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In vitro bone-like nodules generated from patient-derived iPSCs recapitulate pathological bone phenotypes

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The recapitulation of bone formation via the in vitro generation of bone-like nodules is frequently used to understand bone development. However, current bone-induction techniques are slow and difficult to reproduce. Here, we report the formation of bone-like nodules within ten days, via the use of retinoic acid (RA) to induce the osteogenic differentiation of human induced pluripotent stem cells (hiPSCs) into osteoblast-like and osteocyte-like cells that create human bone tissue when implanted in calvarial defects in mice. We also show that the induction of bone formation depends on cell signalling through the RA receptors RARα and RARβ, which simultaneously activate the BMP (bone morphogenetic protein) and Wnt signalling pathways. Moreover, by using patient-derived hiPSCs, the bone-like nodules recapitulated the osteogenesis-imperfecta phenotype, which was rescued via the correction of disease-causing mutations and partially by an mTOR (mechanistic target of rapamycin) inhibitor. The method of inducing bone nodules may serve as a fast and reproducible model for the study of the formation of both healthy and pathological bone.
from hiPSCs to less than ten days. Using this method, we were able to observe the formation process in time-lapse imaging and analyse the nodules in detail. We found that they contained both osteoblast-like and osteocyte-like cells. We also show that the method can be used for disease modelling and for the screening of drugs for treating osteogenesis imperfecta (OI), a genetic bone disease that causes fragile bones\(^3\).

### Results

**Retinoic acid promotes the osteogenic differentiation of hiPSCs and the formation of bone-like nodules in vitro.** hiPSCs (414C2) were cultured in osteogenic induction medium using our previous method\(^2\) with or without RA (1 μM) (Fig. 1a). RA treatment greatly enhanced Alizarin-red-positive nodule formation (Fig. 1b), and quantitative analyses clearly showed the increase of calcium deposition (Fig. 1c). Calcified nodules were detected as soon as day 4 after induction, and this enhancement was reproduced in various on-feeder and feeder-free hiPSCs (Supplementary Fig. 1a,b). Gene-expression analyses over ten days revealed the sequential induction of genes representative of each differentiation stage (Fig. 1d and Supplementary Fig. 2a). Cells without RA treatment remained at the osteoprogenitor stage at day 10 (Fig. 1d and Supplementary Fig. 2a), and cells treated with RA but not in osteogenic medium also showed poor differentiation (Supplementary Fig. 2b). The immunohistochemical results of each stage-related protein were comparable with those obtained by RNA analyses (Fig. 1e and Supplementary Fig. 2c). Particularly, osteocyte markers such as PHEX (phosphate-regulating gene homologues to endopeptidases on the X chromosome) and SOST (sclerostin) were detected only in cells treated with RA.

RA treatment induced the expression of a mesoderm marker (PDGFRα, platelet derived growth factor receptor alpha), but not of marker genes of other lineages such as the ectoderm (SOX1 (sex determining region Y box-1) and PAX6 (paired box protein-6)), the endoderm (SOX17) or the neural crest (SOX10) (Supplementary Fig. 2d). As for other lineage cells in mesenchymal tissues, SOX9 (an osteo-chondro progenitor marker) was upregulated, but aggreccan (ACAN) (a marker of more-differentiated chondrocytes) was not, and the expression of an adipogenic marker (PPARγ, peroxisome proliferator activated receptor gamma) was upregulated, but only transiently (Supplementary Fig. 2d). These data suggest that although RA may induce the differentiation of multiple lineages, its effect in our protocol is specific to the osteogenic lineage.

RA-treated 414C2 cells were isolated from the nodules by collagenase treatment and cultured on dishes. Most of the cells isolated from nodules at day 7 (Fig. 1f) showed a round shape, which is consistent with the morphology of osteoblasts. In contrast, the morphology of cells isolated from nodules at day 10 was heterogeneous and contained dendritic cells (Fig. 1g). Immunostaining also showed heterogeneous populations, including round cells expressing osteocalcin (OCN) strongly (Fig. 1h), and cells with many processes expressing PHEX (Fig. 1i) or SOST (Fig. 1j).

The process of nodule formation was recorded by time-lapse imaging using 317–12 cells (201B7-GFP line)\(^2\) (Supplementary Videos 1 and 2). Nodules appeared at day 4 after induction (Fig. 2a), grew larger with time and fused with other nodules, to make even larger nodules at day 7 (Fig. 2b). Coronal images showed that proliferating cells were stacked and lifted up (day 4, Fig. 2c), from which several cells were pushed out into the space underneath (day 7, Fig. 2d). Cells in nodules at different sites were further analysed by confocal imaging (Fig. 2e). At day 10, cuboidal cells were lined up on the surface of the nodules, beneath which dendritic cells with elongated cell processes were found (Fig. 2f)—although there were only a few of these, as seen in Supplementary Video 2. Cells with similar features were also found at the bottom of the nodules (Fig. 2g), and seemed to have migrated from the surface of the nodules (Supplementary Video 2).

The expression of osteogenic markers was analysed by confocal imaging in horizontal sections near the top of the nodules (Fig. 2h–o). At day 7, type I collagens were detected among cuboidal cells (Fig. 2h), and OCN was detected mainly in the cytoplasm (Fig. 2i). As for osteocyte markers, PHEX was detected, but SOST was not (Fig. 2j,k). At day 10, type I collagen was detected inside the nodules (Fig. 2l), and OCN was detected outside of the cells (Fig. 2m). In addition to PHEX (Fig. 2n), cells became SOST-positive (Fig. 2o). These data suggest that RA promotes the osteogenic differentiation of hiPSCs and contributes to making bone-like nodules that include osteoblast-like and osteocyte-like cells.

**RA induces bone formation in vivo.** 414C2 cells were cultured with or without RA in induction medium, recovered as one mass using temperature-responsive plates\(^4\), and transplanted into bone defects created in the calvariae of NOD/ShiJic-scid Jcl (NOD/SCID) mice (8 weeks old, male) (Supplementary Fig. 3a). Six weeks after transplantation, micro-CT showed little calcified tissue in defects without cell transplantation (Fig. 3a,d), whereas the amount of such tissue was much higher in defects transplanted with RA-treated cells (Fig. 3b,c,d), which were stained by an antibody against human-specific osteopontin (hOPN) (Fig. 3k). The human specificity of anti-hOPN antibody was confirmed by negative staining of the adjacent mouse bone and soft tissues (Fig. 3g and Supplementary Fig. 3b–d). On the other hand, a tumour-like mass was found in defects treated with cells cultured without RA (Fig. 3j), of which the histological features were compatible with those of teratomas containing bone-like structures positive for hOPN (Fig. 3m–o). Such tumour formation suggested the presence of undifferentiated iPSCs in the cells cultured without RA, and was found in three out of eight mice, but was found in none of the eight mice transplanted with RA-treated cells. These results indicate that cells treated with RA have an ability to make membranous bone tissues in vivo without scaffolds. Taken together, we have established a method that can induce hiPSCs to differentiate into cells that display features of osteoblasts and osteocytes within ten days.

**RA induces osteogenesis via BMP and WNT signalling.** Gene expression profiles at day 2 were compared between cells cultured with and without RA by ingenuity pathway analysis (IPA). Canonical pathway analyses identified the osteoblast-related gene set, which included ALPL (alkaline phosphatase, liver/bone/kidney), DLX5 (distal-less homeobox 5), BMP (bone morphogenetic protein), FGFR (fibroblast growth factor receptor) and WNT (wingless-integrase) as a gene set that is upregulated by RA (Supplementary Fig. 4a,b). Additionally, upstream pathway analyses detected ESRI (oestrogen receptor alpha), WNT and BMP (Supplementary Fig. 4c), suggesting the involvement of BMP and WNT as key molecules of RA-induced osteogenesis. Heatmap analyses at day 2 showed a clear upregulation of ligands and downstream molecules of both BMP and WNT signals in cells treated with RA (Fig. 4a). Time course analyses of representative downstream markers showed that the induction of BMP signals (ID1 and ID2 (inhibitor of DNA binding 1 and 2) was constant, whereas that of WNT signals (AXIN2 (axis inhibitor protein 2) and ID1 induced by negative staining of the adjacent mouse bone and soft tissues (Fig. 3g and Supplementary Fig. 3b–d). On the other hand, a tumour-like mass was found in defects treated with cells cultured without RA (Fig. 3j), of which the histological features were compatible with those of teratomas containing bone-like structures positive for hOPN (Fig. 3m–o). Such tumour formation suggested the presence of undifferentiated iPSCs in the cells cultured without RA, and was found in three out of eight mice, but was found in none of the eight mice transplanted with RA-treated cells. These results indicate that cells treated with RA have an ability to make membranous bone tissues in vivo without scaffolds. Taken together, we have established a method that can induce hiPSCs to differentiate into cells that display features of osteoblasts and osteocytes within ten days.

Viewing the role of BMP and WNT signals, we focused on the ALPL gene, which is known to be a downstream gene of both
Fig. 1 | RA promotes the formation of bone-like nodules from hiPSCs. a, Schematic overview of the protocol for bone-like nodule formation from iPSCs with RA. Hereafter, this culture condition is designated RA+. RA− indicates the same protocol but without RA. b, c, Evaluation of bone-like nodules. Nodules were stained by Alizarin red (b), and the deposition of calcium (c) was quantified at the indicated day in RA+ or RA−. d, Heatmap visualization of osteogenic differentiation stage-related genes. RNAs were extracted at the indicated day and assessed for the expression of each gene by quantitative PCR with reverse transcription (qRT-PCR). The expression level of each point was visualized as a value relative to the mean value of each gene during the induction. Minimum (blue) represents downregulation and maximum (red) represents the degree of upregulation. e, Immunostaining of stage-related proteins during the induction. Cells were stained with antibody for each stage-related protein at the indicated day. Scale bars, 100 μm. f, g, Morphology of cells isolated from nodules on day 7 (f) and day 10 (g). Cells were stained with phalloidin (actin), and nuclei were counterstained with DAPI. Scale bars, 100 μm. h–j, Immunostainning of stage-related proteins in isolated cells. Cells at day 10 were isolated, seeded on dishes and stained with antibodies for OCN (red), PHEX (green) and phalloidin (dark blue) (h, i), or antibodies for ALPL (red), SOST (green) and phalloidin (dark blue) (j). Nuclei were stained with DAPI (blue). Scale bars, 20 μm. All experiments were performed using iPSC line 414C2. Experiments were repeated three times (b, e, f, g) or twice (h, i, j) independently with similar results. Results are shown as means ± s.e.m. (n = 9 from three independent experiments in c). RA, retinoic acid; ff, feeder-free; ROCKi, ROCK inhibitor; oi, osteoinduction; N.S., no significant difference. ***P < 0.001 by two-sided Student’s t-test.
BMP and WNT27,28. The upregulation of the ALPL gene was detected only in RA-treated cells at day 2 (Fig. 1d and Supplementary Fig. 2a) and was inhibited by BMP or WNT inhibitor (Fig. 4d). Knockdown of ALPL expression by specific small interfering RNAs (siRNAs) (Supplementary Fig. 5a,b) resulted in the reduction of calcified nodules (Fig. 4e). These results indicate that RA induced both BMP and WNT signals, which in turn upregulated genes related to osteogenesis such as ALPL, and accelerated the process.
RA promotes osteogenesis from iPSCs via RARα and RARβ signalling. RA transmits its signal via RA receptors (RARs), which bind to RARE (RA response element) in the promoter regions of target genes\(^\text{29-31}\). Although all three RARs were expressed in hiPSCs at comparable levels, the expression levels of RARα and RARβ dramatically increased during osteogenic induction, with peaks at day 7, whereas that of RARγ showed no change (Fig. 5a). A pan-RAR antagonist (BMS493) significantly inhibited the formation of calcified nodules and reduced the calcium deposition of cells treated with RA (Fig. 5b,c). siRNAs against RARα, RARβ or RARγ showed specific and significant suppression of each target gene expression (Supplementary Fig. 5c). The treatment of siRNAs against RARα or RARβ reduced nodule formation and calcium deposition by RA, and simultaneous siRNA treatment for both receptors enhanced the inhibitory effects (Fig. 5d), suggesting that in addition to common target genes, each receptor has specific targets related to osteogenesis.

**Fig. 3 | In vivo bone-forming properties of RA-treated cells.** Cells were harvested in a sheet-like structure and transplanted into the bone defects of mice calvariae. Samples were prepared 6 weeks after the transplantation. a, b, 3D reconstructed micro-CT images of calvariae with no cell transplants (a) and with RA+ cells (b). Scale bars, 1 mm. c, Quantification of newly formed calcified tissues in defects. Results are shown as means ± s.e.m. (n=8 from biologically independent animals for each group). ***P < 0.001 by two-sided Student’s t-test. d-o, Vertical reconstructed micro-CT images of calvariae with no transplants (d), RA+ cells (h) and RA− cells (l). Scale bars, 2 mm. Hematoxylin and eosin (H&E) staining of samples with no transplants (e), RA+ cells (i) and RA− cells (m); f,j,n show magnified views of the enclosed areas in e,i,m, respectively; and g,k,o show immunostaining with anti-human osteopontin (hOPN) of the areas in f,j,n, respectively. Nuclei were stained with DAPI (blue). Scale bars, 200 μm. All experiments were performed using iPSC line 414C2 and repeated twice independently with similar results.
Fig. 4 | RA activates BMP and WNT signals. a, Heatmap visualization of the expression of genes related to BMP, WNT and RA signalling. Cells were cultured in RA+ or RA−, and RNAs were extracted on day 0 and day 2. The expression level of each gene was quantified by microarray data and visualized as a value relative to the mean value of all points. Minimum (blue) represents downregulation and maximum (red) represents the degree of upregulation. b, The expression profiles of genes downstream of BMP and WNT. The expression level of each gene is shown as a value relative to that at day 0. c, Effects of BMP or WNT inhibitors on bone-like nodule formation. Cells were cultured in each condition, nodules were stained with Alizarin red, and calcium depositions were quantified at day 10. LDN, BMP inhibitor; IWR1, WNT inhibitor. Images in the top panel correspond to bars directly below in the graph. d, Effects of BMP or WNT inhibitors on the expression of ALPL gene. Cells were cultured in each condition for two days, and the expression level is shown as a value relative to that of RA+ cells. e, Effects of knocking down ALPL gene expression on bone-like nodule formation. Cells were cultured using the protocol described in Supplementary Fig. 5a, nodules were stained with Alizarin red, and calcium depositions were quantified at day 10. Images in the top panel correspond to bars directly below in the graph. All experiments were performed using iPSC line 414C2. Results are shown as the mean ± s.e.m. (n = 3 from three biologically independent experiments in b, d; n = 9 from two biologically independent experiments in c; n = 6 from three biologically independent experiments in e). N.S., no significant difference. *P < 0.05, **P < 0.01, ***P < 0.001 by two-sided Student's t-test.
On the other hand, little effect on RA-induced osteogenesis was found after siRNA treatment against RARY (Fig. 5d).

We also analysed the role of each RAR using specific agonists, and confirmed that RA signals via RARα and RARβ but not RARY promote osteogenic differentiation (Supplementary Fig. 6). These results suggested that RA can be substituted with RARα or RARβ agonists. Indeed, Am580 (RARα agonist) or AC55649 (RARβ agonist) showed effects equivalent with that of RA (Fig. 6a,b), and the expression profiles of downstream genes of BMP (ID1 and ID2), WNT (AXIN2 and CCND1) and stage-related genes (ALPL and SOST) during osteogenic induction with Am580 or AC55649 were comparable to those with RA (Fig. 6c,d). Taken together, RA promoted the process of osteogenesis by its signals via RARα and RARβ.

Establishment of OI-iPSCs and their rescuing. Disease modelling and drug discovery are two major medical applications of iPSCs. OI is a hereditary systemic bone disease and is characterized by fragile bones, low bone mass, short stature and other connective-tissue manifestations34. To demonstrate the feasibility of our current method for the analyses of genetic diseases, we tried to recapitulate the pathological features of OI by using patient-derived iPSCs. OI-specific iPSCs (OI-iPSCs) were established from skin fibroblasts derived from two patients with OI (OI1 and OI2) (Supplementary Table 1b) by the episomal plasmid method35, and two clones from each patient (OI1-1 and 1-2, and OI2-1 and 2-2) were established and characterized (Supplementary Fig. 7a). All clones expressed pluripotent markers, produced teratomas with three germinal components, and showed normal karyotypes (Supplementary Fig. 7a). All clones expressed pluripotent markers, produced teratomas with three germinal components, and showed normal karyotypes (Supplementary Fig. 7a). Whole exome sequencing using DNA from skin fibroblasts identified mutations of the collagen type-1 alpha 1 chain (COL1A1) gene in both cases: a point mutation (c.1066 G>T) causing the substitution of glycine to cysteine (a. 356 G>C) in OI1 and a point mutation at the splicing donor site of intron 32 (c.2235 + 1 G>A) in OI2 (Supplementary Fig. 8a). RT-PCR amplified 611 bp fragments corresponding to the region between exons 29 and 38 of cDNAs from WT and OI1 samples, but an additional fragment with a shorter size was amplified in OI2 samples (Supplementary Fig. 8b), which lacked 108 bases corresponding to the sequence of exon 32 (Supplementary Fig. 8c), indicating that the point mutation caused abnormal splicing, skipping exon 32 to create an in-frame (36 amino acid) deletion (Supplementary Fig. 8d). To obtain ideal control cells, mutated nucleotides in OI-iPSCs were substituted with WT nucleotides via genome-editing technology (see the ‘Gene editing’ section of the Methods), and mutation-rescued OI-iPSCs were established (resOI1-1 and resOI2-2) (Supplementary Fig. 9a–d). In the case of OI2, we confirmed that genome editing also rescued exon skipping (Supplementary Fig. 9e).

In vitro disease modelling using OI-iPSCs. The mutations in OI1 and OI2 create abnormal typeI collagen peptides that inhibit...
the formation of the normal triple helix, which results in the reduction of normal collagen production and the induction of endoplasmic reticulum (ER) stress33,34. Although the respective expression profiles of pluripotent stem cell or osteogenic marker genes showed no significant difference during the induction of WT- and OI-iPSC-derived cells (Fig. 7a), the number of Alizarin-red-positive nodules and the amount of calcium deposition were lower than those of WT-iPSC-derived cells (Fig. 7b,c and Supplementary Fig. 10a,b), and the reduction was recovered in corresponding resOI-iPSC clones (Fig. 7b,c). The level of ER stress was reduced (Fig. 8c). The amount and distribution of type I collagen was partially recovered by mTOR inhibitors (Fig. 8d), and the amount of intracellular type I collagen was reduced (Fig. 8c). The amount and distribution of type I collagen was partially recovered by mTOR inhibitors (Fig. 8d), and the amount of intracellular type I collagen was reduced (Fig. 8c). The amount and distribution of type I collagen was partially recovered by mTOR inhibitors (Fig. 8d), and the amount of intracellular type I collagen was reduced (Fig. 8c).

In previous reports, the inhibition of mTOR (mechanistic target of rapamycin) activity, which results in the activation of autophagy, eliminates misfolded procollagens from ER and can relieve the OI phenotype37,38. Treatment with mTOR inhibitors from day 2 after RA induction increased the formation of Alizarin-red-positive nodules and calcium deposition (Fig. 8a,b), and the level of ER stress was reduced (Fig. 8c). The amount and distribution of type I collagen was partially recovered by mTOR inhibitors (Fig. 8d), and the amount of intracellular type I collagen was reduced (Fig. 8c). One mTOR inhibitor showed a tendency to reduce ER stress and to enhance calcium deposition in normal cells (Supplementary Fig. 11a–c), which suggests that the role of autophagy is to excrete misfolded type I collagen from the cell39,40.

**Discussion**

We have established a rapid and simple method, via the use of RA signalling, to induce the osteogenic differentiation of hiPSCs. The role of RA signalling in osteogenic differentiation is controversial, and its effects depend on the cell type, concentration of RA, and other signals21. Our method uses RA from the stage of pluripotent stem cells (day 0) through to the stage of terminally differentiated cells (day 10) (Fig. 1a). Without the RA signal, osteoinduction medium failed to induce robust gene expression in osteogenic lineages (Fig. 1d and Supplementary Fig. 2a), and the formation of teratoma-like tissues in vivo indicated that the cells maintained characteristics of pluripotent stem cells even after induction. Also, the presence of matrix-making factors such as ascorbic acid...
**Fig. 7** Recapitulation of disease phenotypes using iPSCs derived from patients with osteogenesis imperfecta (OI).

**a.** mRNA expressions of stage-related genes in OI-iPSCs during bone-like nodule formation. Cells were cultured in RA+, and the expression of each gene was analysed at the indicated time points. The expression level of each point is shown as a value relative to that of WT1 at day 0.

**b-e.** Recapitulation and genetic rescue of the pathological phenotype of OI. Cells were cultured in RA+, nodules were stained with Alizarin red (**b**), and calcium depositions were quantified (**c**) at day 7. **d**, Expression of *BiP* gene at day 7. Each value is shown as a value relative to that of WT1. **e, f**, Immunostaining of type I collagen at day 7 (**e**) and induced cells after isolation (**f**). Nuclei were counterstained with DAPI (blue, inset). Scale bars, 100 μm.

**g**, Quantification of intracellular collagen. WT1, 414C2; WT2, 409B2. OI1-1, iPSC clone 1 established from OI1 patient; resOI1-1, mutation-rescued iPSC derived from OI1-1; OI2-1, iPSC clone 1 established from OI2 patient; resOI2-1, mutation-rescued iPSC derived from OI2-1. Experiments in **b, e, f** were repeated three times independently with similar results. Results are shown as the mean ± s.e.m. (**n** = 3 from three biologically independent experiments in **a, g**; **n** = 9 from two biologically independent experiments in **c**; **n** = 6 from three biologically independent experiments in **d**). N.S., no significant difference. *P* < 0.05, **P** < 0.01, ***P** < 0.001 by two-sided Student’s t-test.
and β-glycerophosphates was required for the robust induction of osteogenic genes in RA-treated cells (Supplementary Fig. 1d). These results indicate that the combination of cell–matrix interactions and the osteogenic guidance by RA signalling leads to robust osteogenic differentiation. Indeed, although the bone forming ability of RA-treated iPSCs did not completely repair the defect, the performance matched that of bone marrow stromal cells transplanted using our method (Supplementary Fig. 12a–e). Future modification of the system, such as the incorporation of biomaterials41,42, should improve the results. As for the induction of osteocytes, we were able to obtain dendritic cells positive for the osteocyte-specific marker SOST. The cellular morphology, however, was not like that of osteocytes located in lacunae (which have multiple cellular processes protruding perpendicular to the cell body), suggesting that in vivo factors are required to induce cells with such unique features.

We showed that the induction of both BMP and WNT signalling is a key mechanism of RA action; both of these signals are known to promote osteogenic differentiation43,44. The relationship of RA with BMP or WNT signalling has been reported in several studies45,46. It was also reported that RA and BMP signalling synergistically activate the WNT/β-catenin pathway through the inhibition of glycogen synthase kinase 3β47. In addition to these enhancer roles, we found that RA induced the expression of genes related to BMP and WNT signalling (Fig. 4a). Interestingly, we found that the administration of BMP and/or WNT failed to substitute for the effect of RA (Supplementary Fig. 13), suggesting that RA also plays a direct role in osteogenesis, independently of BMP or WNT signalling. For example, the expression of ALPL, a key gene in our system, is regulated by BMP or WNT, but also directly by RA via RARE in its regulatory region48. Further analyses are required to understand the effect of RA on osteogenesis.

RA exerts its pleiotropic effects by creating complexes with different RARs. Knockout mice of each RAR isoform are almost normal, but RAR double-mutant mice show severe malformations of...
cranium skeletal elements, suggesting the functional redundancy of RAR isoforms. As their role in osteogenesis, RARpotentiates ectopic bone formation by BMP98, whereas inhibitory effects by RARY were reported in mouse models of heterotopic ossification9. These data are consistent with our observation. siRNA experiments suggested that RAR and RAR target common and distinct genes related to osteogenesis. Comparing the binding sites of RAR and RAR may disclose the specific downstream genes, providing greater clarity on RA-mediated osteogenesis. However, other studies have indicated that RAR activation stimulates bone resorption, not growth. For example, all-trans RA stimulated bone resorption in cultured calvariae by inducing RANKL (receptor activator of NF-κB ligand) in osteoblasts through RARα activation4. Future studies should investigate the precise role of each RAR in osteogenesis in vivo by using an osteoblast-specific knockout system.

We showed that our system can recapitulate disease phenotypes and screen drugs for hereditary bone diseases. OI is a syndromic disease characterized by fragile bones. Its clinical phenotypes vary significantly, ranging from perinatal lethality to osteopetrosis, and at least ten genes are listed as causative for OI24,34,52. The clinical phenotype was also significantly different among patients, despite sharing the same responsible mutant genes, such as COL1A1. This heterogeneity may be due to the type of mutation, but the genotype–phenotype correlation is not yet clear. The combination of our method and OI patient-specific iPSCs may provide an answer. The correction of bone production by OI cells was previously reported by knockouting the mutant COL1A1 or COL1A2 allele to leave one copy of normal allele4. However, here we created two normal COL1A1 alleles in OI cells.

The most important advantage of our induction system is that it can be applied to drug screening. To apply the assay system for the screening of a large number of chemicals, the system should be able to perform at a smaller scale, at shorter times, with lower cost, and most importantly with high reproducibility. As shown in this study, our system can assay the various phenotypes of bone cells with minimum requirements (RA and fetal bovine serum (FBS)) within ten days, and the data are highly reproducible. Currently we are able to perform this assay in 96-well plates (Supplementary Fig. 1A.b), but the scale will be further increased in the near future. It would also be desirable to use reporter iPSC cell lines, which can inform of the efficacy of the induction of differentiation. At this point, the data at the final stage, such as the amount of calcium deposition, are only reliable markers of osteogenic differentiation, and there are no biomarkers reflecting the differentiation at earlier time points.

We have shown the process of nodule formation via time-lapse imaging. Nodule formation requires more than three weeks if somatic precursor cells such as bone marrow stromal cells are used, making time-lapse imaging observation difficult40. As shown in Supplementary Video 2, cells proliferated vigorously and stacked within a short period, which may be due to the embryonic potential of these cells. Because of the rapid nodule formation, matrices underneath the cell layer seemed immature, although they could make lamellar bones in vivo. This immaturity, however, may have an advantage for the observation of cells in nodules. Cells at day 10 were composed of those at different stages of differentiation (Fig. 1h–j), which can be isolated and examined. Confocal imaging revealed that osteocyte-like cells localized beneath the layer of osteoblast-like cells, and time-lapse imaging demonstrated the migration of these cells, which can thus be used to investigate the transition from osteoblast to osteocyte. Taken together, our in vitro system could aid the analysis of physiological and pathological processes in bone.

Methods

Generation of iPSCs and cell culture. Human iPSCs (except 317–12 cells) were generated with episomal vectors as reported previously49,50. The 317–12 line was prepared from 201B7 to include constitutively active GFP51. The detailed backgrounds of the iPSCs are shown in Supplementary Table 1a. On-feeder iPSCs were maintained in primate embryonic stem cell medium (ReproCELL) and replenished with 4 μM recombinant human basic fibroblast growth factor (FGF2; Wako) and penicillin-streptomycin (Thermo). Three days before the induction of osteogenic lineage, on-feeder iPSCs were transferred to a matrigel-coated dish and cultured in mTeSR1 medium (STEMCELL Technology). Feeder-free iPSCs (1231A3) were maintained with AK02N (Ajinomoto) on a laminin-coated dish.

Induction method. For the induction of osteogenic lineages from iPSCs, we modified a mineralization protocol44,45 by adding retinoic acid (RA; Wako) at a concentration of 1 μM. In brief, 4 × 10^5 iPSCs were maintained in feeder-free conditions and cultured in a well of gelatin-coated 12-well plates with 20% mTeSR1 and 80% osteogenic induction medium composed of KnockOut DMEM (Thermo) supplemented with 20% FBS (Nichirei), 2 mM Gluta-MAX (Thermo), 10 mM glycerol-2-phosphate (Sigma), 1 mM dexamethasone (Sigma), 0.1 mM 2-mercaptoethanol (Thermo), 50 μg·mL^−1 L-ascorbic acid 2-phosphate, sodium sesquimagnesium salt hydrate (Nacalai Tesque), and 1% non essential amino acids (Thermo) in the presence of Y-27632 (10μM, Wako) and RA. On day 2, the medium was replaced with 100% osteogenic induction medium and changed on days 4 and 7. To isolate the induced cells, the cultured cells were washed twice in phosphate-buffered saline (PBS) and treated with Trypsin–EDTA (Thermo) for 2 min. Next we treated the cells with 0.1% collagenase (Nitta gelatin) for 5 min solution to dissociate the cells from the dish. After repipetting about 10 times and standing for 5 min, the cells were centrifuged and seeded sparsely to a non-coated dish in α-MEM (Thermo) with 10% FBS (Nichirei) and penicillin-streptomycin. Reagents for the modification of signals such as inhibitors, agonists and antagonists were listed in Supplementary Table 3a,b.

Alizarin red staining and calcium deposition measurement. Calcium deposits were visualized by Alizarin red staining. Culture wells were washed twice with PBS and fixed with ethyl alcohol for 10 min at room temperature. Alizarin red solution (40mM, pH 4.2, Wako) was applied to the fixed wells for 10 min at room temperature. Non-specific staining was removed by several washes with water. Calcium was quantified with an o-Cresolphthalein Complexone (OCP/C) method-based kit (N-assay L.Ca, Nittobo medical).

Quantitative PCR analysis. Total RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA, USA) using optional DNase-treatment to remove genomic DNA. Total RNA (0.3μg) was reverse transcribed for single-stranded cDNA using random primers and Superscript III reverse transcriptase (Thermo) according to the manufacturer’s instructions. Quantitative PCR was performed with Thunderbird SYBR qPCR Mix (TOYOBO) and analysed with the StepOnePlus real-time PCR System (Thermo). The primer sequences are described in Supplementary Table 2a.

In vitro immunofluorescence. Cells were fixed with PBS containing 2% paraformaldehyde for 20 min at 4°C, blocked with Blocking One (Nacalai Tesque) for 60 min at 4°C and incubated overnight at 4°C with primary antibodies diluted in 10% (v/v) Blocking One in PBS-T (PBS with 0.2% Triton X-100 solution (Nacalai Tesque)). The samples were then washed 3 times with PBS-T and incubated for 1 h at room temperature with secondary antibodies diluted in 10% Blocking One in PBS-T. Nuclei were stained with 15,000 DAPI (Sigma). The samples were observed with BZ-9000E (Keyence Corporation). Confocal imaging data by FLUOVIEW FV3000 (Olympus) was analysed by FV31S-SW. The antibodies used are listed in Supplementary Table 3c.

Quantitative evaluation of intracellular type I collagen was performed using Image J software (NIH). Briefly, cells at day 7 were isolated, resuspended in sparse condition, and stained with anti-type I collagen (green) and DAPI (blue) as described above. Areas stained green were measured and divided by the number of DAPI in the analysed area to give the average stained area in one cell. The data of three independent experiments are shown.

Time-lapse imaging. Cell culture plates were placed on STRG-WELSX-SET (Tokiha!) equipped with an incubation chamber and motorized stage. The stage incubator was adjusted to the cell culture condition of 37°C, 95% humidity and 5% CO2. Camera settings were identically set for all experiments, and illumination was constant throughout the whole time period. Images were 520 × 520 pixels in resolution and taken at 1 μm depth in the z-direction (120 μm in total) at a 30 min time interval for a total duration of 144 h (from 24–168 h after seeding the cells). Videos were finally visualized in 3D using FV31S-SW (Olympus).

In vivo transplantation. Cells were cultured for seven days on temperature-responsive culture dishes (CellSeed) and harvested as one mass retaining sheet-like structures by changing to culture temperature. Cells were transfected with 4 μm diameter bone defects, of which the size was critical for spontaneous regeneration, made by drilling the calvariae of immune-deficient NOD/ShiSlcIcl (NOD/SCID) mice (CLEA Japan) under anaesthesia. Six weeks later, the
calvariae were harvested, fixed with 4% paraformaldehyde for 24h, and processed for paraffin-embedded sections at the Division of Technical Support, Institute for Frontier Medical Sciences, Kyoto University. Calvariae were scanned using an X-ray CT system (inspeXio SMX-100CT) and analysed by TRIS/PCS-BON (RATOC system engineering) according to the manufacturer’s instructions. All mouse studies were approved by the UCSF Institutional Animal Care and Use Committee or performed in strict accordance with the Regulations on Animal Experimentation at Kyoto University.

**Bone marrow stromal cell culture.** Bone marrow stromal cells from three donors (BM01, BM02 and BM03) were isolated and cultured as previously described. Briefly, BMSMCs were cultured in α-minimal essential medium with Glutamax (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), 0.1% penicillin, and 100 μg ml⁻¹ streptomycin. Expanded cells were characterized with cell surface markers and differentiation capacity (Supplementary Fig. 15a-c). Cells passaged less than five times were used in the experiments. Osteogenic differentiation was induced in growth medium supplemented with 0.1 μM dexamethasone and 50μM ascorbic acid. After 14 days induction, a cell-sheet-like structure was transplanted into the calvarial defect of NOD-SCID mice.

**In vivo immunofluorescence.** Paraffin-embedded sections were deparaffinized. Samples were blocked with Blocking One (Nacalai Tesque) for 60 min and then incubated with human specific Osteopontin (R&D) diluted in PBS and incubated with donkey anti-goat IgG secondary antibody, Alexa Fluor 488 conjugate (Abcam) diluted in Can Get Signal immunostain solution B for 1h at room temperature. DAPI (10 μg ml⁻¹) was added to counterstain nuclei, and the samples were observed by BX-9000E (KEYENCE).

**Microarray experiments.** Total RNA was prepared using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the GeneChip WTT (Whole Transcript) Sense Target Labeling and Control Reagents kit according to the manufacturer’s instructions (Affymetrix). Hybridization to GeneChip Human Gene 1.0 ST expression array, washing, and scanning were performed according to the manufacturer’s protocol (Affymetrix). The raw CEL files were imported into GeneSpring GX 12.6.1 software (Agilent Technologies), and expression values were calculated with the RMA algorithm. The data were analysed by generating heatmaps (GeneSpring GX and R statistics software). Canonical pathways analysis and upstream analysis were performed by Ingenuity Pathway Analysis (IPA; Qiagen).

**Knockdown experiment with small interfering RNAs (siRNAs).** Two siRNAs for each gene (details are shown in Supplementary Table 2b) were obtained from Silencer Select Pre-designed siRNA (Thermo) and transfected into iPSCs at the beginning of the induction using Lipofectamine RNAiMAX (Life Technologies). Silencer Select Negative Control siRNA no. 1 (4390843) was used as a control.

**Teratoma formation.** iPSCs grown in Matrigel-coated 10 cm dishes to 90–100% confluence were harvested and suspended in miSR (supplemented with 10 μM Y-27632). Cells (1×10⁶ in 20 μl) were injected into 8–12-week-old male NOD/SCID mice (CLEA Japan) under the testis capsule with defined media.

**Data availability**

The authors declare that all data supporting the results in this study are available within the paper and its Supplementary Information. Raw data are available from the corresponding author on reasonable request. The affymetrix data have been deposited in the GEO database, under accession number GSE119577.

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**References**


**Acknowledgements**

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**Author contributions**

S.K., H.Y. and J.T. designed the research and wrote the manuscript. J.S. performed imaging studies. C.A., M.L, Y.J, T.A. and S.M. advised on the project. M.H., Y.K. and S.T. established and maintained iPS cells. M.N., K.S. and H.M. helped with animal experiments. S.N. and M.U. helped with in vitro experiments. Y.H. and K.F. provided patient samples and clinical information. All authors provided feedback on the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41551-019-0410-7.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

No software was used.

Data analysis

GeneSpring GX: version 12.6.1
Ingenuity Pathway Analysis: version 01-12
FV31S-SW: version 2.1.1.98

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that all data supporting the results in this study are available within the paper and its Supplementary Information. Raw data are available from the corresponding author on reasonable request. The affymetrix data have been deposited in the GEO database, under accession number GSE119577.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For the in vivo transplantation experiments, we estimated that the effect size, (μ1-μ2)/σ, is 1.5 on the basis of data obtained in preliminary experiments, and that a statistical power of 88.6% for n=8 is sufficient. In other experiments, no pre-specified effect size was calculated, and no statistical method was used to predetermine the sample size. |
| Data exclusions | No data were excluded. |
| Replication | Each experiment was performed at least twice (most of them three times), with similar results. |
| Randomization | The animals were randomly allocated to the groups. |
| Blinding | Mice that received each type of surgery were maintained without further interventions (such as drug treatment), and the results were quantitatively analysed by the imaging software. Therefore, we considered that blinding was not necessary. |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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<th>Methods</th>
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<tr>
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<td>Antibodies</td>
</tr>
<tr>
<td>☑️</td>
<td>Eukaryotic cell lines</td>
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<tr>
<td>☑️</td>
<td>Palaeontology</td>
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<tr>
<td>☑️</td>
<td>Animals and other organisms</td>
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<td>☑️</td>
<td>Human research participants</td>
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<td>☑️</td>
<td>Clinical data</td>
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<td>ChiP-seq</td>
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<td>☑️</td>
<td>Flow cytometry</td>
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<tr>
<td>☑️</td>
<td>MRI-based neuroimaging</td>
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Antibodies

<table>
<thead>
<tr>
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<th>Validation</th>
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<tbody>
<tr>
<td>SC-10758: Santa Cruz Biotechnology, Rabbit Polyclonal Ab, Human, Mouse, Rat Runt-related transcription factor 2</td>
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<td>NB600-408: Novus Biologicals, Rabbit, Human, Mouse, Rat Type I collagen</td>
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<tr>
<td>BAM1448 clone # B4-78: R&amp;D system, Mouse Monoclonal IgG1, Human, liver, bone and kidney Alkaline Phosphatase/ALPL, MAB1419 clone # 190125: R&amp;D system, Mouse Monoclonal IgG1, Human, Rat Osteocalcin</td>
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<td>SAB1300753: Sigma-Aldrich, Rabbit Polyclonal, Human SOST</td>
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<tr>
<td>bs-12313R: BIOSS Antibodies, Rabbit, Polyclonal, Human, Mouse, Rat Phosphate Regulating Endopeptidase Homolog, X-linked</td>
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<td>A22283: Thermo Fisher Scientific, F-actin</td>
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<tr>
<td>D1306: Life Technology, AT regions of DNA (DAPI)</td>
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<tr>
<td>AF1433: R&amp;D system, Goat Polyclonal IgG1, Human Osteopontin/OPN</td>
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<td>MAB4304 clone MC-813-70: MILLIPORE, Mouse Monoclonal, Human, Mouse Stage-Specific Embryonic Antigen-4</td>
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<td>MAB4381 clone TRA-1-81: MILLIPORE, Monoclonal Mouse, Human TRA-1-81</td>
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<td>A22283: None</td>
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<td>D1306: None</td>
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Eukaryotic cell lines

Policy information about cell lines

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<thead>
<tr>
<th>Cell line source(s)</th>
<th>Description</th>
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<tr>
<td>414C2 and 40982: iPS cell lines established from human fibroblasts, purchased from CORIELL INSTITUTE.</td>
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</tr>
<tr>
<td>317-12: iPS cell line derivative of 201B7. 201B7 is established from human fibroblasts, purchased from CORIELL INSTITUTE.</td>
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<tr>
<td>OI#1: iPS cell lines established from skin fibroblasts of an OI patient (female, 17 years old) with informed consent.</td>
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<tr>
<td>OI#2: iPS cell lines established from skin fibroblasts of an OI patient (male, 3 years old) with informed consent.</td>
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<tr>
<td>BM01: primary cultured human bone-marrow stromal cells from a donor (male, 27 years old) with informed consent.</td>
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<tr>
<td>BM02: primary cultured human bone-marrow stromal cells from a donor (male, 29 years old) with informed consent.</td>
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<tr>
<td>BM03: primary cultured human bone-marrow stromal cells from a donor (female, 54 years old) with informed consent.</td>
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</table>

Authentication

None of the cell lines used were authenticated.

Mycoplasma contamination

All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

8-week-old male NOD/ShiJic-scid Jcl (NOD/SCID) mice were used (purchased from CLEA, Japan).

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

Animal experiments were approved by the institutional animal committee of Kyoto University, and performed following the guidance of Regulation on Animal Experimentation at Kyoto University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics

<table>
<thead>
<tr>
<th>Description</th>
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<tbody>
<tr>
<td>OI#1: Type III OI patient (female, 17 years old)</td>
</tr>
<tr>
<td>OI#2: Type III OI patient (male, 3 years old)</td>
</tr>
<tr>
<td>BM01: a donor (male, 27 years old) with informed consent.</td>
</tr>
<tr>
<td>BM02: a donor (male, 29 years old) with informed consent.</td>
</tr>
<tr>
<td>BM03: a donor (female, 54 years old) with informed consent.</td>
</tr>
</tbody>
</table>

Recruitment

OI patients were recruited on the basis of clinical diagnosis as type-III OI, and skin fibroblasts were obtained during the surgical treatments. The clinical phenotype of these patients was compatible for typical type-III OI. Two patients were selected from our patient cohort on the basis of their genotype: one is a missense change at a glycine codon and the other is a splicing mutation. This is to demonstrate that our assay system can be applied irrespective for the type of mutations.

Bone marrow stromal cells (BMSC) donors were recruited from patients who needed autologous bone grafts, and bone stromal cells were obtained during graft harvesting. Three donors were randomly selected from our stocks of BMSCs. Our previous study (Shibata, et al. Stem Cells 2007;25:2371-82) on the growth and differentiation potentials of BMSCs showed no remarkable differences among donors.

Ethics oversight

The experimental protocols for the human subjects were approved by the Ethics Committee of the Department of Medicine and Graduate School of Medicine, Kyoto University, and the Ethics Committee of Shiga Medical Center for Children.

Note that full information on the approval of the study protocol must also be provided in the manuscript.