure 7). After 3 weeks of maturation, the construct reached a compressive modulus of 0.59 MPa, which was within the 0.1–2 MPa range for native articular cartilage [22]. The construct achieved a compressive modulus of 0.77 MPa after 4 weeks of maturation, which corresponded with the previously reported 1 MPa for primary chondrocytes self-assembling to produce articular cartilage [25]. Interestingly, a further increase in stiffening and elasticity was not accompanied by additional GAGs or type II collagen deposition. This could be due to increased collagen covalent cross-linking, which has been recognized as an improving factor for tissue-stiffening properties and suture retention of cultured constructs [26, 27].

Quantitative evaluations obtained for type II collagen reflected gradually increasing mechanical properties of constructs; however, they were far behind those predicted for native articular cartilage and were more relative than absolute in value. Although a comparison of type II collagen deposition in constructs fabricated using different cell sources would be an ideal way to appreciate the potential of iNCMSCs, fabrication of control constructs was almost impossible due to difficulties in obtaining the high number of cells at the same passage essential for printing comparable constructs.

The topography of cartilage defects is patient specific. The potential to precisely recreate this variability via bioprinting due to computer-controlled manufacturing software has been presented by many groups, despite the inability to maintain high cell viability during the final shape arrangement process.

The scaffold-free Kenzan method has been successfully used to fabricate a wide variety of tissue, including blood vessels [28], cardiac tissue [29], diaphragm [30], and trachea [31], suggesting its applicability for cartilage regeneration.

The current study attempted to fabricate a construct for large defect repair, exclusively using cells in an effort to reproduce the precision of bio-ink technology. The first construct, which confirmed the method’s potential to sustain shape, was L-shape with a 90° angle (supplementary movie 1). After removal from the Kenzan, the construct was able to maintain the applied design and withstand mechanical manipulation regardless of its 2 cm² total area. Although unintentionally created, an empty space appeared between the two layers of spheroids, implied severe consequences of inadequate medium flow for complete maturation of the construct.

Applying the 7 mm round Kenzan, we were able to increase the construct size up to 6 cm² total area (figure 10). A characteristic half-pipe-shaped structure achieved after unfolding the tubular construct may be utilized to treat a wide, curved area such as the patellar surface. Despite impressive size enlargement, the round Kenzan dimensions limited the possible area of fabricated tissue. Combining smaller sub-blocks (constructs) to build bigger and more complex structures allowed us to address large defects with patient-detailed topography (figure 11, supplementary movie 2). In this new approach, separately engineered constructs were assembled into one solid structure during the maturation period. Although scanned and translated porcine femoral groove data reflected groove geometries with great precision in surface-shape construct, and overall integrity of joined constructs was successfully met, constructs revealed poorly matured areas, which indicates a need (figure 11) to build a bioreactor system for improved
medium penetration around the construct in the future.

Using iPSC-derived cells as a source material has many benefits, but it remains extremely expensive. For this reason, further optimization and improvement of the maturation system will be crucial before conducting even more time- and money-consuming studies in vitro.

5. Conclusion

This study demonstrated the successful fabrication of completely scaffold-free, highly homogeneous, iPSC-derived iNMSC-based cartilage construct with properties that may facilitate resurfacing larger chondral defects. We also provide new findings in iNMSC construct-specific properties that may be useful for future studies targeting OA treatment.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Conflict of interest

Co-author K Nakayama is a co-founder and shareholder of Cyfuse Biomedical K K and an inventor/designee designated on the patent for the Bio-3D printer. Patent title: Method for production of three-dimensional structure of cell; patent number: JP4517125. Patent title: Cell structure production device; patent number; JP5896104. The other authors have declared that no competing interests exist.

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