

# Rapid and efficient generation of cartilage pellets from mouse induced pluripotent stem cells by transcriptional activation of BMP-4 with shaking culture

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## Abstract

Induced pluripotent stem cells (iPSCs) offer an unlimited source for cartilage regeneration as they can generate a wide spectrum of cell types. Here, we established a tetracycline (tet) controlled *bone morphogenetic protein-4* (BMP-4) expressing iPSC (iPSC-*Tet/BMP-4*) line in which transcriptional activation of *BMP-4* was associated with enhanced chondrogenesis. Moreover, we developed an efficient and simple approach for directly guiding iPSC-*Tet/BMP-4* differentiation into chondrocytes in scaffold-free cartilaginous pellets using a combination of transcriptional activation of *BMP-4* and a 3D shaking suspension culture system. In chondrogenic induction medium, shaking culture alone significantly upregulated the chondrogenic markers *Sox9*, *Col2a1*, and *Aggrecan* in iPSCs-*Tet/BMP-4* by day 21. Of note, transcriptional activation of *BMP-4* by addition of tet (doxycycline) greatly enhanced the expression of these genes. The cartilaginous pellets derived from iPSCs-*Tet/BMP-4* showed an oval morphology and white smooth appearance by day 21. After day 21, the cells presented a typical round morphology and the extracellular matrix was stained intensively with Safranin O, alcian blue, and type II collagen. In addition, the homogenous cartilaginous pellets derived from iPSCs-*Tet/BMP-4* with 28 days of induction repaired joint osteochondral defects in immunosuppressed rats and integrated well with the adjacent host cartilage. The regenerated cartilage expressed the neomycin resistance gene, indicating that the newly formed cartilage was generated by the transplanted iPSCs-*Tet/BMP-4*. Thus, our culture system could be a useful tool for further investigation of the mechanism of *BMP-4* in regulating iPSC differentiation toward the chondrogenic lineage, and should facilitate research in cartilage development, repair, and osteoarthritis.

## Keywords

BMP-4, cartilage regeneration, iPS cells, shaking culture, transcriptional activation

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## Introduction

Induced pluripotent stem cells (iPSCs), which can be generated via reprogramming of somatic cells by forced expression of several defined pluripotency factors, possess extensive proliferative ability and multipotent differentiation ability.<sup>1</sup> These cells provide a promising opportunity to develop cell-based tissue/organ regeneration for degenerative diseases.<sup>2</sup> Genetic manipulation is useful for accessing the roles of genes of interest in directing iPSC differentiation toward particular cell lineages. To obtain efficient and stable transgene expression, various gene delivery vectors have been used in pluripotent stem cells.<sup>3–5</sup> A transposon DNA vector, the *piggyBac* (PB) transposon-based gene delivery system, provides several benefits over classic viral and nonviral gene delivery systems, such as efficient delivery of DNA fragments into target cells and reversible insertion.<sup>6</sup> In addition, this system can be combined with tet-controlled transcriptional regulation to achieve spatiotemporal control of transgene expression during iPSC differentiation.<sup>7–9</sup> This can be advantageous for iPSC research.<sup>10</sup> Given its capacity for efficient and reversible gene transfer, the PB transposon system is a promising vector for gene delivery.

Among the diverse cell types derived from iPSCs, those of chondrogenic lineage present considerable interest because of their potential use in treating osteoarthritis (OA), a common clinical degenerative disease.<sup>11,12</sup> Considering the poor regenerative and reparative ability of articular cartilage in response to injury or degenerative disease, engineering of cell-based cartilage offers a new strategy for replacing the damaged tissue.<sup>13,14</sup> One of the most important considerations in using iPSCs for OA therapy is establishing a simple and convenient induction system to achieve uniform and sufficient numbers of chondrocytes. Previous studies with mouse and human pluripotent stem cells including embryonic stem cells (ESCs) and iPSCs demonstrated that chondrogenic differentiation of these cells can be initiated by exposure to several growth factors, such as the transforming growth factor (TGF)- $\beta$ s<sup>15,16</sup> and their family members bone morphogenetic proteins (BMPs) and activins.<sup>17,18</sup> Several techniques such as cell sorting<sup>12</sup> are used to achieve uniform cell lineage; 3D culture systems including embryoid bodies (EBs),<sup>19–21</sup> micro-masses,<sup>22,23</sup> pellets<sup>24,25</sup> and scaffolds<sup>21,26</sup> are also used to further enhance the chondrogenesis of pluripotent stem cells (Supplemental Table S1). However, the complicated induction procedure, which requires several stages and a long culture period, may hinder potential application of pluripotent stem cells in cartilage tissue engineering.

Simplifying the induction procedure and shortening the culture period is necessary for employing pluripotent stem cells in regenerative medicine. Efficient generation of mesoderm-derived articular chondrocytes from iPSCs will depend on finding key factors regulating the development

of cartilage. We recently reported that shaking culture as a simple approach enhances chondrogenesis<sup>20</sup> and osteochondrogenesis<sup>19</sup> of iPSCs. However, the shaking culture itself was not sufficient to induce chondrogenic lineage commitment of iPSCs; therefore, an additional approach to activate key molecules in the commitment process would be necessary to achieve successful induction of homogeneous chondrocytes.

BMP-4 guides pluripotent stem cell differentiation toward mesoderm cell lineages via brachyury (also known as T), which encodes a T-box transcription factor that plays an essential role in mesoderm formation, and SMAD signaling.<sup>27–29</sup> In addition, BMP-4 plays an important role in regulating cartilage development and is considered to be a stimulator of chondrogenesis and potential therapeutic agent for cartilage repair.<sup>30</sup> Thus, BMP-4 would be a key factor that can accelerate iPSC differentiation toward chondrogenic mesoderm lineages. Indeed, a chondrogenic mesodermal lineage of mouse iPSCs can be efficiently induced through regulation of recombinant BMP-4 and FGF-2.<sup>31</sup> However, the high running cost is a potential issue with the usage of multiple recombinant proteins for differentiation assays. In addition, transcriptional activation properties of BMP-4 in the chondrogenesis of iPSCs remain unclear.

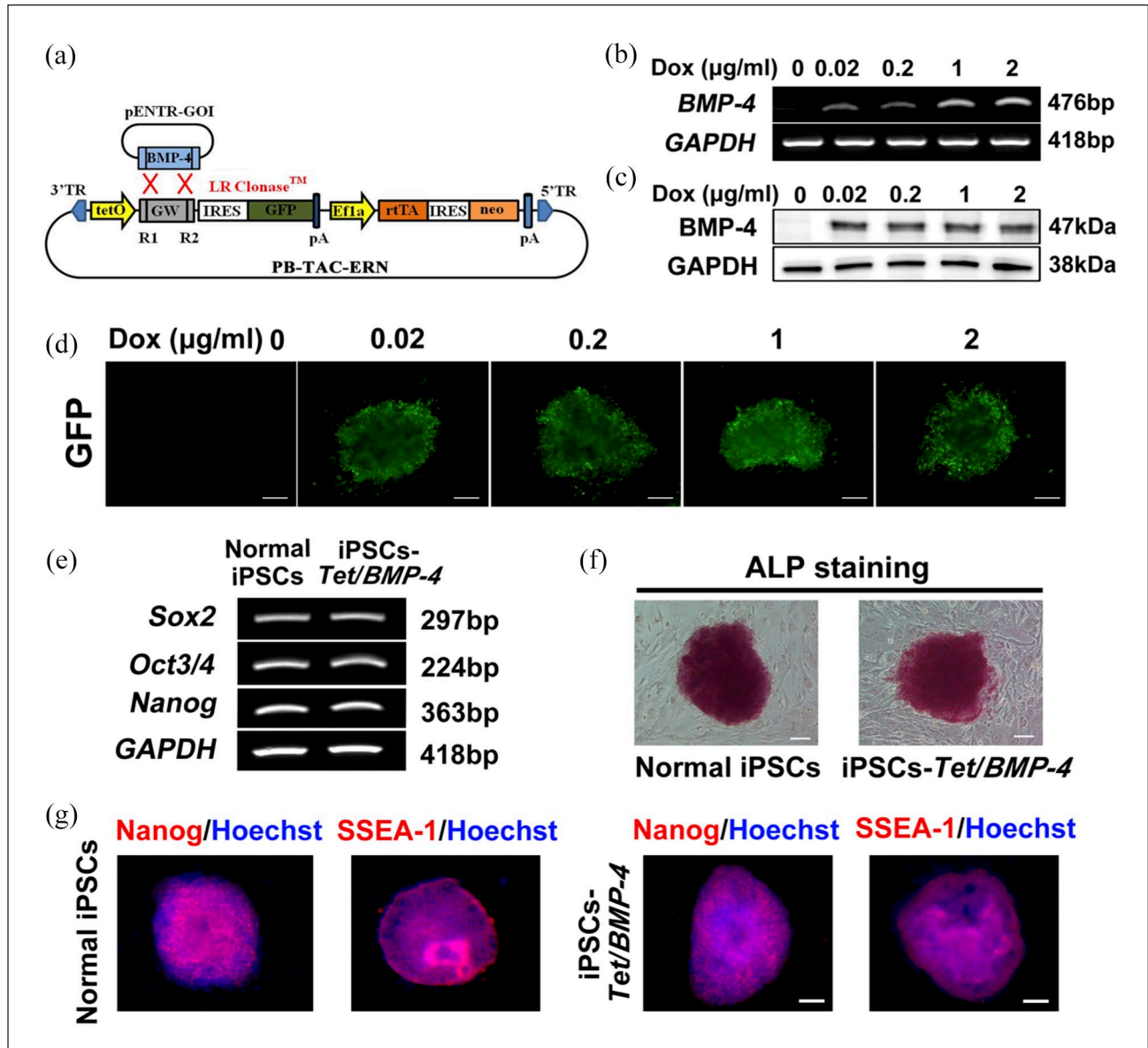
In this study, we established a PB vector-based tet-controlled *BMP-4* gene regulation system for iPSCs (iPSCs-*Tet/BMP-4*) in which BMP-4 expression can be spatiotemporally controlled, and then explored the role of BMP-4 in regulating iPSCs lineage commitment. On this basis, a simple and convenient approach for direct guiding of iPSC differentiation into chondrogenic mesodermal lineages was established by controlled *BMP-4* gene expression using a 3D shaking suspension culture system. The cartilage regeneration potential of iPSC-derived chondrocytes was also examined using an osteochondral defect model.

## Materials and methods

### Cell culture

SNLP76.7-4 feeder cells were supplied by Dr. Allan Bradley of the Sanger Institute (London, UK) and cultured in Dulbecco's modified Eagle medium (DMEM, Nacalai Tesque) supplemented with 7% FBS (Japan Bioserum), 2 mM L-glutamine (Wako, Japan), and 0.5% penicillin/streptomycin (Wako, Japan). When cells reached 90% confluence, they were inactivated by treatment with mitomycin-C (Nacalai Tesque) for 2.5 h. The mitomycin-C treated SNL cells were re-plated on 0.1% gelatin-coated 6-well plates for the following iPSC culture.

Mouse gingiva-derived iPSCs<sup>32</sup> were maintained on mitomycin-C treated SNLP76.7-4 feeder cells in ES medium (DMEM with 15% FBS, 2 mM L-glutamine,  $1 \times 10^{-4}$  M non-essential amino acids,  $1 \times 10^{-4}$  M 2-mercaptoethanol, 50 U penicillin, and 50  $\mu$ g/ml streptomycin).<sup>33</sup>



**Figure 1.** Establishment of iPSCs-Tet/BMP-4 and pluripotency evaluation. (a) Construction of a tetracycline (tet)-inducible *BMP-4*-expressing piggyBac vector. (b and c) *BMP-4* expression was detected using RT-PCR (b) and western blotting (c) after 24 h of culture with or without doxycycline (Dox). (d) Fluorescence microscopy images of GFP expression in iPSCs-Tet/BMP-4 after 24 h of culture with or without the administration of Dox. (e) Expression of pluripotency marker genes in iPSCs-Tet/BMP-4 and original iPSCs (normal iPSCs) was investigated using RT-PCR. (f and g) To assess the undifferentiated status of iPSCs-Tet/BMP-4, alkaline phosphatase (ALP) staining. (f) and immunocytochemistry for Nanog and SSEA-1 (g) were performed. Nuclei were stained with Hoechst 33258 (blue). Scale bars: 100  $\mu\text{m}$ .

### Generation of a tetracycline (tet)-inducible *BMP-4* iPSCs

This study was approved by the Center and Committee of Gene Research, Tohoku University (approval number: 2015DnLMO-008). The PB-TAC-ERN (KW200) vector (All-in-One PB transposon) and pCAG-PBase expression vector (KW158) were kindly provided by Dr. Knut Woltjen of CiRA (Kyoto University, Japan). The PB-TAC-ERN vector

constitutively expresses the neomycin (G418) resistance gene along with the reverse tet transactivator (rtTA) element, regulating tet-dependent activation of target genes controlled by the tet-operator (tetO) (Figure 1(a)). By using this system, target gene expression can be indirectly monitored by GFP activation.<sup>34</sup>

The Gateway entry vector (pENTR221) containing full-length human *BMP-4* cDNA (GenBank: EU176183.1) was purchased from Open Biosystems. The *BMP-4* cDNA was

transferred into the PB-TAC-ERN vector using the LR recombination reaction provided by the Gateway system. The Neon transfection system (Invitrogen) was used to yield the *BMP-4* iPSC line (iPSCs-*Tet/BMP-4*). Briefly, 1  $\mu$ g of destination vector and 1  $\mu$ g of PBase were added to 100  $\mu$ l of iPSC suspension ( $1 \times 10^7$  cells/ml); then, the cell/DNA mixture was aspirated into the electroporation cuvette. The appropriate parameters (pulse voltage, 1300v; pulse width, 20ms; pulse no. 2) were selected for electroporation. At 48h after electroporation, 150mg/ml neomycin (G418; Wako, Japan) was added to the culture medium to select the positive clones. After 5–7 days of drug selection, appropriate iPSC-*Tet/BMP-4* clones with high GFP expression in the presence of doxycycline (Dox: tet derivative) were selected for further use. To confirm the optimal concentration of Dox, different concentrations of Dox (0.02–2.0  $\mu$ g/ml, Sigma) were added to the culture medium to induce *BMP-4* and GFP expression. *BMP-4* expression was detected using reverse transcription polymerase chain reaction (RT-PCR) and western blotting; GFP expression was detected with a fluorescence microscope.

For pluripotency analysis, ALP staining as described in a previous study<sup>32</sup> and immunofluorescence were performed. For immunofluorescence analysis, both normal iPSCs and iPSCs-*Tet/BMP-4* were fixed with 4% paraformaldehyde. The fixed cells were washed with PBS and then incubated with 1% bovine serum albumin (BSA) and 0.1% TritonX-100 for 20 min at room temperature. After blocking and permeabilization, the cells were incubated with primary antibodies against Nanog (Cell Signaling, Danvers, MA) and SSEA-1 (Bioss, Woburn, MA) at 4°C overnight. Then, the cells were incubated with anti-rabbit secondary antibody (Abcam) at room temperature for 60 min. The cell nuclei were stained with Hoechst 33258 (Invitrogen, Thermo Fisher Science). The results were observed using a fluorescence microscope. The expression of pluripotency markers such as *Sox2*, *OCT3/4*, and *Nanog* was detected using RT-PCR.

### Investigating the role of *BMP-4* in iPSC lineage commitment

Using the established iPSCs-*Tet/BMP-4*, we performed a differentiation assay by controlling the expression *BMP-4* to investigate its role in iPSC lineage commitment. iPSCs-*Tet/BMP-4* were first cultured in ES medium to form embryoid body (EBs). Then, Dox (1  $\mu$ g/ml) was added to induce spontaneous differentiation of iPSCs-*Tet/BMP-4* by forced expression of *BMP-4*. Total RNA was extracted on days 3 and day 7 and semi-quantitative RT-PCR was performed to investigate the expression of three germ-related marker genes. Subsequently, the cell aggregates of each group on day 14 were embedded in growth factor-reduced Matrigel (Corning, NY) and subcutaneously transplanted into 5-week-old immunodeficient mice (CB-17 SCID; Clea Japan). All implants were harvested after

30 days. The extracted samples were embedded in paraffin. Then, cross-sections were generated and stained with hematoxylin-eosin (HE) and toluidine blue (Wako, Japan) for histological analysis. The cartilage area that stained positive for toluidine blue (the percentage of cartilage area within the whole implant) was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### *In vitro* chondrogenic differentiation

iPSCs-*Tet/BMP-4* were dissociated into single cells with 0.25% trypsin and quickly aggregated low-adhesion U-bottom 96-well plates (Greiner bio-one, Germany) in ES medium (30,000 cells/well, 100  $\mu$ l). After 24h of culture, cell pellets were cultured in chondrogenic medium consisting of high-glucose DMEM supplemented with 10ng/ml TGF $\beta$ -3 (Oncogene Research Products, Cambridge, MA), 100nM dexamethasone (Sigma, St. Louis, MO), 50  $\mu$ g/ml ascorbic acid (Wako Pure Chemicals, Tokyo, Japan), 100  $\mu$ g/ml sodium pyruvate, 40  $\mu$ g/ml proline, and ITS-plus (Collaborative Biomedical Products, Cambridge, MA; final concentrations: 6.25  $\mu$ g/ml bovine insulin, 6.25  $\mu$ g/ml transferrin, 6.25  $\mu$ g/ml selenous acid, 5.33  $\mu$ g/ml linoleic acid, and 1.25 mg/ml BSA)<sup>20</sup> and Dox (1  $\mu$ g/ml). After 4 days in culture, cell pellets were transferred into low-adhesion 6-well plates (Thermo Fisher Scientific) and subjected to suspension shaking culture<sup>33</sup> using a seesaw shaker at 0.5 Hz for 28 days. The culture medium was changed every 2 days. The feret's diameter of the cell pellets ( $n=3$ , each time point, each group) on days 4, 7, 14, and 21 was measured using ImageJ software. The cell pellets at days 14, 21, and 28 were embedded in paraffin for histological analysis.

### RT-PCR analysis

Total RNA was extracted with Trizol Reagent (Life Technologies) and quantified with a Thermo Scientific NanoDrop 1000 ultraviolet-visible spectrophotometer (NanoDrop Technologies, Wilmington, DE). After treatment with DNase I (Invitrogen, ThermoFisher Scientific), 1  $\mu$ g of total RNA was used to synthesize cDNA using a reverse transcription system (Promega). For semi-quantitative RT-PCR analysis, the genes of interest were amplified using Taq DNA polymerase (Promega) according to the manufacturer's instructions. PCR products were analyzed using 2% agarose gel electrophoresis with ethidium bromide staining and detected under UV illumination. The PCR primer pairs used are presented in Supplemental Table S2. For quantitative real-time RT-PCR analysis, a SYBR Green assay was performed using Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) on a StepOnePlus real-time PCR system (Applied Biosystems).<sup>33</sup> Target gene expression was quantitatively measured using the  $\Delta\Delta C_t$  method.<sup>35</sup> The corresponding primer sequences are displayed in Supplemental Table S3.

### Western blot analysis

Total protein was collected using Nonidet P-40 (NP-40) lysis buffer supplemented with protease inhibitor cocktail (Nacalai Tesque) from iPSCs-*Tet/BMP-4* after incubation with Dox for 24 h. The protein concentration was determined using a BCA protein assay. Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Wako, Japan). The blots were blocked with 5% nonfat milk, and then incubated with primary antibodies against BMP-4 (1:1000, Abcam, Cambridge, MA) or GAPDH (1:5000, Millipore) at 4°C overnight. After washing with Tris-buffered saline with Tween (TBST), the membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Finally, the signals were visualized using an ImmunoStar Zeta kit (Wako, Japan).

### Animal experiments

All animal experiments conducted in this study were approved by Animal Research and Care committee of Tohoku University (approval number: 2015DnA-022).

### Teratoma formation assay

Six iPSCs-*Tet/BMP-4* pellets obtained on days 14 and 28 were subcutaneously transplanted into 5-week-old immunodeficient mice (CB-17 SCID; Clea Japan, Tokyo, Japan). The mice were sacrificed after 4 weeks. The transplants were extracted, fixed in 4% paraformaldehyde, and embedded in paraffin for histological analysis.

### Rat osteochondral defect model

Ten-week-old male Sprague-Dawley rats (Nippon SLC) were used in this study. The skin and joint capsules were opened to expose the knee joints. An osteochondral defect (diameter: 2 mm; depth: 2 mm) was created in the trochlear groove of the femur using a drill with an outer diameter of 2 mm.<sup>36</sup> The iPSC-*Tet/BMP-4* pellets were implanted in the osteochondral defects, and then the joint capsule and skin were closed. The rats were treated with 14 mg/kg/day cyclosporine for immunosuppression. After 4 weeks, the rats were sacrificed and knee samples were collected for gross and histological examination.

### Histology and immunohistochemistry

For histological analysis, samples were fixed with 10% neutral buffered formalin, embedded in paraffin and sectioned at a thickness of 8 μm. The slides were deparaffinized and stained with HE, Safranin O, and Alcian blue following procedures previously described.<sup>17,37</sup> For

immunohistochemistry, the slides were incubated with anti-type II collagen antibody (1:50, Bioss), anti-type X collagen antibody (1:100, eBioscience), and anti-aminoglycoside 3'-phosphotransferase (APH 3' II, 1:100; Abcam) antibody overnight at 4°C, respectively. Then, the slides were incubated with the corresponding HRP-conjugated secondary antibody (1:100, Santa Cruz Biotechnology), and a diaminobenzidine (DAB) kit (Sigma) was used for visualization. Cartilage repair was statistically calculated using a histological grading scale (Supplemental Table S4).<sup>26</sup>

### Data analysis and statistical methods

The experiment data are presented as the mean ± standard deviation (SD). Statistical analysis in this study was performed using one-way ANOVA with Tukey's multiple comparison test to assess differences among multiple experimental groups. For comparison between two groups, the Mann-Whitney *U* test was used.  $p < 0.05$  was used considered statistically significant.

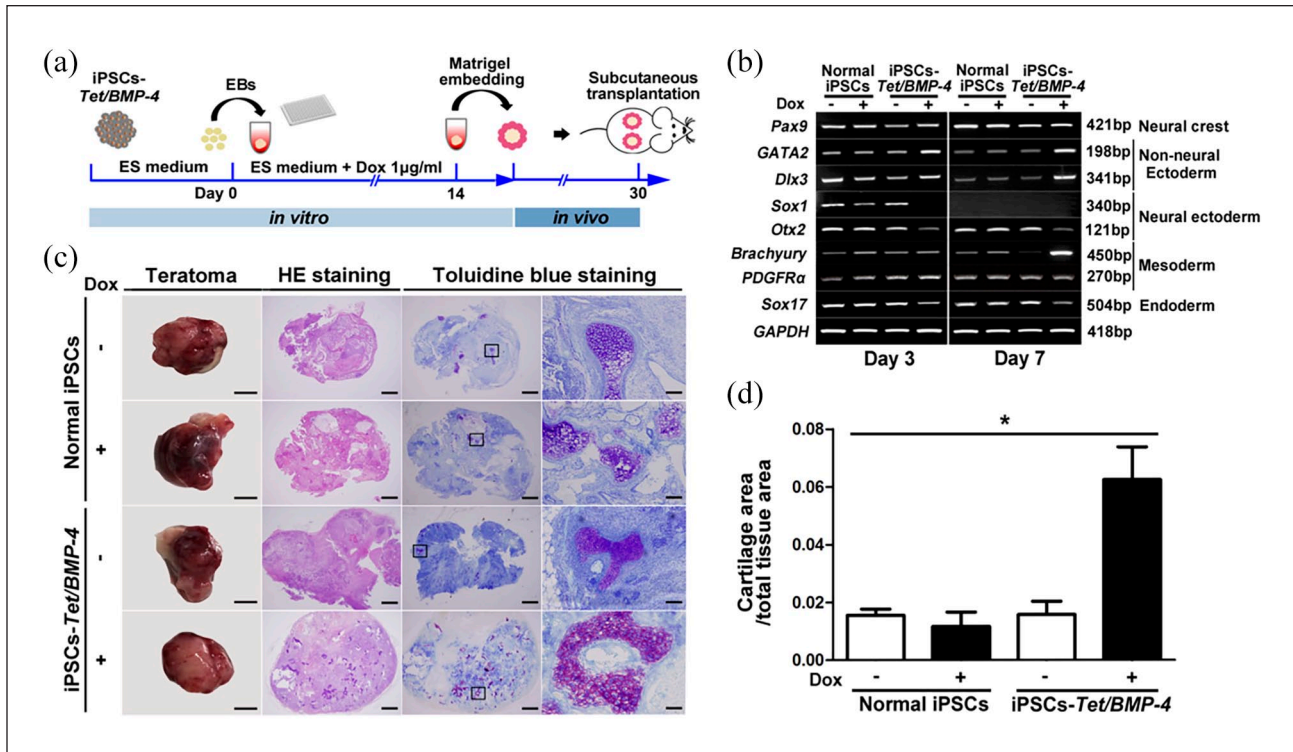
## Results

### Generation of iPSCs-*Tet/BMP-4*

In the established iPSCs-*Tet/BMP-4*, administration of Dox at different concentrations for 24 h robustly induced the expression of *BMP-4* as determined by *BMP-4* mRNA and protein levels and GFP fluorescence (Figure 1(b)–(d)). The Dox concentration of 1 mg/ml was selected for the following study. Next, the pluripotency of iPSCs-*Tet/BMP-4* was evaluated. The expression of the pluripotency marker genes *Sox2*, *Oct3/4*, and *Nanog* in iPSCs-*Tet/BMP-4* was equal to that in normal iPSCs (Figure 1(e)). ALP staining and immunofluorescence analysis for Nanog and SSEA-1 showed no difference between these two groups (Figure 1(f) and (g)). These results indicate that iPSCs-*Tet/BMP-4* maintained their pluripotency.

### Effects of BMP-4 on regulating the lineage commitment of iPSCs

To study the roles of BMP-4 in the lineage commitment of iPSCs, a differentiation assay was performed using the established iPSC-*Tet/BMP-4* clones (Figure 2(a)). The EBs were cultured in ES medium and treated with Dox to induce the expression of the *BMP-4* gene. The expression of representative marker genes of the three germ layers was detected using RT-PCR at days 3 and 7. Forced expression of *BMP-4* upregulated non-neural ectoderm-related markers, including *GATA2* and *Dlx3*, and downregulated endoderm-related markers, such as *Sox17*, at day 7. Furthermore, forced expression of *BMP-4* dramatically



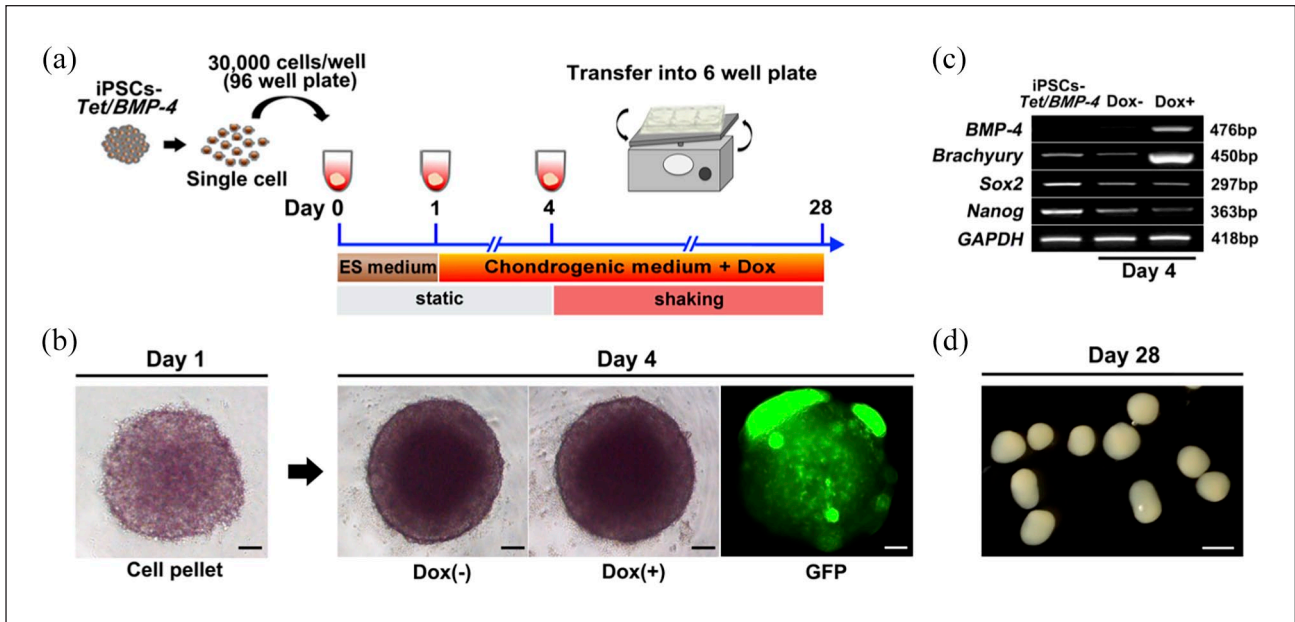
**Figure 2.** Effects of BMP-4 on regulating iPSC lineage commitment. (a) Schematic representation of the protocol for investigating the role of BMP-4 in regulation iPSC differentiation. (b) RT-PCR results for gene expression related to each of the three germ layers after treatment with Dox on days 3 and 7. (c) Images of teratomas (scale bars: 1 cm); HE staining (scale bars: 100  $\mu$ m) and toluidine blue staining (scale bars: left panel 100  $\mu$ m, right panel 50  $\mu$ m) for cartilage tissue in the teratomas. (d) Toluidine blue-positive cartilage area was calculated based on histomorphometric analysis using ImageJ software ( $n = 3$ ;  $*p < 0.05$ , ANOVA with Tukey's multiple comparison test).

upregulated the mesoderm-related marker *brachyury* and downregulated the neural ectoderm-related markers *Sox1* and *Otx2* (Figure 2(b)). These results suggest that transcriptional activation of *BMP-4* was associated with enhanced non-neural ectodermal and especially mesodermal lineage differentiation of iPSCs.

After culture for 14 days and administration of Dox, the cell aggregates were subcutaneously transplanted into SCID mice and the transplants were harvested on day 30 for histological evaluation. HE staining showed that all teratomas derived from normal iPSCs and iPSCs-Tet/BMP-4 contained several types of tissue (Figure 2(c)). However, the tissue component ratio was different among the four groups, especially with respect to the content of cartilage tissue. HE and toluidine blue staining showed that the Dox-plus group of iPSCs-Tet/BMP-4 contained more cartilage tissue than the other groups (Figure 2(c) and (d)). This result is consistent with RT-PCR results shown in Figure 2(b), as cartilage is derived from mesodermal lineage. These results indicate that iPSCs-Tet/BMP-4 can be used to investigate the mechanism of *BMP-4* in regulating the lineage commitment of iPSCs and regeneration of mesodermal derivatives, especially cartilage tissue.

### Establishment of a 3D shaking suspension culture method to generate iPSCs-Tet/BMP-4 derived chondrogenic pellets

To generate scaffold-free cartilaginous tissue using iPSCs-Tet/BMP-4, a shaking suspension 3D culture method was examined (Figure 3(a)). First, iPSCs-Tet/BMP-4 were cultured in 96-well low-attachment U-bottom plates with ES medium for 1 day. The cells quickly aggregated at the bottom of the plates and formed cell pellets (Figure 3(b)). Then, the ES medium was exchanged for serum-free chondrogenic induction medium, coupled with administration of Dox, and the pellets were cultured for an additional 3 days in 96-well plates. The size of the iPSCs-Tet/BMP-4 pellets increased. The pellets displayed strong GFP fluorescence after administration of Dox (Figure 3(b)). RT-PCR showed that *BMP-4* expression significantly increased in the presence of Dox. *BMP-4* dramatically upregulated the *brachyury* gene (Figure 3(c)), which encodes a T-box transcription factor that plays an essential role in mesoderm formation.<sup>29</sup> The expression of the pluripotency markers *Nanog* and *Sox2* gradually decreased in both the Dox-minus and Dox-plus groups compared with iPSCs-Tet/BMP-4 (Figure 3(c)).



**Figure 3.** Protocol for differentiating iPSCs-Tet/BMP-4 toward the chondrogenic lineage. (a) Schematic representation of the protocol for inducing iPSC differentiation into chondrocytes. (b) Phase and GFP fluorescence images of cell pellets on days 1 and 4. Scale bars: 100  $\mu$ m. (c) Expression of pluripotency marker genes *Sox2* and *Nanog* as well as mesodermal marker genes *BMP-4* and *brachyury* in undifferentiated iPSCs and cell pellets with or without Dox. (d) Images of iPSC-Tet/BMP-4-derived cartilage pellets on day 28. Scale bar: 1 mm.

After 4 days of culture in 96-well plates, the 3D cell pellets were transferred to low-adhesion 6-well plates (Figure 3(a)). Some of the cell pellets were subjected to shaking culture using a seesaw shaker at 0.5 Hz; the rest of the pellets were maintained in static culture. Under shaking culture in chondrogenic medium, the pellets derived from iPSCs-Tet/BMP-4 with Dox treatment showed oval morphology and white cartilaginous appearance on day 28 (Figure 3(d)).

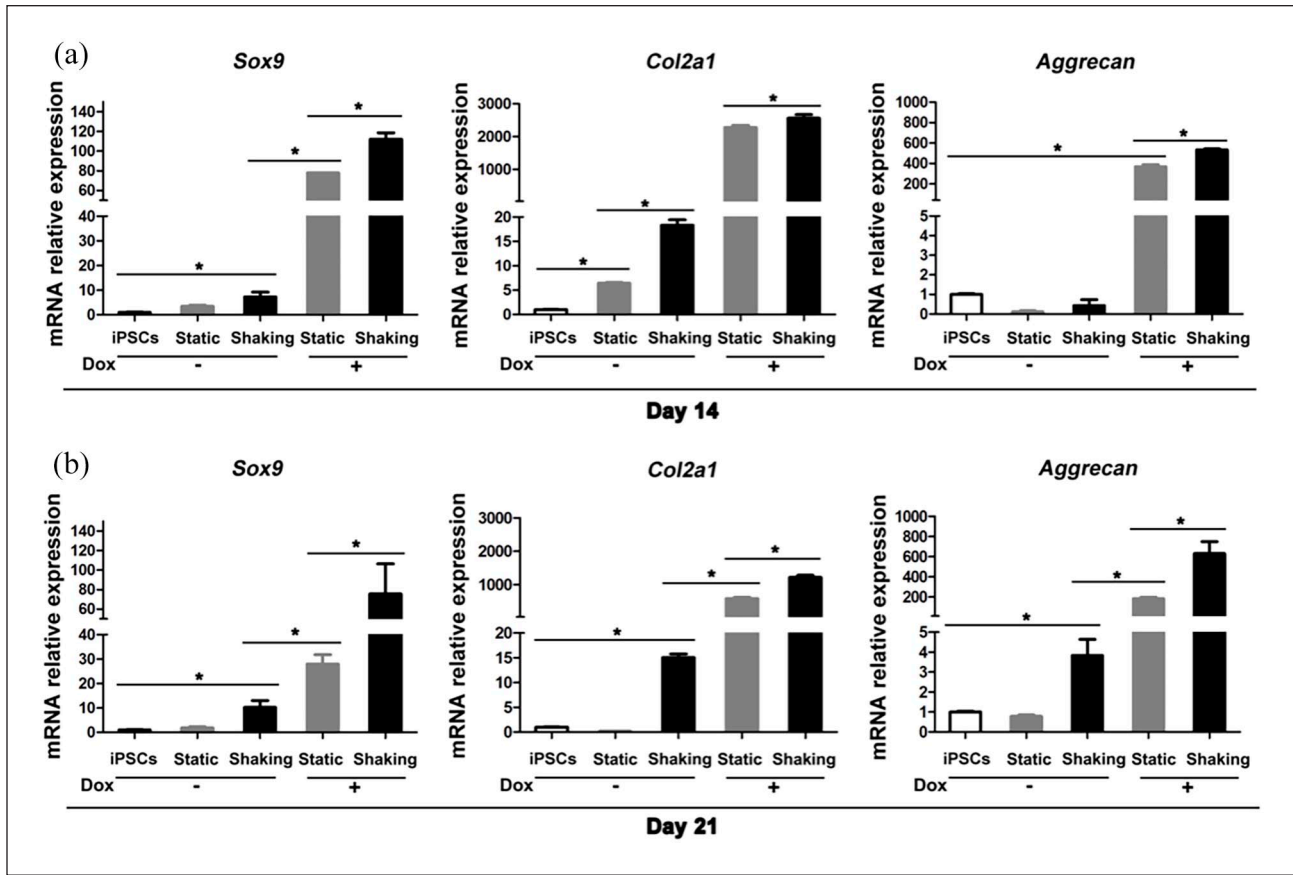
### Characteristics of cartilaginous pellets

During chondrogenic induction, the size of the pellets of the Dox-minus groups did not show a significant change from day 4 to day 21 (Supplemental Figure S1A and B). By contrast, the size of the pellets cultured in either the static or shaking condition in the Dox-plus group progressively increased over the culture period. In both static and shaking cultures, Feret's diameter significantly increased in the Dox-plus groups compared with the Dox-minus groups (Supplemental Figure S1B). In the presence of Dox, *BMP-4* mainly guided iPSC differentiation toward a mesodermal lineage, these cells survived well and continuously proliferated.

To confirm chondrogenesis, total RNA was extracted from the cell pellets at days 14 and 21. Then, the expression of chondrogenic genes was investigated using real-time RT-PCR. The expression of *Sox9*, a key transcriptional factor

that plays an essential role during chondrocyte differentiation,<sup>38</sup> was significantly increased in both static and shaking cultures in the Dox-plus groups compared with the Dox-minus groups and normal iPSCs at days 14 and 21 (Figure 4(a) and (b)). Two other key chondrogenic markers, *Col2a1* and *Aggrecan*, showed the same expression pattern. In the Dox-plus groups, all three key chondrogenic genes showed higher expression in the shaking culture group compared with the static culture group. These results indicate that *BMP-4* significantly induced the expression of *Sox9*, *Col2a1*, and *Aggrecan* during iPSC differentiation toward the chondrogenic lineage. Shaking culture further promoted this effect.

To further determine whether these iPSCs differentiated into chondrocytes, a histological analysis was performed at days 14, 21, and 28. In the Dox-minus groups without forced expression of *BMP-4*, cell pellets from both static and shaking cultures stained negatively with Safranin O and Alcian blue on day 14; these cultures showed poor formation of smooth-surfaced cartilaginous pellets (Figure 5(a) and (b)). These results agreed with the real-time PCR data shown in Figure 4(a). In the Dox-plus groups, the cell pellets from both static and shaking cultures stained positively with Safranin O and Alcian blue, indicating deposition of glycosaminoglycans (GAGs) (Figure 5(a) and (b)). The staining was more intense in the Dox-plus shaking culture group than in the Dox-plus



**Figure 4.** Expression of chondrogenic marker genes in iPSC-*Tet/BMP-4*-derived cartilaginous pellets. Expression of *Sox9*, *Col2a1*, and *Aggrecan* was evaluated by real-time RT-PCR on days 14 (a) and 21 (b). The data represent the mean values  $\pm$ SD ( $n=3$ ). (\* $p < 0.01$ , ANOVA with Tukey's multiple comparison test).

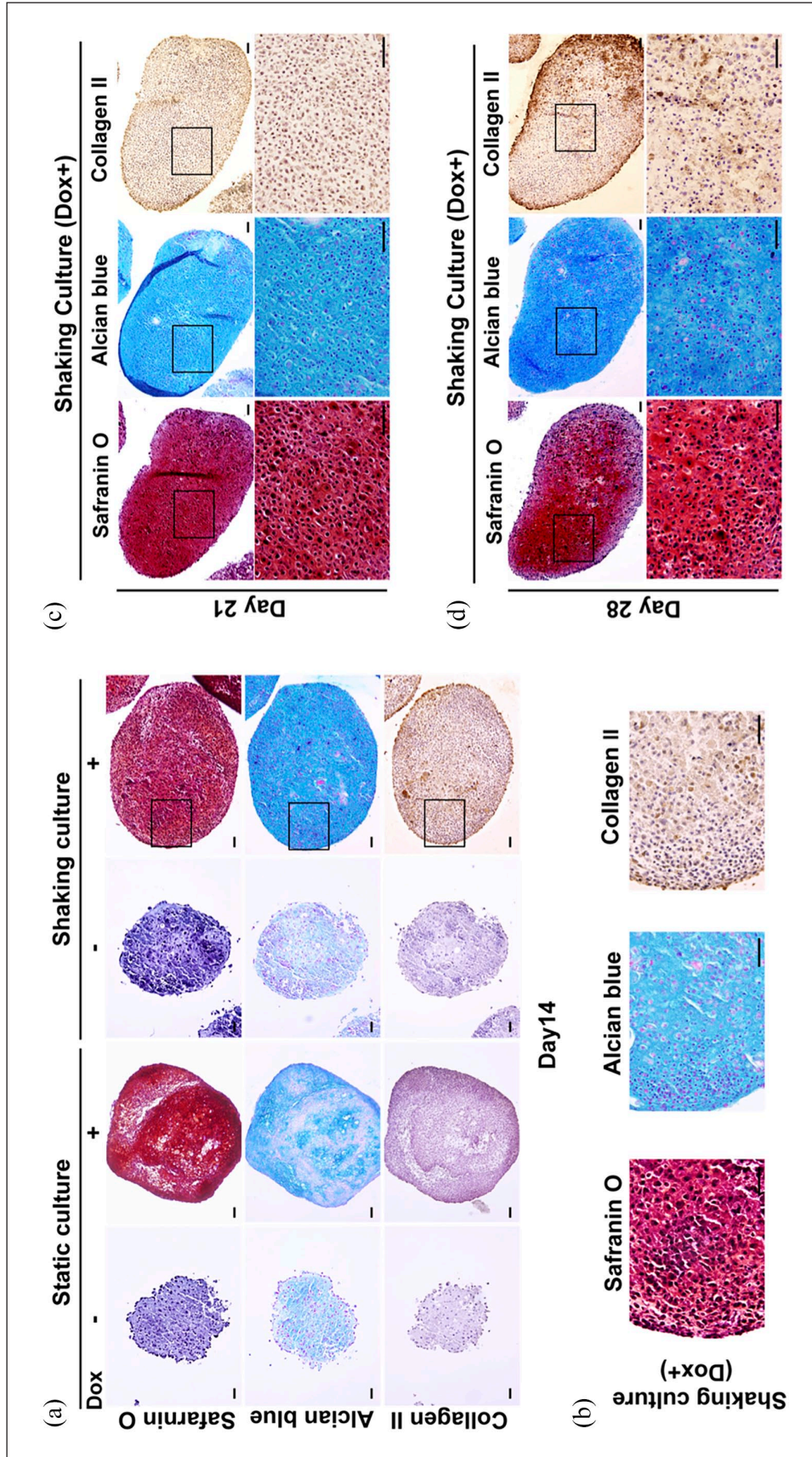
static culture group. The pellets of the Dox-plus shaking culture group exhibited extensive GAG staining throughout the whole pellet. However, the pellets of the Dox-plus static culture group did not show uniform GAG production. Shaking culture may contribute to the formation of homogenous mesodermally derived cartilage pellets. Only the shaking culture of the Dox-plus group stained positively for type II collagen at day 14 (Figure 5(a) and (b)). Thereafter, the shaking culture method was selected in combination with Dox treatment for driving iPSC-*Tet/BMP-4* differentiation into chondrocytes. After continuous culture, the cartilaginous pellets sampled on days 21 and 28 showed more intensely positive staining for Safranin O and Alcian blue, and increased expression of collagen II (Figure 5(c) and (d)), compared with samples from day 14 of culture (Figure 5(b)). The cells embedded in the extracellular matrix (ECM) showed round morphology more typical of chondrocytes, without obvious hypertrophy, on days 21 and 28 as compared with their morphology on day 14. Maintaining the 3D pellets in shaking culture, combined with controlled expression of *BMP-4*, was sufficient and effective for inducing iPSC differentiation into mature chondrocytes.

#### Assessment of functional maturation of cartilaginous pellets derived from iPSCs-*Tet/BMP-4*

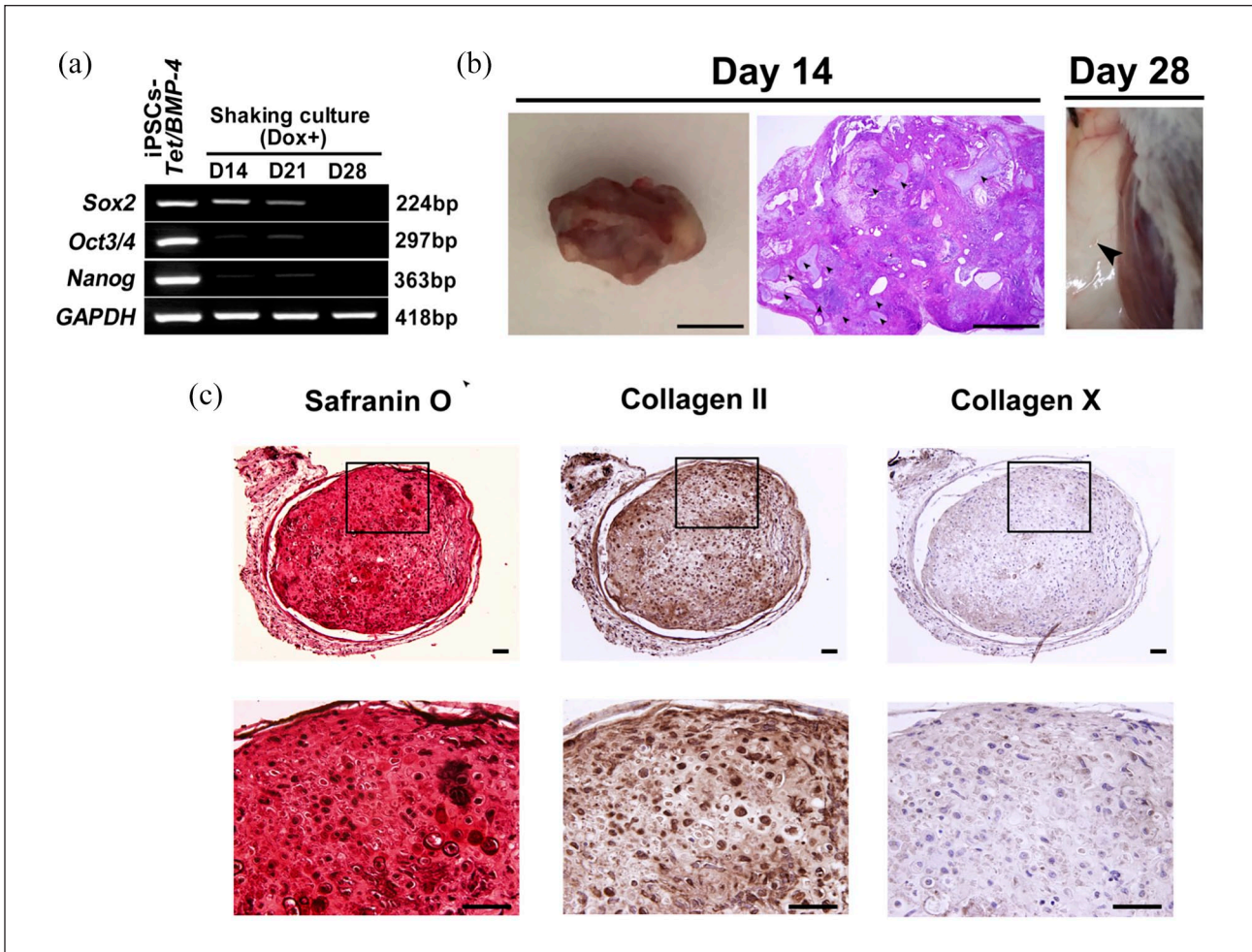
RT-PCR analysis was performed to investigate the expression of pluripotency markers in cell pellets on days 14, 21, and 28 before transplantation. The expression of the pluripotency markers *Sox2*, *Oct3/4*, and *Nanog* was significantly decreased in cartilaginous pellets compared with undifferentiated iPSCs-*Tet/BMP-4* (Figure 6(a)). However, the pluripotency markers were still expressed at low levels in cartilaginous pellets on days 14 and 21. By contrast, the expression of these markers was nearly undetectable in the cartilaginous pellets on day 28.

To assess the teratoma formation and chondrogenic activity of iPSC-*Tet/BMP-4* cartilaginous pellets, the iPSC-*Tet/BMP-4* derived cartilaginous pellets from days 14 and 28 were subcutaneously transplanted into SCID mice. The pellets from day 14 formed teratomas after 4 weeks (Figure 6(b)). HE staining showed that the tumors contained several types of tissue, including abundant cartilage tissue. In contrast, there were no signs of tumor formation at any of the transplantation sites for the day 28





**Figure 5.** Histological and immunohistochemical analysis of cartilaginous pellets. iPSCs-*Tet/BMP-4* were cultured in static or shaking culture condition with or without Dox for 28 days. Cartilaginous pellets at days 14 (a and b), 21 (c) and 28 (d) were stained with Safranin O/-Fast Green/-iron hematoxylin and Alcian blue/Fast Red, and assessed by immunohistochemical detection of type II collagen. (b) Magnified images of boxed regions in a. (c and d) lower panels are magnifications of the boxed regions. Scale bars: 50  $\mu$ m.



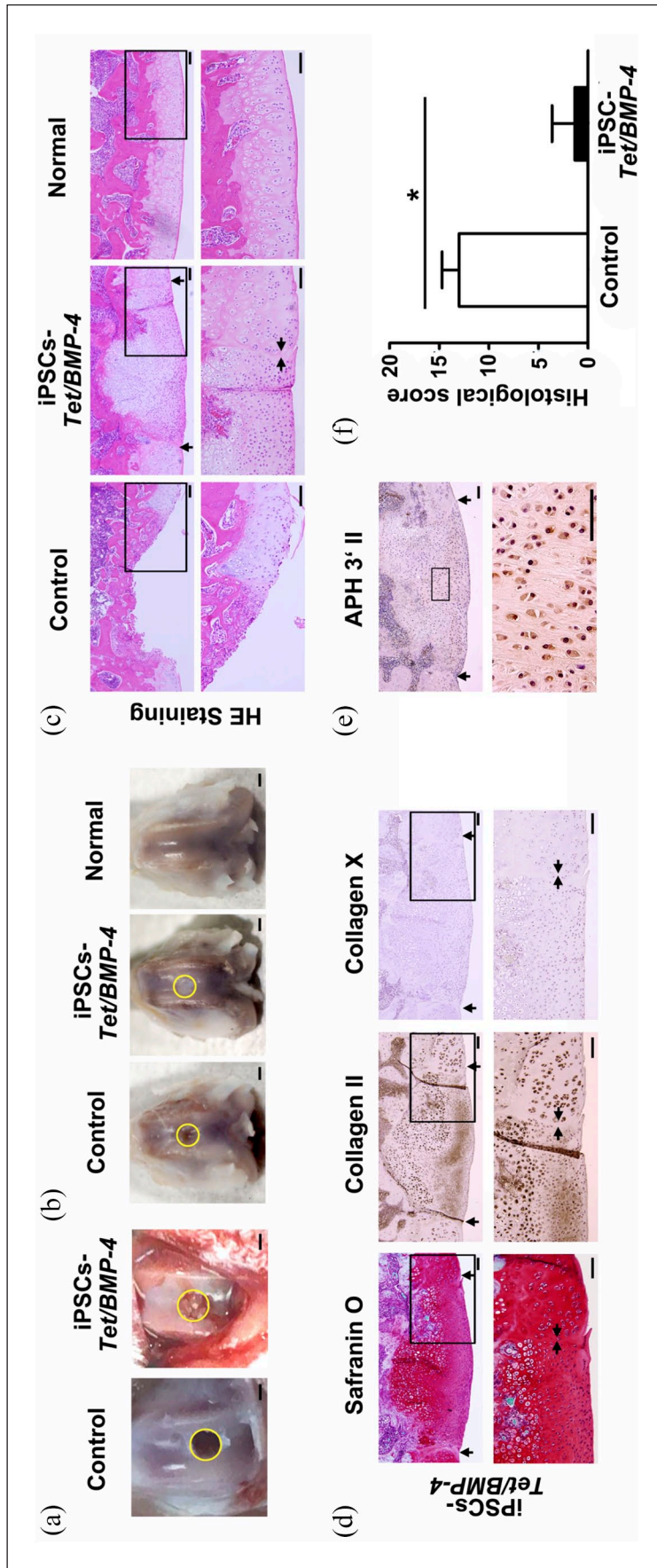
**Figure 6.** Tumorigenicity and chondrogenesis of iPSC-Tet/BMP-4 cartilaginous pellets. (a) Expression of pluripotency marker genes (Sox2, Oct3/4 and Nanog) in chondrogenically-induced iPSC-Tet/BMP-4 pellets by shaking culture with Dox for 14 (D14), 21 (D21) and 28 (D28) days. Undifferentiated iPSCs-Tet/BMP-4 were used as a control. GAPDH expression was used as an internal control. (b) Monitoring of tumor formation 4 weeks after subcutaneous transplantation of chondrogenically-induced iPSC-Tet/BMP-4 pellets, which were cultured in shaking condition with Dox for 14 (Day 14) and 28 days (Day 28). Scale bars, 1 cm. The black arrow indicates the transplanted pellet (no tumor formation). (c) Histological analysis of transplanted iPSC-Tet/BMP-4 pellets, which were cultured in shaking condition with Dox for 28 days before subcutaneous transplantation. Sections were stained with Safranin O-Fast Green-iron hematoxylin, and immunostained with anti-type II and type X collagen antibody. Scale bars, 50 μm.

pellets after 4 weeks (Figure 6(b)). The transplanted day-28 pellets formed cartilage-like tissue that stained intensely with Safranin O and Alcian blue, and was immunopositive for type II collagen and negative for type X collagen (Figure 6(c)).

#### Orthotopic cartilage regeneration using iPSCs-Tet/BMP-4 derived cartilaginous pellets

To evaluate whether iPSC-Tet/BMP-4-derived cartilaginous pellets can contribute to cartilage repair, the pellets were transplanted into osteochondral defects created on the patellar groove of immunosuppressed rats (Figure 7(a)). Macroscopic observation showed that the defects filled with iPSC-Tet/BMP-4-derived cartilaginous pellets

were repaired with smooth, glossy, and firm tissue resembling normal articular cartilage after 4 weeks post transplantation (Figure 7(b)). HE staining indicated that the defects had been completely repaired, with highly organized cartilage-like tissue similar to adjacent host cartilage and normal cartilage (Figure 7(c)). Integration between the cartilage regenerated by transplanted cells and the adjacent rat articular cartilage was successfully obtained. The chondrocytes of the articular cartilage formed by the transplanted cells showed a typical round morphology without obvious hypertrophy, similar to the cells of the adjacent host cartilage. The inner cartilage tissue, below the articular cartilage, showed a similar morphology resembling epiphyseal cartilage, with chondrocytes gradually undergoing hypertrophy (Figure 7(c)). We speculate that the



**Figure 7.** Repair of osteochondral defects using iPSC-Tet/BMP-4-derived cartilaginous pellets. (a) iPSC-Tet/BMP-4 cartilaginous pellets, which were cultured in shaking condition with Dox for 28 days, were transplanted into osteochondral defects in the trochlear groove of the rat's femur. Yellow circles indicate the margin of the defects. Scale bars: 1 mm. (b) Gross appearance of the defects 4 weeks after transplantation. Scale bars: 1 mm. (c–e) Histological analysis using HE, Safranin O, and immunohistochemical staining for type II collagen, type X collagen, and aminoglycoside 3'-phosphotransferase (APH 3'II). Scale bars: 100  $\mu$ m. (f) Histological scoring for reparative cartilage tissues. (\* $p < 0.05$ , Mann-Whitney U test;  $n = 4$ ).

bone marrow environment may stimulate hypertrophy of transplanted cartilaginous pellets. Safranin O staining of the newly formed cartilage was of similar intensity to the adjacent articular cartilage (Figure 7(d)). The newly regenerated articular cartilage was positive for type II collagen and negative for type X collagen. Next, we investigated whether the regenerated cartilage was indeed derived from the transplanted cartilaginous pellets. The iPSCs-*Tet/BMP-4* continuously expressed the neomycin resistance gene (neo) encoding APH 3'II, and the chondrocytes inside the regenerated cartilage were indeed positive for APH 3'II (Figure 7(e)). Moreover, there were no signs of tumor or other ectopic tissue formation in the transplanted sites. Histological scoring of the regenerated cartilage showed significantly increased regeneration by chondrogenic pellets formed from iPSCs-*Tet/BMP-4* compared with the control group (Figure 7(f)).

## Discussion

Steering iPSCs efficiently into defined cell lineages is crucial for cell-based regenerative medicine. This process may be hindered by low differentiation efficiency, heterogeneous cell populations,<sup>39</sup> complicated induction methods, and moreover the persistence of undifferentiated iPSCs, which are associated with risk of teratoma formation in vivo.<sup>40</sup> Thus, it is critical to identify the key regulators that govern specific cell lineage commitment to facilitate and shorten iPSC differentiation.<sup>41</sup> The derivation of specific cell types from iPSCs could be achieved by mimicking natural developmental processes. Cartilage is mainly derived from two embryonic mesodermal lineages: the paraxial mesoderm and lateral plate mesoderm.<sup>42</sup> BMP-4 plays an important role in both mesoderm and cartilage development,<sup>27,30</sup> and may thus be a key regulator of iPSC differentiation toward chondrogenic lineages. In this study, we successfully established a PB-based tet regulated gene expression system for delivery of the *BMP-4* gene into iPSCs to directly investigate the role of BMP-4 in regulating iPSC differentiation. Because *BMP-4* expression can be controlled, the established iPSCs-*Tet/BMP-4* were identical to normal iPSCs and still maintained their pluripotency.

The role of BMP-4 in iPSC lineage commitment was investigated using the established iPSCs-*Tet/BMP-4* through controlled *BMP-4* expression. Forced expression of *BMP-4* mainly enhanced the expression of the mesoderm-related gene *brachyury* and downregulated the neural ectoderm-related genes *Sox1* and *Otx2*.<sup>43,44</sup> In vivo, the Dox-plus group of iPSCs-*Tet/BMP-4* contained more cartilage tissue than other groups. These results indicate that iPSCs-*Tet/BMP-4* are a good candidate for investigating the mechanism by which BMP-4 regulates the lineage commitment of iPSCs and also for regeneration by mesodermal derivatives, especially that of cartilage tissue.

Current approaches for chondrogenic differentiation of pluripotent stem cells are hampered by low differentiation efficiency and complicated induction procedures.<sup>45–47</sup> Therefore, it is necessary to develop a simple and efficient induction method for chondrogenic differentiation of pluripotent stem cells. Mechanical forces, such as hydrodynamic shear and tension, play an important role in the maintenance of native articular cartilage and also have stimulatory effects on stem cells used in cartilage tissue engineering.<sup>48–50</sup> We recently found that shaking or rotary suspension culture enhances chondrogenic differentiation in mouse iPSCs.<sup>20,31</sup> On this basis, in this study, we established a novel approach for directly guiding iPSC differentiation toward chondrogenic lineages that avoids the use of multiple recombinant proteins; rather, our new method utilizes tet-controlled *BMP-4* gene expression with a 3D shaking suspension culture system. This culture system directly induces iPSC differentiation into chondrocytes without complicated induction stages and shortens the culture period compared with previous approaches (Supplemental Table S1).

The forced expression of *BMP-4* in the early stage of chondrogenic induction dramatically upregulated the mesodermal marker *brachyury*, and mainly committed iPSCs to the chondrogenic mesodermal lineage. Overexpression of *brachyury* promotes chondrogenesis in mesenchymal stem cells,<sup>51</sup> which partly explains the effective lineage commitment in the present results. Over time, chondrogenic genes including *Sox9*, *Col2a1*, and *aggrecan* showed higher expression in Dox-plus groups compared with Dox-minus groups on day 14. Moreover, the Dox-plus group with shaking suspension culture showed the highest expression. Using the same shaking culture method, we previously demonstrated that mechanically generated signals, including TGF- $\beta$  expression and Wnt signaling, play an important role in enhancing the chondrogenesis of iPSCs<sup>20</sup>; however, shaking culture on its own was insufficient to achieve complete chondrogenesis. In this study, our results indicate that BMP-4 significantly induces *Sox9*, *Col2a1*, and *aggrecan* expression during iPSC differentiation toward the chondrogenic lineage, and shaking culture further promotes this effect. The cell pellets of the Dox-plus groups with shaking suspension culture were larger and had a smooth, lubricious appearance. The cell pellets were positively stained with Safranin O/Alcian blue and expressed type II collagen, and the cells in the pellets showed typical round chondrocyte-like morphology. These results indicate that the newly established approach was sufficient and effective for directly inducing iPSC differentiation into mature chondrocytes. To our knowledge, this is the first report of directly guiding iPSC differentiation into chondrocytes without multiple induction stages.

Teratoma formation is one of the major challenges hindering iPSC application.<sup>40,52</sup> The teratoma-forming potential of iPSC-*Tet/BMP-4* derived cartilaginous pellets was

evaluated using immune-deficient mice. The day-14 pellets formed teratomas, indicating that there were still undifferentiated cells present. In contrast, there were no more signs of teratoma formation for the day-28 cell pellets at 4 weeks after transplantation. The longer culture may have helped to deplete incompletely differentiated cells. The transplanted day-28 pellets formed cartilage tissue that stained intensely with Safranin O, was immunopositive for type II collagen. These results suggest that the combined use of transcriptional activation of *BMP-4* and shaking culture facilitates chondrogenic differentiation and functional maturation of iPSCs.

To further evaluate the cartilage regeneration ability of iPSC-*Tet/BMP4*-derived cartilaginous pellets in an orthotopic site, the cartilaginous pellets were transplanted into osteochondral defects created on the patellar groove of immunosuppressed rats. At 4 weeks after transplantation, the defects had been completely repaired, with highly organized cartilage-like tissue similar to adjacent native cartilage. The chondrocytes in the newly formed articular cartilage showed a typical round morphology without obvious hypertrophy. Newly formed cartilage could be generated by the transplanted iPSCs because genetically modified iPSC-*Tet/BMP4*, but not recipient cells, express neomycin resistance gene, which produce APH 3'II.

Integration between repaired cartilage and native cartilage has been considered a critical step in cartilage tissue engineering, as it is necessary for biomechanical competence.<sup>53</sup> In this study, the cartilaginous matrix produced by iPSCs-*Tet/BMP-4* fused well with the surrounding host cartilage, and effective integration was successfully obtained. In addition, iPSCs-*Tet/BMP4*-derived cartilaginous pellets did not produce teratoma or other ectopic tissue formation in the orthotopically transplanted site. It should be noted that the inner cartilage tissue, below the articular cartilage, showed a similar morphology resembling epiphyseal cartilage, with chondrocytes gradually undergoing hypertrophy. We speculate that the recipient's bone marrow environment might recognize the transplanted pellets as a functionally matured cartilaginous tissue to stimulate their hypertrophy. Fabrication of functionally matured cartilaginous tissues from mouse iPSCs, as demonstrated in this study, could be advantageous to develop human iPSC-derived cartilage organoids for regenerative therapy, disease modeling and drug screening.

Stem-cell-based cartilage regeneration is sometimes accompanied by undesired hypertrophy, which may lead to calcification and ultimately, cartilage damage. In our study, iPSCs-*Tet/BMP-4*-derived cartilaginous pellets did not exhibit a hypertrophic phenotype when cultured in a 3D shaking suspension system. In addition, the cartilaginous pellets successfully repaired the cartilage defect and did not undergo hypertrophy or mineralization at the transplanted site. Our previous studies showed

that mechanical stimulation from shaking cultures enhanced the expression of TGF- $\beta$ -signaling- and Wnt-signaling-related genes during the chondrogenic differentiation of iPSC constructs,<sup>20</sup> which may contribute to the inhibition of chondrogenic hypertrophy and pathological calcification. It should be noted that *BMP-4* treatment for iPSC chondrogenesis leads to cartilage hypertrophy and mineralization.<sup>18</sup> Although the tet-controlled system in our study does not upregulate *BMP-4* in the absence of Dox, the potential risks of cartilage hypertrophy and mineralization need to be further evaluated in future studies.

## Conclusions

In summary, we successfully established a tet-controlled *BMP-4* gene regulation system for iPSCs in which transcriptional activation of *BMP-4* was associated with enhanced chondrogenesis. On this basis, we developed a simple approach for directly guiding iPSC differentiation into chondrocytes capable of cartilage regeneration in vivo, without teratoma or ectopic tissue formation, through controlled *BMP-4* gene expression using a 3D suspension shaking culture system. This culture system may be a useful tool for further investigation of the mechanism by which *BMP-4* regulates iPSC differentiation toward chondrogenic lineages and also will contribute to research related to cartilage development, repair, and osteoarthritis.

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## Author contributions

HE and K N designed and supervised the study. MZ, KN, and HE conceived the idea; MZ, KN, TK, PL, and MX carried out cell culture and RT-PCR experiments. YK carried out vector construction. MZ, HO, and MY performed histological analyses. MZ, KN, TK, and PL carried out animal experiments. MZ, KN, XJ, and HE: evaluated the data. MZ and HE drafted the manuscript. XJ critically revised the manuscript. All authors read and approved the final manuscript.

## Declaration of conflicting interests

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**Supplemental material**

Supplemental material for this article is available online.

**Availability of data and materials**

The original contributions presented in the study are included in the article/Supplementary Materials, and further inquiries can be directed to the corresponding authors.

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