Title

Spheroid culture for chondrocytes triggers the initial stage of endochondral ossification

Authors

Jeonghyun Kim[a,*], Kosei Tomida[a], Takeo Matsumoto[a], and Taiji Adachi[b]

[a] Department of Mechanical Systems Engineering, Nagoya University, Nagoya 464-8603, Japan

[b] Institute for Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

***Corresponding author**: Jeonghyun Kim, Ph.D.

Address: Department of Mechanical Systems Engineering, Nagoya University, Nagoya 464-8603, Japan

Tel: +81 52 789 4486 E-mail address: jkim@nagoya-u.jp

Abstract

Endochondral ossification is the process of bone formation derived from growing cartilage during skeletal development. In previous studies, we provoked the osteocyte differentiation of osteoblast precursor cells under a three-dimensional (3D) culture model. In order to recapitulate the endochondral ossification, the present study utilized the self-organized scaffold-free spheroid model reconstructed by pre-chondrocyte cells. Within 2-day cultivation in the absence of the chemically induced chondrogenesis supplements, the chondrocyte marker was greatly expressed in the inner region of the spheroid, whereas the hypertrophic chondrocyte marker was strongly detected in the surface region of the spheroid. Notably, we found out that the gene expression levels of osteocyte markers were also greatly up-regulated compared to the conventional 2D monolayer. Moreover, after long-term cultivation for 28 days, it induced morphological changes in the spheroid, such as cellular hypertrophy and death. In this study, in order to recapitulate the initial stage of the endochondral ossification, we highlighted the potentials of the 3D culture method to drive the hypertrophic chondrocyte differentiation of the pre-chondrocyte cells

Keywords

3D culture; spheroid; chondrocyte; hypertrophic chondrocyte; osteocyte; endochondral ossification; ATDC5.

1. Introduction

For the last decade, a three-dimensional (3D) culture technology has been widely applied to various organs in the field of mammalian development and organ regeneration in the name of organoids (Eiraku et al., 2008; Eiraku et al., 2011; Huch and Koo, 2015; Spence et al., 2011; Takebe et al., 2013). The 3D culture models are believed to exhibit a suitable culture environment with an appropriate cell-cell interaction (Ghezelayagh et al., 2021), whereas the conventional two-dimensional (2D) culture models inevitably provide such a greater cell-substrate interaction with a hard plastic or glass substrate via generating the tight filaments of actin cytoskeletons, which is different from an *in vivo* situation (Birgersdotter et al., 2005; Edmondson et al., 2014). Since the 3D culture models enable to recapitulate the developmental process of various organs *in vitro* (Kim et al., 2020; Rossi et al., 2018), it broadened our knowledge to make new insights on the cell behaviors beyond the findings occurred on the conventional 2D culture method.

Endochondral ossification during the development process is the bone formation event initiated from the mesenchymal condensation process via cartilage formation, followed by apoptosis of hypertrophic chondrocytes (Mackie et al., 2008; Usmani et al., 2012). On the other hand, the intramembranous ossification is another bone developmental process which is directly derived from the mesenchymal condensation (Aghajanian and Mohan, 2018; Berendsen and Olsen, 2015). As various 3D culture models have been introduced in the field of cartilage tissue engineering (Furukawa et al., 2003; Furukawa et al., 2008; Tsumaki et al., 2015), most of them focused on the differentiation capability into chondrocytes or cartilage regeneration after transplantation. To date, however, there is no experimental *in vitro* model to study the endochondral ossification due to the lack of understanding in the cell behaviors inside the 3D culture model. In previous studies, our group reported that pre-osteoblast cells and mesenchymal stem cells in the 3D scaffold-free culture model provoked *in vitro* osteocyte differentiation (Kim et al., 2020; Kim and Adachi, 2021, 2020, 2019). Particularly, we recently reported that the mesenchymal condensation process can be recapitulated from the 3D spheroid model reconstructed by human bone marrow derived mesenchymal stem cells (MSCs) (Kim and Adachi, 2021). As a result, compared to the 2D model, the MSC spheroid under the osteogenesis supplements exerted the osteocyte-likeness within 2 days, which implied the potentials of MSC spheroid strategy as bone organoids to recapitulate the intramembranous ossification process. In this present study, we aimed to mimic the endochondral ossification process from 3D scaffold-free spheroids reconstructed by mouse chondrocyte precursor ATDC5 cells.

2. Materials and methods

2.1 Cell culture

Mouse chondrocyte precursor cell line, ATDC5 cell, was provided by RIKEN Cell Bank (Japan). The ATDC5 cells were routinely cultured in DMEM/F12 (Gibco, USA) supplemented with 5% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic (Nacalai Tesque, Japan) in a humified incubator at 37℃ with 5% CO_2. A cell passage was performed every 3 – 4 days when the cells become 80 – 90% confluent. To fabricate a self-organized spheroid, 2,500 cells were subcultured in Nunclon Sphera U-bottom plate (ThermoFisher, USA) for 2 days as illustrated in Fig. 1 (A). For a conventional 2D monolayer model, 200,000 cells were subcultured in the 35 mm diameter petri dish to become confluent as previously described (Kim et al., 2020a).

2.2 Real time-PCR

After the samples were collected in Isogen II (Nippon Gene, Japan), total RNA was extracted using PureLink RNA Mini kit (Invitrogen, USA). Then, cDNA was synthesized from the extracted RNA using Transcriptor Universal cDNA Master

(Roche, Switzerland) according to the manufacturer's protocol. We subsequently performed real-time PCR using PowerUp SYBR Green Master mix (ThermoFisher, USA) in a StepOnePlus Real-Time PCR system. To investigate the differentiation markers for chondrocytes, we examined the mRNA expressions of SRY-box transcription factor 9 (*Sox9*), aggrecan (*Acan*), and collage type II alpha 1 chain (*Col2*). For hypertrophic chondrocyte markers, we examined mRNA expressions of fibroblast growth factor receptor 3 (*Fgfr3*), collagen type X alpha 1 chain (*Col10*), and matrix metallopeptidase (*Mmp13*). For osteocyte marker, we examined mRNA expressions of osteopontin (*Opn*), dentin matrix acidic phosphoprotein 1 (*Dmp1*), and sclerostin (*Sost*). All the mRNA expressions were normalized to those of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The relative mRNA expressions were subsequently calculated by the Delta-Delta Ct method. All the sets of gene-specific oligonucleotide primers were described in Supplementary Table 1.

2.3 Immunostaining

The spheroids were fixed in the 4% paraformaldehyde. After permeabilization using 0.1% triton X-100 for 30 min, the spheroid sample was blocked with 4% bovine serum albumin for 1 h and then treated with the primary antibody for 1 h. For the primary antibodies, we utilized COL2 (anti-collagen II antibody; Abcam, UK), COL10 (anti-collagen X antibody; Abcam, UK), and DMP1 (Anti-DMP1 antibody; Abcam, UK) as chondrocyte, hypertrophic chondrocyte, and osteocyte markers, respectively. After washing with PBS, the samples were treated with the Alexa fluor 546 secondary antibody (Invitrogen, USA), Alexa fluor 488 phalloidin (Invitrogen, USA), and DAPI (Sigma, USA) for 1 h. The samples were subsequently observed by FLUOVIEW FV3000 (Olympus, Japan).

2.4 Histochemical assay

After collecting the spheroids, they were fixed in 10% formalin neutral buffer solution (Nacalai, Japan). Then, the samples were treated with 30% sucrose in PBS for 2 h and embedded in O.C.T. compound (Sakura Tissue-TEK, Japan) for frozen block. The cryosections were prepared at 10 μm thickness using the Cryostat. The O.C.T. compound in the cryosections was removed by water immersion for 5 min prior to histological stains. According to the manufacturer's protocol, the spheroids were stained with hematoxylin and eosin (HE) (Sigma, USA), Safranin-O (Merck, Germany).

2.5 Statistics

The bars in real-time PCR graph indicate the mean ± standard error values. To examine the statistical significance, Student's *t*-test or one-way ANOVA with Tukey's HSD *post-hoc* test was performed. If a *p*-value calculated is less than 0.05, the difference is considered statistically significant.

3. Results

3.1 Fabrication of spheroids

In this study, we fabricated self-organized spheroids reconstructed by ATDC5 cells in the ultra-low attachment dish. After subculturing 2,500 cells for 2 days in the dish, the cells were aggregated under the dish and eventually formed about 150 μm diameter of spheroid as shown in the Fig. 1(B). For the comparative model, we utilized a 2D monolayer that had a fully confluent condition formed by ATDC5 cells in Fig. 1(C).

3.2 ATDC5 spheroid exhibited greater chondrocyte differentiation markers in the inner region of the spheroid.

After 2-day culture, we examined the mRNA expression changes in the ATDC5 spheroid measured by real-time PCR. To confirm the chondrocyte differentiation capability in the spheroid, we evaluated mRNA expression of chondrocyte markers in the spheroid normalized to those in the monolayer in Fig. 2(A). As a result, all the chondrocyte markers in the spheroid were greatly up-regulated within 2 days; *Sox9* (7.18-fold change; $p < 0.05$), *Acan* (20.3-fold change; $p < 0.005$), and *Col2* (68.7-fold change; $p < 0.005$). We also conducted the immunostaining to detect the protein expression of chondrocyte marker, type II collagen (COL2), for the ATDC5 spheroid in Fig. 2(B). The staining results represented that the COL2 expression was strongly detected, particularly in the inner region of the spheroid, whereas the surface region of the spheroid relatively exhibited a weak level of COL2 expression.

3.3 ATDC5 spheroid exhibited greater hypertrophic chondrocyte differentiation markers in the surface region of the spheroid.

To evaluate the hypertrophic chondrocyte markers in the ATDC5 spheroid culture, we also performed real-time PCR and immunostaining. Figure 3(A) represented that there were significant and great expression changes in the hypertrophic chondrocyte markers in the spheroid compared to the monolayer; *Fgfr3* (7.00-fold change; $p < 0.005$), *Col10* (102.6-fold change; $p < 0.005$), and *Mmp13* (22.4-fold change; $p < 0.05$). We then carried out the immunostaining to detect type X collagen (COL10) expression for the spheroid in Fig. 3(B). The immunostaining results represented that COL10 expression was strongly detected in the surface region of the spheroid where the F-ACTIN were highly stained. Compared to the surface of the spheroid, the COL10 expression was relatively weak in the inner region of spheroid. Hence, it induced the hypertrophic chondrocyte differentiation within 2 days in the surface region of the spheroid.

3.4 ATDC5 spheroid remarkably up-regulated osteocyte markers in the spheroid.

We then evaluated the osteocyte markers in the ATDC5 spheroid. The real-time PCR result in Fig.4(A) showed that the all the osteocyte markers were remarkably up-regulated in the spheroid compared to the monolayer; *Opn* (87.7-fold change; $p < 0.05$), *Dmp1* (182.0-fold change; $p < 0.005$), and *Sost* (7.17-fold change; $p < 0.005$). The immunostaining was then conducted to detect DMP1 expression as osteocyte marker. From the results shown in Fig. 4(B), the ATDC5 spheroid also exhibited the DMP1 expression entirely, but its protein level of expression was weak compared to the expression of COL2 and COL10.

3.5 3D spheroid culture is essential for up-regulations of chondrocyte, hypertrophic chondrocyte, and osteocyte markers.

To evaluate the effect of 3D spheroid structure, we fabricated a new model termed sph-mono model as illustrated in Fig. 5(A). The 2-day-old spheroid in the ultra-low attachment dish was transferred to the normal cell adhesive dish. Immediate after the spheroid was transferred, the spheroid initiated to attach and slowly spread over the flat dish. We subcultured the spheroids for another 4 days until the spheroids lost its initial 3D shape as represented in the Fig. 5 (B) and (C), respectively.

We then collected the sph-mono model and compared its gene expression changes against monolayer and spheroid models subcultured for 6 days in Fig.5(D). As a result of real-time PCR, similar to the previous experiment for 2 days, the spheroids subcultured for 6-day exhibited much greater gene expressions level of chondrocyte (*Sox9*, *Acan*, and *Col2*), hypertrophic chondrocyte (*Fgfr3* and *Col10*) markers than monolayer, whereas *MMP13* expression was non-significantly altered. Regarding the osteocyte markers, while the increase in the early osteocyte markers (*Opn* and *Dmp1*) in the spheroid were diminished, the mature osteocyte marker, *Sost*, was greatly up-regulated compared to the monolayer model. In the sph-mono model, on the other hand, mRNA expressions for chondrocyte (*Sox9*, *Acan*, and *Col2*), hypertrophic chondrocyte (*Fgfr3* and *Col10*), and osteocyte markers (*Sost*) were altered. Particularly, mRNA gene expressions in the sph-mono model became similar level to the those of monolayer. In other words, the up-regulated gene expressions in the spheroid were reversed to the level of monolayer in the sph-mono. The results indicate that the 3D structure acquired from the spheroid culture contributed to the up-regulation of chondrocyte, hypertrophic chondrocyte, and osteocyte markers.

3.6 Spheroids became hypertrophied after 4 days

We then conducted a long-term culture up to 28 days as represented in Figure 6(A). In Figure 6(B), the projected area of spheroid became smaller until Day 4 compared to the 2-day spheroid (92% of 2-day spheroid area), but the spheroid became enlarged from Day 4 up to Day 28 (Day 7: 102% of 2-day spheroid area; Day 14: 132% of 2-day spheroid area; Day 21: 181% of 2-day spheroid area).

As a result of HE staining in Figure 6(C), we found the morphologic change of the cells in the spheroids. The cell nuclei in the 28-day spheroid were hypertrophied compared to the 2-day spheroid. Furthermore, the distances between the nuclei in the 28-day spheroid were greater than those of the 2-day spheroid. We also quantified the nuclear size from images stained by Hoechst 33342 as shown in Figure S1, the results showed an increase in the size of 28-day spheroid compared to 2-day spheroid (1.68-fold change; 2-day spheroid: 23.4 ± 4.3 μm^2; 28-day spheroid: 39.3 ± 5.4 μm^2; mean ± SD). On the other hand, it represented a slight reduction of the cell number in the 28-day spheroid compared to the 2-day spheroid (0.89-fold change; 2-day spheroid: 73.6 ± 8.9 cells; 28-day spheroid: 65.6 ± 14.7 cells; mean ± SD). The results indicate that the cells in the spheroid were hypertrophied after 28-day incubation. Moreover, severe cell death was also observed in the 28-day spheroid, whereas it did not have any vacant space inside in the 2-day spheroid. To examine proteoglycans as the main component of the extracellular matrix in the cartilage, Safranin-O staining was carried out for the ATDC5 spheroids. In Figure 6(D), the cells in both 2-day and 28-day spheroids were stained pink, which represented that the ATDC5 cells in the spheroids produced the proteoglycan even after 28-day incubation. In Fig. 6(E), COL10 protein expression, hypertrophic chondrocyte marker, was entirely detected in the 28-day spheroid.

4. Discussion

To date, a pellet model was regarded as one of mainstreams of the 3D scaffold-free model in the field of cartilage tissue engineering due to its ease of handling in the assay process including the sectioning for staining (Kawanishi et al., 2007; Yamashita et al., 2015). In this study, to examine chondrocyte differentiation capability in the scaffold-free culture, we fabricated a smaller size of 3D scaffold-free spheroids (about 150 μm) than the conventional pellet model (0.5 – 2 mm). The smaller size of the spheroid is believed to be a low risk of cellular necrosis in the center of the spheroid due to the lack of nutrients and gasses, whereas the diameter of the pellet became reaches up to several mm. Particularly, we fabricated the self-organized spheroid model reconstructed by ATDC5 cells without recruiting any scaffolds as previously studied (Kim et al., 2020).

By comparing the spheroid model to the conventional 2D monolayer model, we suggested that the ATDC5 spheroid exerted the greater expressions of chondrocyte differentiation marker inside the spheroid and hypertrophic chondrocyte marker outside the spheroid. Moreover, these local protein expressions of chondrocyte-related markers including COL2 and COL10 expressions were detected in the same manner as previous studies (Marlovits et al., 2003; Yamashita et al., 2015). We also first found out the transient increase in the gene expressions of osteocyte markers in the ATDC5 spheroid model, compared to the monolayer. As the transient up-regulations of osteocyte markers for pre-osteoblast cells or mesenchymal stem cells in the 3D culture model were reported by our group (Kim et al., 2020; Kim et al., 2021; Kim and Adachi, 2021, 2020, 2019), the chondrocyte precursor cells in the spheroid culture also triggered the transient increase in osteocyte gene expressions. The results represented that the significance of 3D culture to provoke the up-regulations of chondrocyte/hypertrophic chondrocyte/osteocyte markers, which might imply that the spheroid reconstructed by chondrocytes reconstituted the earl stage of the endochondral ossification process.

In this study, to examine the effect of spheroid culture, we recruited the sph-mono model by transferring the spheroids from the ultra-low attachment dish to the cell adhesive normal culture dish as previously done using pre-osteoblast cells and mesenchymal stem cells(Kim et al., 2020a; Kim and Adachi, 2019). Since the mouse chondrocyte precursor ATDC5 cells has a lower cell mobility than mouse osteoblast precursor MC3T3-E1 cells, it took 4 days to dissociate the cells in the spheroid to lose its 3D spheroid structure while the MC3T3-E1 cells took 2 days(Kim et al., 2021). Despite the longer time of dissociation for ATDC5 spheroids, the up-regulated chondrocyte and hypertrophic chondrocyte genes in the 6-day spheroid were dramatically suppressed in the sph-mono model. The results indicate that the cell condensed condition acquired in the 3D spheroid culture is essential for facilitation of the chondrocyte and hypertrophic chondrocyte differentiation. Moreover, we reported that up-regulation of osteocyte markers were also dependent on the 3D cellular structure, which was also observed in other spheroid model using pre-osteoblast cells and mesenchymal stem cells (Kim and Adachi, 2019; Kim and Adachi, 2021).

Beyond the transient up-regulated hypertrophic chondrocyte marker in the gene expression level, the spheroid reconstructed by ATDC5 cells underwent the hypertrophic chondrocyte differentiation in the long-term experiment. After the self-organized spheroid was formed, the size of spheroid became shrunk up to 4-day due to the cell-cell interaction in the spheroid like other spheroid models reconstructed by pre-osteoblast cells or mesenchymal stem cells (Kim and Adachi, 2021). While those previous 3D osteocytic spheroid model using pre-osteoblast cells or mesenchymal stem cells became smaller after a long-term incubation, the ATDC5 spheroid exhibited a different behavior from the previous osteocytic models and was gradually hypertrophied from 4-day incubation. The reduction of the spheroid size reconstructed by pre-osteoblast cells or mesenchymal stem cells was thought to occur due to their strong cell-cell interaction force, whereas the cell-cell interaction force of ATDC5 cell was relatively weak, which induced the different cellular behavior of the spheroids. The increase in the size of ATDC5 spheroid was provoked by the hypertrophied cells as confirmed in the histochemical staining. Moreover, we also found the severe cell death inside the spheroid after 28-day incubation. This cell death is known to occur during the endochondral ossification of the developmental process. The chondrocytes in the growth cartilage undergo hypertrophy and then programmed cell death to provide a vacant space for the ossification after blood vessels penetrated. Since our model does not have any other cell source, the space derived from the cell death in the 28-day spheroids is remained vacant. By conjugating the microelectromechanical systems in our present model, it might be able to induce the angiogenesis and bone formation in order to reconstruct the late phase of the endochondral ossification *in vitro*. Nevertheless, our results might indicate the possibility that the spheroid culture for pre-chondrocyte ATDC5 cells recapitulated the hypertrophic chondrocyte differentiation as well as cell death, early events of the endochondral ossification. Most importantly, our findings highlighted the significance of 3D culture environment to induce the hypertrophic chondrocytes beyond the chemically induced approach on the conventional 2D model. Hence, this type of cell condensation acquired from the structural change might become a triggering factor for the pre-chondrocyte cells in the bone collar to undergo the endochondral ossification *in vivo*.

Transdifferentiation is that a fully differentiated cell in the tissue lineage converts into one of a distinct lineage (Pesaresi et al., 2019). This phenomenon is known to occur in multiple types of tissue such as neurons (Tanabe et al., 2015), hepatocytes(Schaub et al., 2018), chondrocytes (Zhou et al., 2014), etc. Particularly, the model of chondrocyte-to-osetoblast/osteocyte transdifferentiation has been recruited in the ossification process, but the detailed mechanism remains veiled (Aghajanova et al., 2010). In the endochondral ossification process, the chondrocyte-to-osteoblast transdifferentiation has been often mentioned followed by terminal differentiation into osteocytes(Aghajanian and Mohan, 2018). Several studies reported the validity evidence of chondrocyte-to-osteoblast transdifferentiation using *in vivo* model (Hu et al., 2017; Ruscitto et al., 2020). Our present study supported that the chondrocyte in 3D culture model provoked the chondrocyte-to-osteocyte transdifferentation.

In conclusion, we fabricated the self-organized scaffold-free spheroids using mouse chondrocyte precursor cells. In the spheroids, the chondrocyte differentiation was induced in the inner region of the spheroid, whereas the hypertrophic chondrocyte differentiations were mainly detected in the surface region of the spheroid. Notably, we found out that the osteocyte markers were also greatly up-regulated in the spheroid reconstructed by pre-chondrocyte ATDC5 cells. Moreover, the cells in the spheroid underwent the hypertrophic chondrocyte differentiation after the long-term cultivation. Therefore, we suggested that our 3D spheroid culture has potentials to be utilized as a new *in vitro* model to recapitulate the early stage of endochondral ossification.

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Conflicts of interest

The authors declare no competing interests.

Author contributions

J.K. designed the experiments; J.K. and K.T. performed the experiments and analyzed the data; J.K., T.M., and T.A. contributed reagents or other essential materials; J.K. wrote the manuscript; T.M. and T.A. made the manuscript revision.

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References

Aghajanian P, Mohan S. 2018. The art of building bone: Emerging role of chondrocyte-to-osteoblast transdifferentiation in endochondral ossification. *Bone Research* **6**, 19.

Aghajanova L, Horcajadas JA, Esteban FJ, Giudice LC. 2010. The Bone Marrow-Derived Human Mesenchymal Stem Cell: Potential Progenitor of the Endometrial Stromal Fibroblast1. *Biology of Reproduction* **82**:1076–1087.

Berendsen AD, Olsen BR. 2015. Bone development. *Bone* **80**, 14–18.

Birgersdotter A, Sandberg R, Ernberg I. 2005. Gene expression perturbation in vitro - A growing case for three-dimensional (3D) culture systems. *Seminars in Cancer Biology* **15**, 405–412.

Edmondson R, Broglie JJ, Adcock AF, Yang L. 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and Drug Development Technologies* **12**, 207–218.

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y. 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472**:51–58.

Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y. 2008. Self-Organized Formation of Polarized Cortical Tissues from ESCs and Its Active Manipulation by Extrinsic Signals. *Cell Stem Cell* **3**:519–532.

Furukawa KS, Imura K, Tateishi T, Ushida T. 2008. Scaffold-free cartilage by rotational culture for tissue engineering. *Journal of Biotechnology* **133**:134–145.

Furukawa KS, Suenaga H, Toita K, Numata A, Tanaka J, Ushida T, Sakai Y, Tateishi T. 2003. Rapid and Large-Scale Formation of Chondrocyte Aggregates by Rotational Culture. *Cell Transplantation* **12**:5, 475–479.

Ghezelayagh Z, Zabihi M, Kazemi Ashtiani M, Ghezelayagh Z, Lynn FC, Tahamtani Y. 2021. Recapitulating pancreatic cell–cell interactions through bioengineering approaches: the momentous role of non-epithelial cells for diabetes cell therapy. *Cellular and Molecular Life Sciences* **78**, 7107–7132.

Hu DP, Ferro F, Yang F, Taylor AJ, Chang W, Miclau T, Marcucio RS, Bahney CS. 2017. Cartilage to bone transformation during fracture healing is coordinated by the invading vasculature and induction of the core pluripotency genes. *Development (Cambridge)*.

Huch M, Koo BK. 2015. Modeling mouse and human development using organoid cultures. *Development (Cambridge)* **144** (2), 221–234.

Kawanishi M, Oura A, Furukawa K, Fukubayashi T, Nakamura K, Tateishi T, Ushida T. 2007. Redifferentiation of dedifferentiated bovine articular chondrocytes enhanced by cyclic hydrostatic pressure under a gas-controlled system. *Tissue Engineering* **13**:957–964.

Kim J, Adachi T. 2019. Cell Condensation Triggers the Differentiation of Osteoblast Precursor Cells to Osteocyte-Like Cells. *Frontiers in Bioengineering and Biotechnology* **7**, 288.

Kim J, Adachi T. 2020. Modulation of Sost gene expression under hypoxia in 3D scaffold-free osteocytic tissue. *Tissue Engineering Part A* **27** (15-16), 1037–1043..

Kim J, Adachi T. 2021. Cell-fate decision of mesenchymal stem cells toward osteocyte differentiation is committed by spheroid culture. *Scientific Reports* **11**:13204.

Kim J, Kigami H, Adachi T. 2020a. Characterization of self-organized osteocytic spheroids using mouse osteoblast-like cells. *Journal of Biomechanical Science and Engineering* **15**:1–8.

Kim J, Kigami H, Adachi T. 2021. Comparative gene expression analysis for pre-osteoblast MC3T3-E1 cells under non-adhesive culture toward osteocyte differentiation. *Journal of Bioscience and Bioengineering* **132** (6), 651–656.

Kim J, Koo BK, Knoblich JA. 2020b. Human organoids: model systems for human biology and medicine. *Nature Reviews Molecular Cell Biology* **21**, 571–584.

Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. 2008. Endochondral ossification: How cartilage is converted into bone in the developing skeleton. *International Journal of Biochemistry and Cell Biology* **40**, 46-62.

Marlovits S, Tichy B, Truppe M, Gruber D, Vécsei V. 2003. Chondrogenesis of Aged Human Articular Cartilage in a Scaffold-Free Bioreactor. *Tissue Engineering* **9**, *6.*

Pesaresi M, Sebastian-Perez R, Cosma MP. 2019. Dedifferentiation, transdifferentiation and cell fusion: in vivo reprogramming strategies for regenerative medicine. *FEBS Journal* **286** (6), 1074-1093.

Rossi G, Manfrin A, Lutolf MP. 2018. Progress and potential in organoid research. *Nature Reviews Genetics* **19**:671–687.

Ruscitto A, Morel MM, Shawber CJ, Reeve G, Lecholop MK, Bonthius D, Yao H, Embree MC. 2020. Evidence of vasculature and chondrocyte to osteoblast transdifferentiation in craniofacial synovial joints: Implications for osteoarthritis diagnosis and therapy. *FASEB Journal* **34** *(3),* 4445–4561.

Schaub JR, Huppert KA, Kurial SNT, Hsu BY, Cast AE, Donnelly B, Karns RA, Chen F, Rezvani M, Luu HY, Mattis AN, Rougemont AL, Rosenthal P, Huppert SS, Willenbring H. 2018. De novo formation of the biliary system by TGFβ-mediated hepatocyte transdifferentiation. *Nature* **557**, 247–251.

Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko V v., Wells SI, Zorn AM, Shroyer NF, Wells JM. 2011. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* **470**:105–110.

Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N, Aoyama S, Adachi Y, Taniguchi H. 2013. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499**:481–484.

Tanabe K, Haag D, Wernig M. 2015. Direct somatic lineage conversion. *Philosophical Transactions of the Royal Society B: Biological Sciences*.

Tsumaki N, Okada M, Yamashita A. 2015. iPS cell technologies and cartilage regeneration. *Bone* **70**:48–54.

Usmani SE, Pest MA, Kim G, Ohora SN, Qin L, Beier F. 2012. Transforming growth factor alpha controls the transition from hypertrophic cartilage to bone during endochondral bone growth. *Bone* **51**:131–141.

Yamashita A, Morioka M, Yahara Y, Okada M, Kobayashi T, Kuriyama S, Matsuda S, Tsumaki N. 2015. Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. *Stem Cell Reports* **4**:404–418.

Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. 2014. Chondrocytes Transdifferentiate into Osteoblasts in Endochondral Bone during Development, Postnatal Growth and Fracture Healing in Mice. *PLoS Genetics* **10** (2), e1004820.

Figure legends

Fig. 1 Fabrication of scaffold-free spheroid reconstructed by ATDC5 cells. (A) Schematic diagram of fabrication method for self-organized scaffold-free spheroid in the U-bottom ultra-low attachment dish after 2-day. Microscopic images of (B) spheroid and (C) monolayer after 2-day. Black bars indicate 200 μm.

Fig. 2 Evaluation of chondrocyte markers in the spheroid after 2-day cultivation. (A) Relative mRNA expressions of chondrocyte markers (*Sox9*, *Acan*, and *Col2*) in ATDC5 spheroids normalized to monolayer samples. The bars represent the mean± standard error ($n = 8$; p-value was calculated from Student's t-test; * $p < 0.05$, ** $p < 0.005$). (B) Immunostaining images of the ATDC5 spheroid; nucleus (DAPI), actin filaments (ACTIN), type II collagen (COL2), and merged image (MERGE). White bars indicate 100 μm.

Fig. 3 Evaluation of hypertrophic chondrocyte markers in the spheroid after 2-day cultivation. (A) Relative mRNA expressions of hypertrophic chondrocyte markers (*Fgfr3*, *Col10*, and *Mmp13*) in ATDC5 spheroids normalized to monolayer samples. The bars represent the mean± standard error ($n = 8$; p-value was calculated from Student's t-test; * $p < 0.05$, ** $p < 0.005$). (B) Immunostaining images of the ATDC5 spheroid; nucleus (DAPI), actin filaments (ACTIN), type X collagen (COL10), and merged image (MERGE). White bars indicate 100 μm.

Fig. 4 Evaluation of osteocyte markers in the spheroid after 2-day cultivation. (A) Relative mRNA expressions of osteocyte markers (*Opn*, *Dmp1*, and *Sost*) in ATDC5 spheroids normalized to monolayer samples. The bars represent the mean± standard error ($n = 8$; p-value was calculated from Student's t-test; * $p < 0.05$, ** $p < 0.005$). (B) Immunostaining images of the ATDC5 spheroid; nucleus (DAPI), actin filaments (ACTIN), dentin matrix protein 1 (DMP1), and merged image (MERGE). White bars indicate 100 μm.

Fig. 5 Evaluation of gene expression change in cells dissociated from spheroid (sph-mono). (A) Schematic diagram of fabrication method for sph-mono. The spheroids subcultured for 2-day in the ultra-low attachment dish were transferred to the normal cell attachment dish and subcultured for another 4-day. Microscopic images of the 2-day-old spheroid transferred to the normal attachment dish followed by incubation for another (B) 2-day and (C) 4-day (sph-mono). Black bars indicate 200 μm. (D) Relative mRNA expressions of osteocyte markers (*Opn*, *Dmp1*, and *Sost*) in ATDC5 spheroids normalized to monolayer samples. The bars represent the mean± standard error ($n = 4$; p-value was calculated from one-way ANOVA with Tukey's HSD *post-hoc* test ($\alpha = 0.05$); Bar indicates the significance between groups; $p < 0.05$)

Fig. 6 The spheroid was hypertrophied after a long-term cultivation up to 28-day. (A) Microscopic images of the spheroid after 2-day, 4-day, 7-day, 14-day, 21-day, and 28-day cultivation. White bars indicate 100 μm. (B) Projected area of the spheroids during the long-term cultivation. The error bars indicate the mean± standard deviation (n = 34 from 3 independent experiments). (C) HE staining and (D) Safranin-O staining images of 2-day and 28-day spheroids. Black bars indicate 50 μm. (E) COL10 immunostaining image of 28-day spheroid. White bar indicates 50 μm.

Figure S1 Fluorescence image stained by Hoechst 33342 of (A) 2-day and (B) 28-day spheroids. Binarization of fluorescence image using ImageJ for (C) 2-day and (D) 28-day spheroids. White bars indicate 50 μm.