

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 RAR α and RAR β , 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.

Bone is a dynamic tissue that constantly undergoes formation and resorption (bone remodelling)^{1,2}. Defects in either process, in which a number of cellular and humoral factors play distinctive roles, may impair the production of healthy bone^{3,4}. Because of this complexity, understanding the physiological and pathological conditions of bone requires in vivo animal studies⁵. These studies have effectively disclosed the fundamental cellular and molecular mechanisms of the development and maintenance of bone tissues. Yet in vivo studies have disadvantages: the precise analyses of individual cell types are difficult, transient events such as the transition from osteoblast to osteocyte are hard to observe, and large-scale drug screening is unrealistic. Also, in some cases, differences among species may lead to contradictory results⁶.

Several in vitro approaches mimic in vivo events, among which the formation of bone-like nodules is the gold standard^{7,8}. This process starts with the condensation of precursor cells and their induction to differentiate into osteoblasts, which make bone-matrix protein and excrete matrix vesicles containing alkaline phosphatase^{9,10}. The supplementation of reagents such as β -glycerophosphate and ascorbic acid facilitates calcified-nodule formation from stem cells^{11,12}. Few studies, however, have demonstrated this process in a sequential manner via imaging, particularly using human cells, which is in part due to the lack of a rapid and reproducible induction system employing somatic precursor cells. Human pluripotent stem cells, particularly induced pluripotent stem cells (iPSCs), are promising

precursor cells for this tissue because of their unlimited growth ability and pluripotency¹³. There are two methods for engineering bone-like nodules from iPSCs. The first employs embryoid-body formation, through which some cells spontaneously differentiate into mesenchymal cell lineages containing the precursors of bone cells¹⁴. After obtaining adherent cells with fibroblastic morphology, osteogenic induction is performed through conventional methods to make bone-like nodules. This type of method is relatively simple, but the efficacy of the induction is not stable. The other method faithfully recapitulates the developmental process of bone tissues, and uses multiple steps to induce bone-like nodules^{15–17}. Although these methods are valuable for understanding bone development, they require a relatively long time, and the quality and quantity of the final products fluctuates. Therefore, they are not suitable for extensive analyses such as drug screening.

In addition, the acquisition of terminally differentiated cells (namely osteocytes) in vitro is difficult irrespective of the method (including those that form bone-like nodules), and requires three-dimensional (3D) culture and the use of a biomaterial as a scaffold¹⁸, or the use of cell sorting¹⁹.

We have previously reported a relatively simple method for osteogenic differentiation²⁰—and because of the role of retinoic acid (RA) in osteogenesis^{21–23}, we have focused this present study on RA signalling to improve that method. We found that the addition of RA dramatically accelerated the formation of bone-like nodules

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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 bones²⁴.

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 method²⁰ with or without RA (1 μ M) (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 with homologies 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 (PDGFRA, 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 aggrecan (ACAN) (a marker of more-differentiated chondrocytes) was not, and the expression of an adipogenic marker (PPAR γ , peroxisome proliferator activated receptor gamma) was upregulated, but only transiently (Supplementary Fig. 2d). These data suggests 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)²⁵ (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 plates²⁶, 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,h). Histological analyses showed fibrous tissues in defects without cell transplantation (Fig. 3e,f), whereas dense collagenous structures were found in those that received transplanted RA-treated cells (Fig. 3i,j), 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. 3l), 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 *ESR1* (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 *CCND1* (cyclin D1)) was relatively transient with a peak at day 2 (Fig. 4b). Inhibitors for each signal (*LDN-193189* for BMP and *endo-IWR1* for WNT) decreased the mineralization of RA-treated cells (Fig. 4c).

To further analyse the role of BMP and WNT signals, we focused on the *ALPL* gene, which is known to be a downstream gene of both

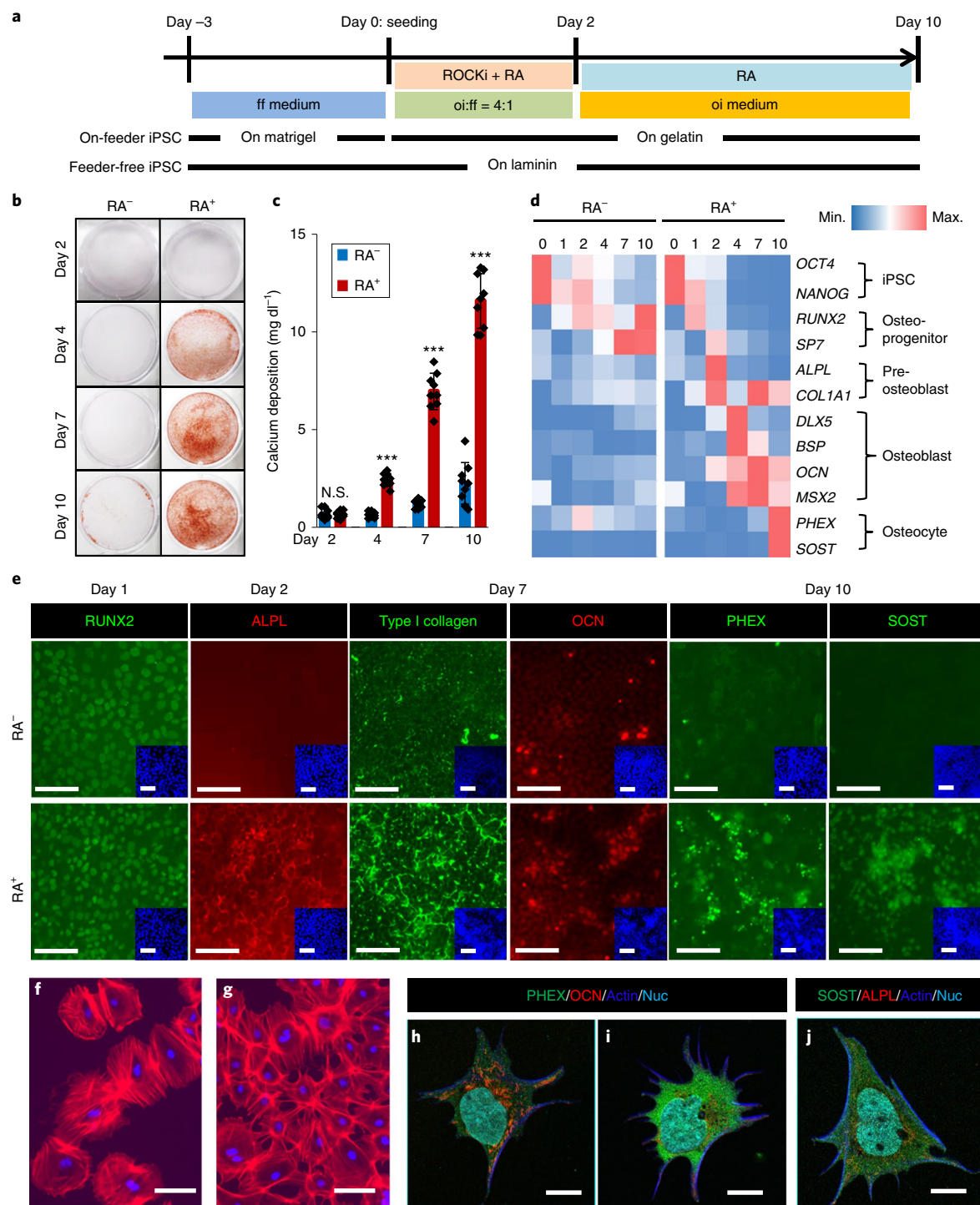


Fig. 1 | RA promotes the formation of bone-like nodules from hiPSCs. a, Schematic overview of the protocol for bone-like nodule formation from iPSCs with RA. Hereafter, this culture condition is designated RA⁺. RA⁻ indicates the same protocol but without RA. **b,c**, Evaluation of bone-like nodules. 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 μ m. The presence of cells was confirmed by staining nuclei with DAPI (blue, inset). **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 μ m. **h-j**, Immunostaining 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 μ m. All experiments were performed using iPSC line 414C2. Experiments were repeated either three times (b,e,f,g) or twice (h-j) independently with similar results. Results are shown as means $\pm$ 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.

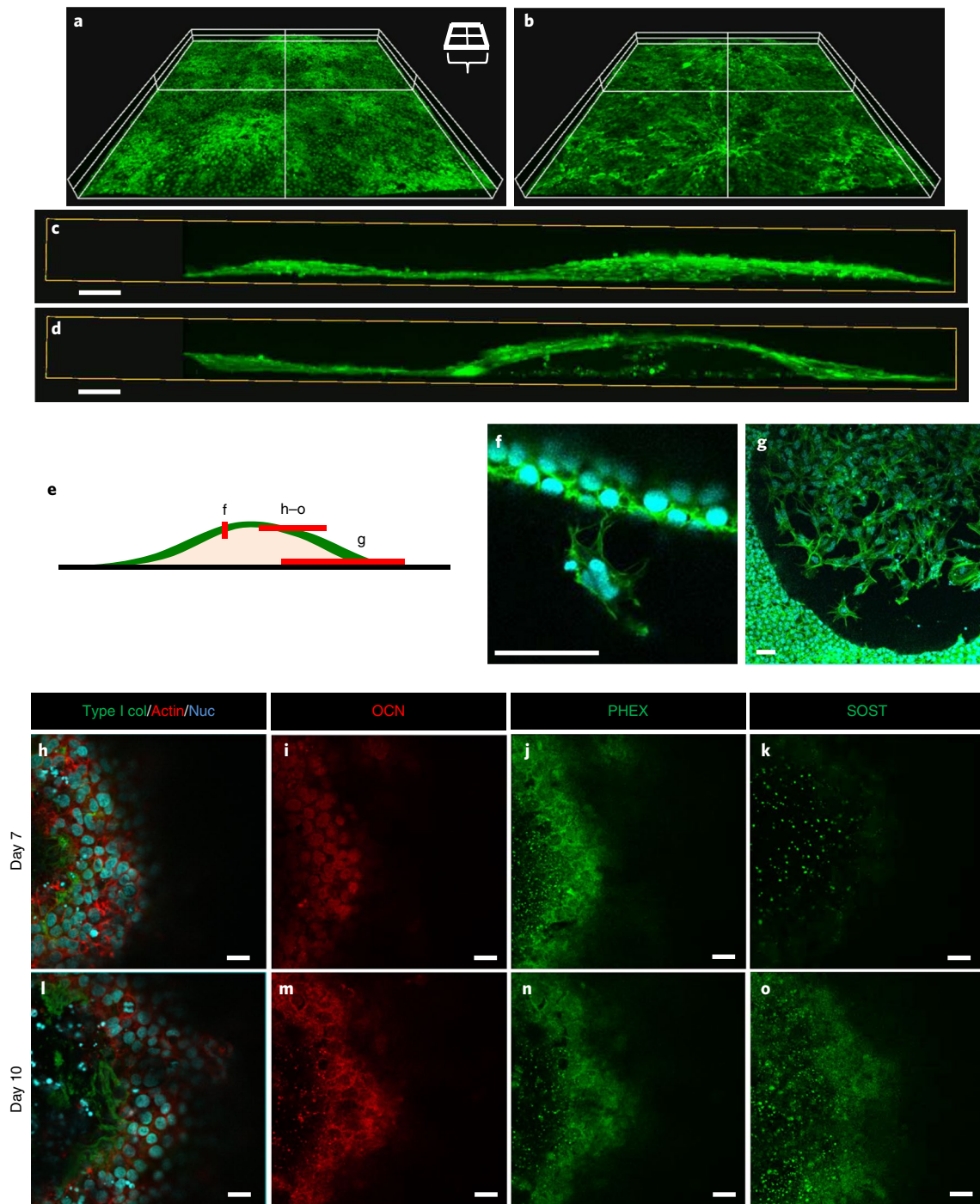


Fig. 2 | RA induces bone-like nodules containing osteoblast- and osteocyte-like cells. **a–d**, Snapshots of time-lapse imaging. 317–12 iPSCs were cultured with RA for seven days, and stacked images at a 30 min interval were gathered from day 1 to day 7. Snapshot images were taken in 3D view at day 4 (**a**) and day 7 (**b**) and in vertical view at day 4 (**c**) and day 7 (**d**). One side of the square measures 1,840 μm (**a,b**). Scale bars, 100 μm (**c,d**). Images were reconstructed in 3D and horizontal sections. **e–o**, Schematic presentation (**e**) of the site, showing the directions of the images in **f–o**. **f,g**, Confocal images of nodules at day 10. Cells were stained with actin (green) and DAPI (blue) in the vertical view at the top (**f**) and the horizontal view at the bottom (**g**). Scale bars, 20 μm . **h–o**, Immunofluorescence analyses at the top of the nodules. Nodules were stained by the indicated antibody on day 7 and day 10, and images were obtained from three different samples (OCN and PHEX were stained in a same sample) on each day. They were stained with: type I collagen (green), phalloidin (actin) (red) and DAPI (nuclei) (blue) (**h,i**); OCN (red) and PHEX (green) (**j,m,n**); and SOST (green) (**k,o**). Scale bars, 20 μm . Experiments in **a–d** were performed using iPSC line 317–12 and others were performed using iPSC line 414C2. All experiments were repeated twice independently with similar results.

BMP and WNT^{27,28}. 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.

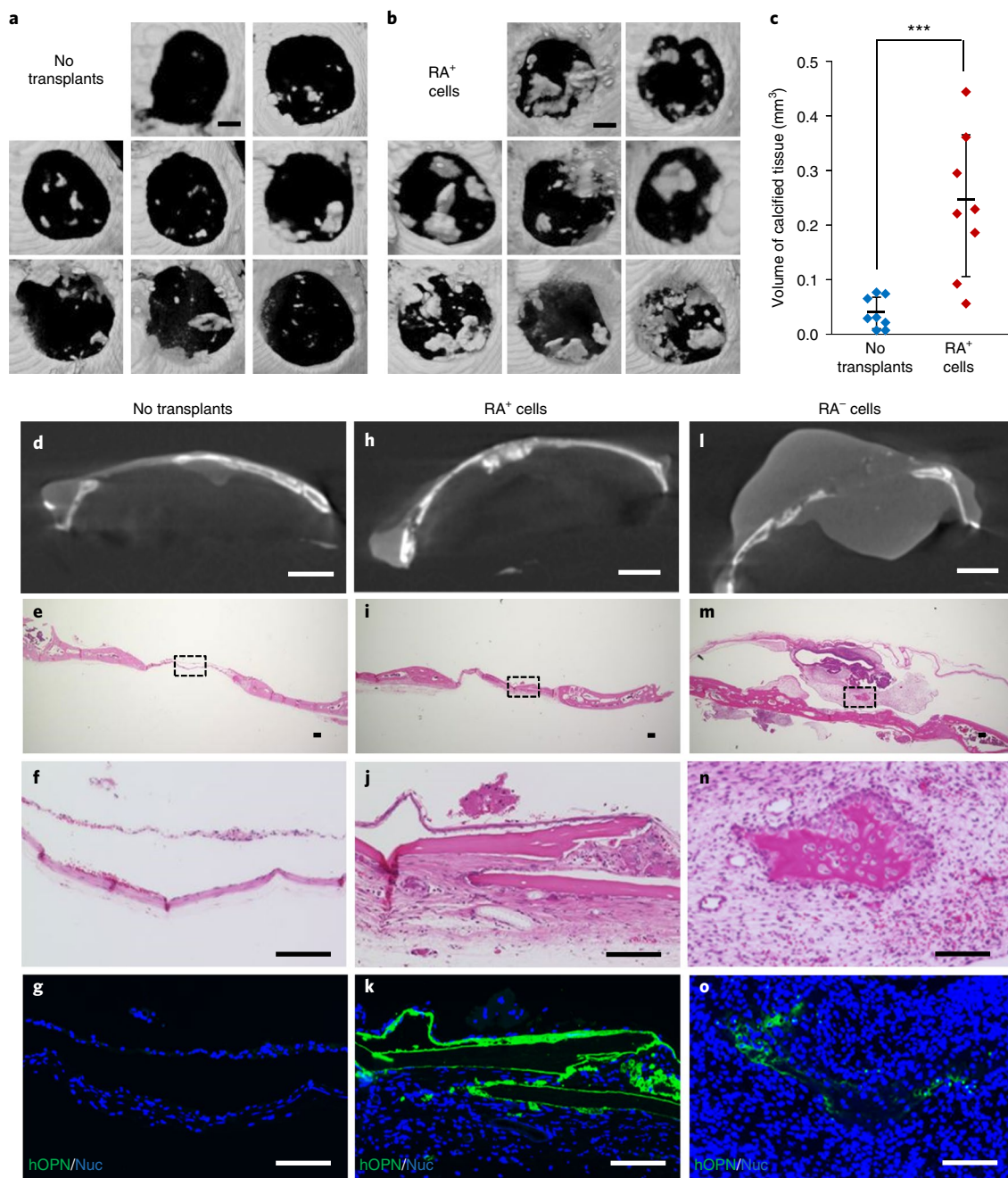


Fig. 3 | In vivo bone-forming properties of RA-treated cells. Cells were harvested in a sheet-like structure and transplanted into the bone defects of mice calvariae. Samples were prepared 6 weeks after the transplantation. **a,b**, 3D reconstructed micro-CT images of calvariae with no cell transplants (**a**) and with RA⁺ cells (**b**). Scale bars, 1 mm. **c**, Quantification of newly formed calcified tissues in defects. Results are shown as means $\pm$ 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 (**i**). 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 μ m. All experiments were performed using iPSC line 414C2 and repeated twice independently with similar results.

RA promotes osteogenesis from iPSCs via RAR α and RAR β signalling. RA transmits its signal via RA receptors (RARs), which bind to RARE (RA response element) in the promoter regions of target genes^{29–31}. Although all three RARs were expressed in hiPSCs at comparable levels, the expression levels of RAR α and RAR β dramatically increased during osteogenic induction, with peaks at day7, whereas that of RAR γ 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 RAR α , RAR β or RAR γ showed specific and significant suppression of each target gene expression (Supplementary Fig. 5c). The treatment of siRNAs against RAR α or RAR β 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.

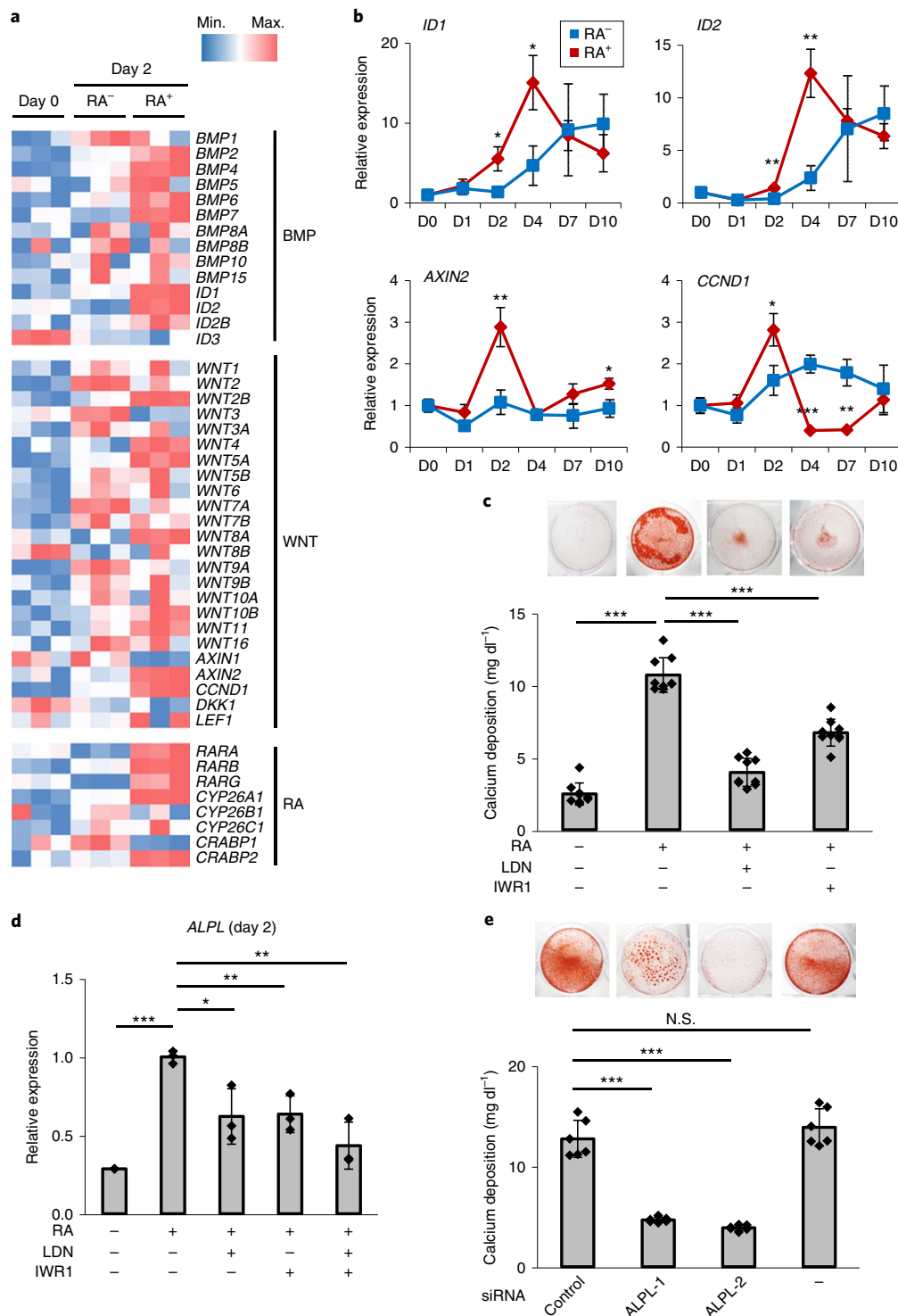


Fig. 4 | RA activates BMP and WNT signals. **a**, Heatmap visualization of the expression of genes related to BMP, WNT and RA signalling. Cells were cultured in RA⁺ or RA⁻, and RNAs were extracted on day 0 and day 2. The expression level of each gene was quantified by microarray data and visualized as 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 $\pm$ 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.

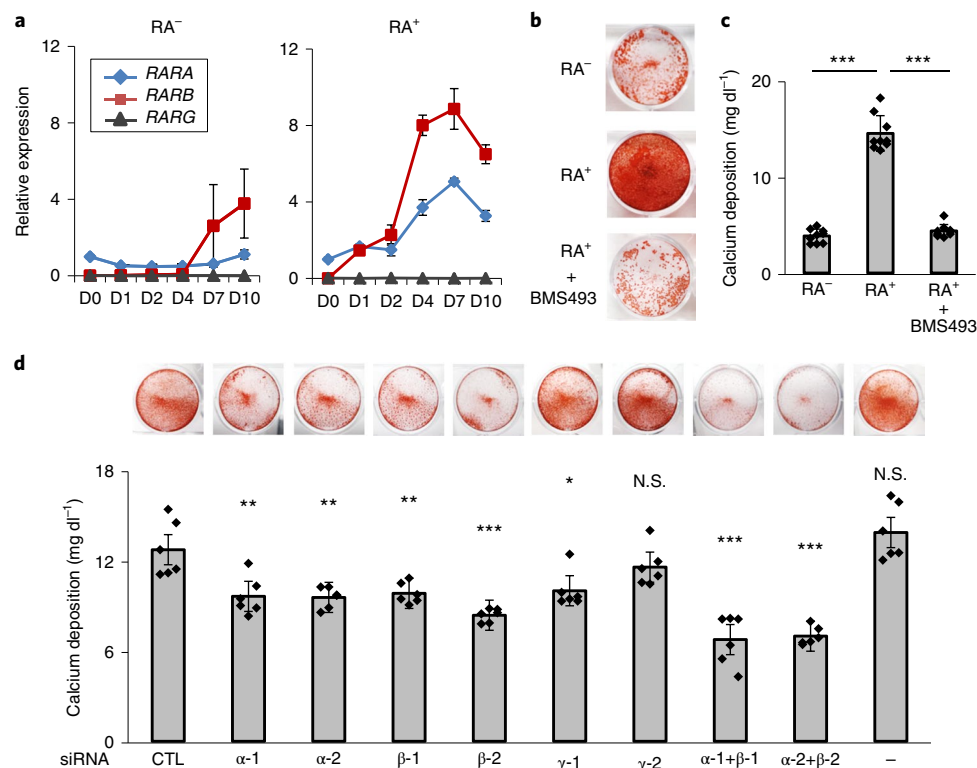


Fig. 5 | RA promotes osteogenic differentiation of iPSCs via *RARα* and *RARβ*. **a**, Expression of *RAR* genes (*RARA*, *RARB* and *RARG*) during induction in RA^+ or RA^- . The expression level of each point is shown as a value relative to the expression level of the *RARA* gene at day 0. **b,c**, Effects of a pan-antagonist of *RAR* (BMS493) on bone-like nodule formation. Cells were cultured in each condition for 10 days, nodules were stained with Alizarin red (**b**), and calcium depositions were quantified (**c**). **d**, Effects of knocking down each *RAR* gene on bone-like nodule formation ($\alpha-1$ and $\alpha-2$ for *RARA*; $\beta-1$ and $\beta-2$ for *RARB*; $\gamma-1$ and $\gamma-2$ for *RARG*). 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. Statistical differences were compared with control (CTL). Images in the top panel correspond to bars directly below in the graph. All experiments were performed using iPSC line 414C2. Experiments in **b** were repeated twice independently with similar results. Results are shown as the mean $\pm$ s.e.m. ($n=3$ from three biologically independent experiments in **a**, $n=9$ 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.

On the other hand, little effect on RA-induced osteogenesis was found after siRNA treatment against *RARγ* (Fig. 5d).

We also analysed the role of each *RAR* using specific agonists, and confirmed that RA signals via *RARα* and *RARβ* but not *RARγ* 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 manifestations²⁴. 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 method³², 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. 7b–d). 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.1066G>T) causing the substitution of glycine to cysteine (a. 356G>C) in OI1 and a point mutation at the splicing donor site of intron 32 (c.2235+1G>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 type I collagen peptides that inhibit

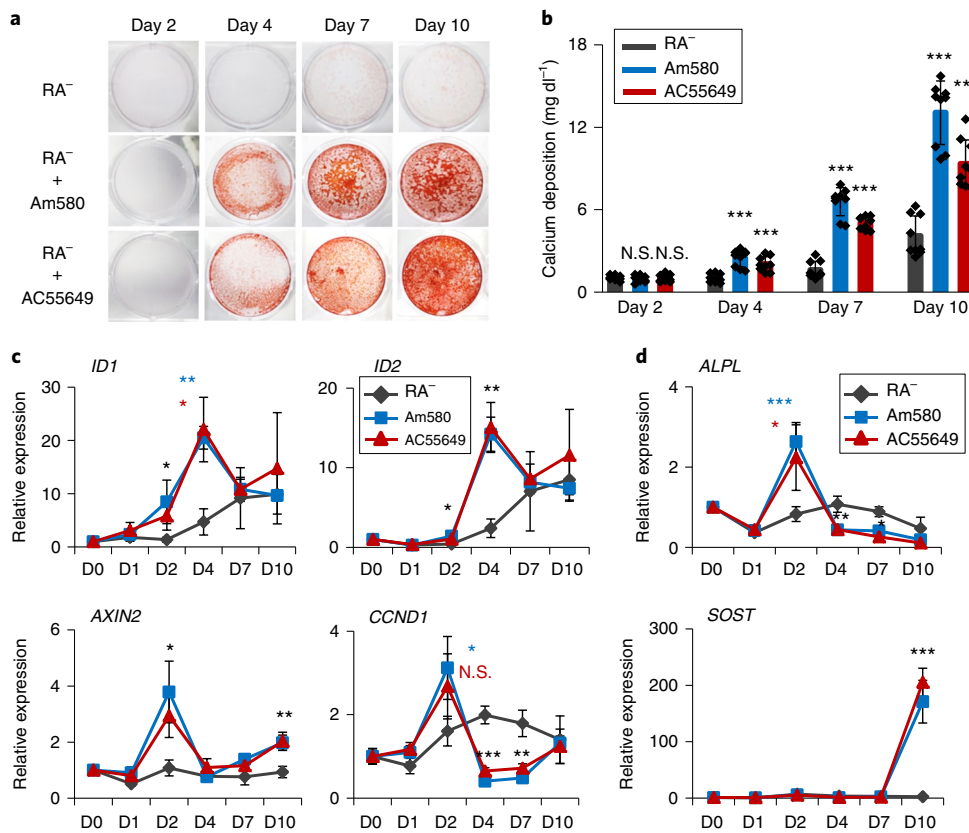


Fig. 6 | Agonists specific for RAR α and RAR β promote osteogenic differentiation. **a, b**, Effects of RAR agonists on bone-like nodule formation. Cells were cultured in each condition, nodules were stained by Alizarin red (**a**), and calcium depositions were quantified (**b**) at the indicated time points. Am580, RAR α agonist; AC55649, RAR β agonist. **c, d**, Gene expression profiles of BMP- and WNT-downstream genes (**c**) and stage-related genes (**d**). The expression level at each time point is shown as a value relative to RA⁻ at day 0. All experiments were performed using iPSC line 414C2. Experiments in **a** were repeated twice independently with similar results. Results are shown as the mean $\pm$ s.e.m. ($n=3$ from three biologically independent experiments in **c, d**; $n=9$ from three biologically independent experiments in **b**). N.S., no significant difference. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by two-sided Student's t -test.

the formation of the normal triple helix, which results in the reduction of normal collagen production and the induction of endoplasmic reticulum (ER) stress^{33,34}. 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 of OI-iPSC-derived cells 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 expression level of the ER stress-related chaperone gene *BiP* (binding immunoglobulin protein) was increased in OI-iPSC-derived cells (Fig. 7d and Supplementary Fig. 10c). Immunohistochemical staining of typeI collagen around the nodules induced from WT-iPSC-derived cells showed uniform distribution, whereas for OI-iPSC-derived cells the amount was much lower and its distribution was quite uneven (Fig. 7e and Supplementary Fig. 10d). To investigate this abnormality, we evaluated intracellular typeI collagen (Fig. 7f and Supplementary Fig. 10e), and found that the amount retained was much larger in OI-iPSC-derived cells than in WT-iPSC-derived cells (Fig. 7g). These abnormal features were recovered in resOI-iPSCs (Fig. 7b–g). Moreover, they were comparable with those reported in primary cells derived from both patients with OI and model mice^{35,36}, indicating that the combination of our induction method using RA and patient-derived iPSCs successfully recapitulated the phenotype of OI in vitro.

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 phenotype^{37,38}. Treatment with mTOR inhibitors from day2 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 typeI collagen was partially recovered by mTOR inhibitors (Fig. 8d), and the amount of intracellular typeI collagen was reduced (Fig. 8e,f). 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 typeI collagen from the cell^{39,40}.

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 signals²³. Our method uses RA from the stage of pluripotent stem cells (day0) through to the stage of terminally differentiated cells (day10) (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

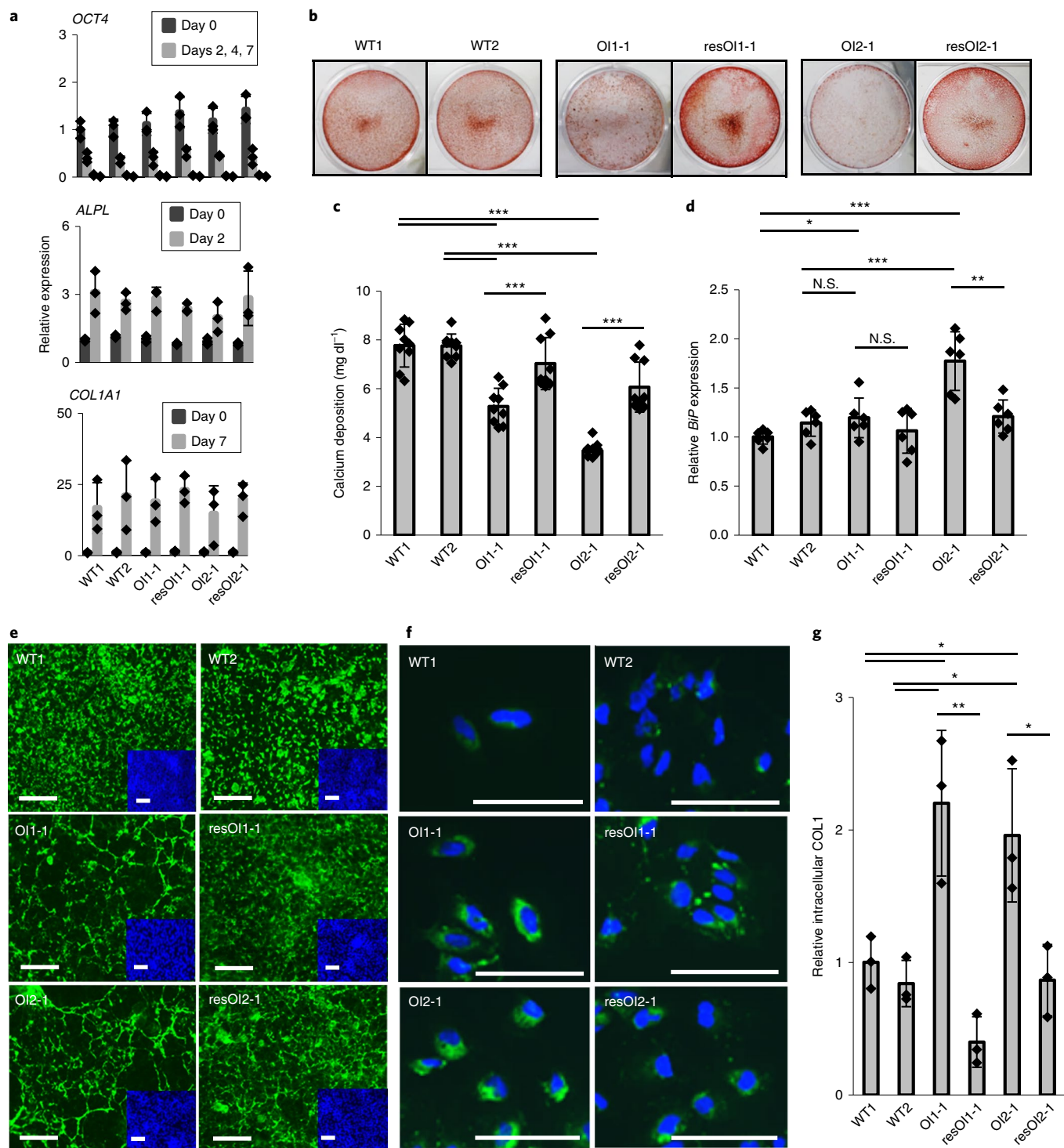


Fig. 7 | Recapitulation of disease phenotypes using iPSCs derived from patients with osteogenesis imperfecta (OI). **a**, mRNA expressions of stage-related genes in OI-iPSCs during bone-like nodule formation. Cells were cultured in RA⁺, and the expression of each gene was analysed at the indicated time 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 μ m. **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 $\pm$ 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.

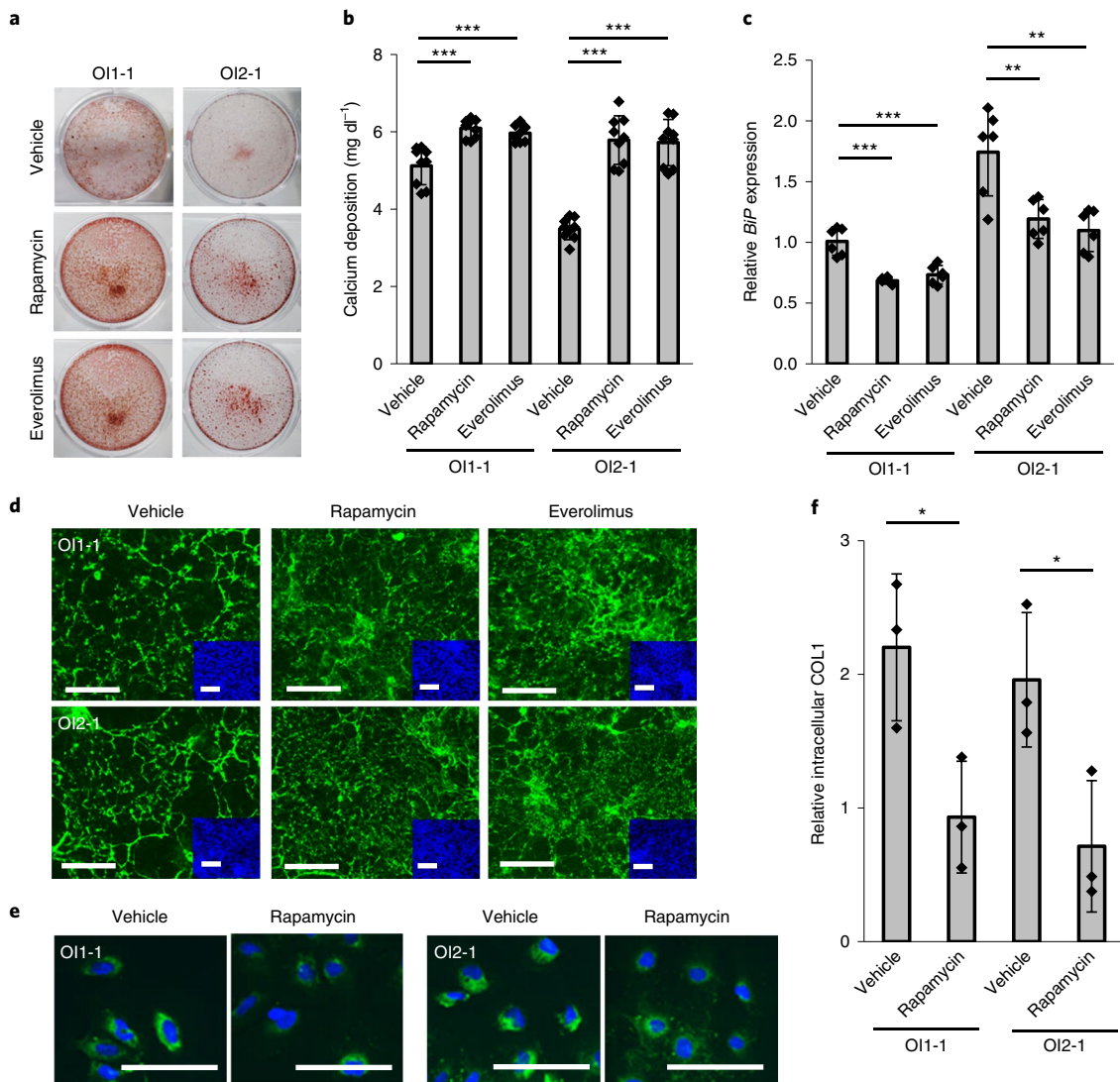


Fig. 8 | mTOR inhibitors rescue OI phenotypes. Effects of mTOR inhibitors on the bone-like nodule formation of OI-iPSCs. **a,b**, Cells were cultured in RA⁺ with rapamycin (10 nM), everolimus (100 nM) or vehicle, nodules were stained with Alizarin red (**a**), and calcium depositions were quantified (**b**) at day 7. **c**, Expression of *BiP* gene at day 7. Each value is shown as a value relative to that of WT1. **d,e**, Immunostaining of type I collagen at day 7 (**d**) and induced cells after isolation (**e**). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m. **f**, Quantification of intracellular collagen. OI1-1, iPSC clone 1 established from OI1 patient; OI2-1, iPSC clone 1 established from OI2 patient. Experiments in **a,d,e** were repeated three times independently with similar results. Results are shown as the mean $\pm$ s.e.m. ($n=3$ from three biologically independent experiments in **f**; $n=9$ from two biologically independent experiments in **b**; $n=6$ from three biologically independent experiments in **c**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ 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 biomaterials^{41,42}, 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 differentiation^{43,44}. The relationship of RA with BMP or WNT signalling has been reported in several studies^{45,46}. It was also reported that RA and BMP signalling synergistically activate the WNT/ β -catenin pathway through the inhibition of glycogen synthase kinase 3 β ⁴⁷. 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

cranial skeletal elements, suggesting the functional redundancy of RAR isoforms. As for their role in osteogenesis, RAR α potentiates ectopic bone formation by BMP9⁴⁹, whereas inhibitory effects by RAR γ were reported in mouse models of heterotopic ossification⁵⁰. These data are consistent with our in vitro results. siRNA experiments suggested that RAR α and RAR β target common and distinct genes related to osteogenesis. Comparing the binding sites of RAR α and RAR β 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- κ B ligand) in osteoblasts through RAR α activation⁵¹. 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 osteoporosis, and at least ten genes are listed as causative for OI^{24,34,52}. The clinical phenotype was also significantly different among patients, despite sharing the same responsible mutant genes, such as *COL1A1*⁵³. 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 knocking out the mutant *COL1A1* or *COL1A2* allele to leave one copy of normal allele⁵⁴. 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. 14a,b), but the scale will be further increased in the near future. It would also be desirable to use reporter iPS 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 difficult⁵⁵. 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 previously^{32,56}. The 317–12 line

was prepared from 201B7 to include constitutively active GFP²⁵. The detailed backgrounds of the iPSCs are shown in Supplementary Table 1a. On-feeder iPSCs were maintained in primate embryonic stem cell medium (ReproCELL) supplemented with 4 ng ml⁻¹ 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 dish⁵⁶.

Induction method. For the induction of osteogenic lineages from iPSCs, we modified a mineralization protocol^{30,57} by adding retinoic acid (RA; Wako) at a concentration of 1 μ M. 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 (Nishirei), 2 mM Gluta-MAX (Thermo), 10 mM glycerol-2-phosphate (Sigma), 1 nM dexamethasone (Sigma), 0.1 mM 2-mercaptoethanol (Thermo), 50 μ g ml⁻¹ L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Nacalai Tesque), and 1% non essential amino acids (Thermo) in the presence of Y-27632 (10 μ M, 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 to dissociate the cells from the dish. After pipetting 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 (Nishirei) 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 (40 mM, 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 (OCPC) 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 (0.3 μ g) 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 hr at room temperature with secondary antibodies diluted in 10% Blocking One in PBS-T. Nuclei were stained with 1:5,000 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, reseeded 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 (Tokaihit) 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 320 $\times$ 320 pixels in resolution and taken at 1 μ m depth in the z-direction (120 μ m 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 transplanted into 4 mm-diameter bone defects, of which the size was critical for spontaneous regeneration, made by drilling the calvariae of immune-deficient NOD/ShiJic-scid Jcl (NOD/SCID) mice (CLEA Japan) under anaesthesia. Six weeks later, the

calvariae were harvested, fixed with 4% paraformaldehyde for 24 h, 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/FCS-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 described⁵⁸. Briefly, BMSCs were cultured in α -minimal essential medium with GlutaMAX (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), 100 U ml⁻¹ penicillin, and 100 mg 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 μ M dexamethasone and 50 μ M 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 Can Get Signal immunostain solution B (TOYOBO) for 16–18 h at 4°C. Next, the samples were washed several times in 0.2% Tween 20 (Sigma-Aldrich) 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 1 h at room temperature. DAPI (10 μ g ml⁻¹) was used to counterstain nuclei, and the samples were observed by BZ-9000E (KEYENCE).

Microarray experiments. Total RNA was prepared using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the GeneChip WT (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 arrays, 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 released with Accutase (LifeTech) for 5 min and resuspended in mTeSR1 supplemented with 10 μ M Y-27632. Cells (1×10^6 in 20 μ l) were injected into 8–12-week-old male NOD/SCID mice (CLEA Japan) under the testis capsule as described previously²⁹. A minimum of six testes were injected per iPSC line. Tumours were collected 8–12 weeks after injection, fixed with 4% PFA for 24 h, and processed for paraffin-embedded sections at the Division of Technical Support.

DNA extraction and sequencing. Primer sets were designed to amplify regions containing mutated sites (exon 17 for OI1 and exon 32 for OI2), which we found by exome sequencing.

PCR products were then sequenced on a 3500XL Genetic Analyzer (Applied Biosystems) with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to standard procedures.

Gene editing. ssODN (100 bp) for the rescue of OI mutation was designed to include a restriction enzyme site with silent mutation to confirm the insertion of ssODN (Supplementary Table 2c). Mutation-specific target site sequences for guide RNA (Supplementary Table 2c) were inserted into pX459, a SpCas9(BB)-2A-Puro expressing vector (Addgene). Electroporation was performed as previously described⁶⁰ with some modifications. For OI1, we electroporated pX459 (5 μ g) and ssODN (10 μ g) into 1×10^6 cells using NEPA 21 (NEPA GENE). For OI2, we electroporated pX459 (0.5 μ g) and ssODN (10 μ g) into 2×10^5 cells using Nucleofector (Lonza). Cells were plated onto one well of a six-well plate with feeders in the presence of 10 mM Y-27632 and treated with 0.5 μ g ml⁻¹ puromycin (Thermo) from 24–54 h after electroporation for the selection of pX459-transfected cells. Seventy-two colonies for each iPSC line were randomly chosen and expanded for genomic DNA extraction and genotyping by PCR using primers. Amplified PCR bands were further analysed by restriction enzyme (XhoI for OI1 and ApaI for OI2) and Sanger sequencing to confirm the expected correction. Primer sequences are described in Supplementary Table 2c.

Study approval. The experimental protocols dealing with 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. Written informed consent was provided by each donor.

Statistical analysis. For the in vivo transplantation experiments, we estimated that the effect size, $(\mu_1 - \mu_2)/\sigma$, 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. All results are biological replicas from two or three independent experiments and are presented as the mean $\pm$ s.e.m. P values were calculated using the Student's t -test or Dunnett multiple comparison test, and P values less than 0.05 were considered significant. The precise values of each comparison are provided in Supplementary Table 4.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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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Author contributions

S.K., H.Y. and J.T. designed the research and wrote the manuscript. J.S. performed imaging studies. C.A., M.I., Y.J., T.A. and S.M. advised on the project. M.H., Y.K. and S.T. established and maintained iPSCs. 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

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Software and code

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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

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Life sciences study design

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Sample size	For the in vivo transplantation experiments, we estimated that the effect size, $(\mu_1 - \mu_2)/\sigma$, is 1.5 on the basis of data obtained in preliminary experiments, and that a statistical power of 88.6% for $n=8$ s 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.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	SC-10758: Santa Cruz Biotechnology, Rabbit Polyclonal Ab, Human, Mouse, Rat Runt-related transcription factor 2 NB600-408: Novus Biologicals, Rabbit, Human, Mouse, Rat Type 1 collagen BAM1448 clone # B4-78: R&D system, Mouse Monoclonal IgG1, Human, liver, bone and kidney Alkaline Phosphatase/ALPL, MAB1419 clone # 190125: R&D system, Mouse Monoclonal IgG1, Human, Rat Osteocalcin SAB1300753: Sigma-Aldrich, Rabbit Polyclonal, Human SOST bs-12313R: BIORX Antibodies, Rabbit, Polyclonal, Human, Mouse, Rat Phosphate Regulating Endopeptidase Homolog, X-linked A22283: Thermo Fisher Scientific, F-actin D1306: Life Technology, AT regions of DNA (DAPI) AF1433: R&D system, Goat Polyclonal IgG1, Human Osteopontin/OPN MAB4304 clone MC-813-70: MILLIPORE, Mouse Monoclonal, Human, Mouse Stage-Specific Embryonic Antigen-4 MAB4360 clone TRA-1-60: MILLIPORE, Mouse Monoclonal, Human TRA-1-60 MAB4381 clone TRA-1-81: MILLIPORE, Monoclonal Mouse, Human TRA-1-81
Validation	SC-10758: Deckelbaum, R. A., et al Development 2012;139(7):11346-58. NB600-408: Kasetti RB, et al. Sci Rep 2017;7(1):14951 BAM1448 clone # B4-78: Drake MT, et al. Bone 2011;49(3):349-55. MAB1419 clone # 190125: Xia H, et al. Am J Pathol 2014;184(5):1369-83. SAB1300753: None bs-12313R: Choi H, et al. Cell Tissue Res 2016;364(1):95-103. A22283: None D1306: None AF1433: Liersch R, et al. Blood. 2012;119(22):5215-20. MAB4304 clone MC-813-70: Debowski K, et al. PLoS One. 2015;10(3):e0118424

Eukaryotic cell lines

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Cell line source(s)	414C2 and 409B2: iPS cell lines established from human fibroblasts which is purchased from CORIELL INSTITUTE. 1231A3: iPS cell line established from human PBMC which is purchased from CORIELL INSTITUTE. 317-12: iPS cell line derivative of 201B7. 201B7 is established from human fibroblast purchased from CPORIELL INSTITUTE. OI#1: iPS cell lines established from skin fibroblasts of an OI patient (female, 17 years old) with informed consent. OI#2: iPS cell lines established from skin fibroblasts of an OI patient (male, 3 years old) with informed consent. BM01: primary cultured human bone-marrow stromal cells from a donor (male, 27 years old) with informed consent. BM02: primary cultured human bone-marrow stromal cells from a donor (male, 29 years old) with informed consent. BM03: primary cultured human bone-marrow stromal cells from a donor (female, 54 years old) with informed consent.
Authentication	None of the cell lines used were authenticated.
Mycoplasma contamination	All cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	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	OI#1: Type III OI patient (female, 17 years old) OI#2: Type III OI patient (male, 3 years old) BM01: a donor (male, 27 years old) with informed consent. BM02: a donor (male, 29 years old) with informed consent. BM03: a donor (female, 54 years old) with informed consent.
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.

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