Supporting Information (SI Appendix)

Neofunction of ACVR1 in fibrodysplasia ossificans progressiva

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SI Tables

SI Materials and Methods

TGF-β superfamily ligands and related reagents. All TGF-β superfamily ligands with the exception of Inhibin-A (Raybiotech Inc., Norcross, GA, USA), growth differentiation factor-15 (GDF-15; Abnova Corporation, Taipei, Taiwan) and GDF-6 and -7 (GeneTex Inc., Irvine, CA, USA) were purchased from R&D Systems Inc. (Minneapolis, KA, USA). FLRG, FST, Anti-Activin-A antibody, ACVR1-Fc, BMPR1-Fc, ACVR2A-Fc, ACVR2B-Fc and BMPR2-Fc were purchased from R&D Systems. DMH1 and CD437 were purchased from Tocris Bioscience (Bristol, UK). SB-431542, FK506, and R667 were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), Apollo Scientific Ltd. (Cheshire, UK) and Toronto Research Chemicals Inc. (Ontario, Canada), respectively. Activin-A and BMP-7 were used at 100 ng/ml, and TGF-β3 at 10 ng/ml unless otherwise noted.

Cell culture. iPSCs were maintained in primate ES cell medium (ReproCELL Incorporated, Tokyo, Japan) supplemented with 4 ng/mL recombinant human FGF2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The induction and maintenance of iNCCs and iMSCs were previously described (1). Briefly, iNCCs were maintained in chemically defined medium (CDM) supplemented with FGF2 and recombinant human EGF (R&D Systems), and we used up to 20 passages in this study. iMSCs were induced and maintained in αMEM (Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) FBS (Nichirei Inc., Tokyo, Japan), 5 ng/mL FGF2 and 0.5% penicillin and streptomycin (Invitrogen). FOP-iPSCs used in this study (FOP-iPSCs from patient 1 and 2, previously described as vFOP4-1 and vFOP5-22 (2), respectively) harbor the R206H heterozygous mutation in ACVR1, and gene-corrected resFOP-iPSCs were generated by

BAC-based homologous recombination (3). All experiments shown in Fig. 1-5 were performed using FOP-iPSCs from patient 1 and resFOP-iPSCs (cl1) (3). Other clones' data are shown in SI Appendix, Fig. S2. COS (monkey kidney cells), C3H10T1/2 (murine multipotent mesenchymal cells), U2OS (human osteosarcoma cells), HEK293 (human embryonic kidney cells) and HepG2 (human Hepatocellular carcinoma cells) were obtained from ATCC, and LentiX293T was obtained from Takara Bio Inc. (Shiga, Japan). Cells were maintained in DMEM （Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% (v/v) FBS and 1 mM Na-pyruvate (Invitrogen) (COS and C3H10T1/2) or in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) FBS (U2OS, HEK293, HepG2 and LentiX293). To prepare C3H10T1/2-expressing Dox-inducible hINHBA (C3H-DoxOn-hINHBA), we inserted hINHBA cDNA into PB-TAC-ERN (KW111), which constitutively expresses the neomycin (G418) resistance gene along with the rtTA transactivator element and mediates doxycycline (Dox)-dependent activation of hINHBA controlled by tetO promoter (PB-TAC-ERN-hINHBA) (4, 5). After transfecting PB-TAC-ERN-hINHBA vector into C3H10T1/2, the neomycin-resistant population was selected. Activin-A production in vitro was confirmed by ELISA (SI Appendix, Fig. S17).

Luciferase assay. The BRE-Luc reporter was purchased from Addgene (Cambridge, MA, USA) (6). The CAGA-Luc reporter was constructed as previously described (7). The pRL-CMV renilla luciferase reporter (Promega Corporation, Madison, WI, USA) was transiently transfected and used for normalization. Expression vectors for FOP mutant receptors were constructed using the KOD-Plus-Mutagenesis Kit (TOYOBO CO., LTD., Osaka, Japan) and inserted into a pcDNA^{TM}-DEST40 vector. For transient expression,

FuGene® HD (Promega) or Lipofectamine® 2000 (Invitrogen, for RNAi experiments) were used according to the manufacturer's instructions. Luciferase activity was measured using the dual luciferase reporter assay system (Promega), and the luminescence signal was measured on EnVision® Multilabel Reader (PerkinElmer Co., Ltd, Waltham, MA, USA) according to the manufacturer's instructions. siRNAs specific for type I or II receptors were purchased from Thermo Fisher Scientific Inc. (Silencer® Select Pre-designed siRNA, Waltham, MA, USA) . Sequence information is shown in Table S1. For inhibition assay with Fc-fusion receptors, Activin-A (final 30 ng/mL) was incubated with Fc-fusion receptors (final concentrations of 0.1, 1 and 10 μg/mL) for 2 hours at 37 °C under 5% CO_2, and Activin-A and Fc-fusion receptors were added to FOP-iMSCs transiently transfected with BRE-Luc and CMV-Renilla.

Western Blotting. SDS-PAGE and blotting with whole-cell lysates were performed by standard procedures. Protein bands were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and visualized using BIO-RAD Molecular Imager® Chemi-Doc™ XRS+ with Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The antibodies used in this study are described in Table S2.

Quantitative PCR analysis. Total RNA was purified with the RNeasy Kit (Qiagen Inc., Valencia, CA, USA) and treated with the DNase-one Kit (Qiagen) to remove genomic DNA. Total RNA (0.3 μg) was reverse transcribed for single-stranded cDNA using random primers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR was performed with Thunderbird SYBR

qPCR Mix (TOYOBO) and analyzed with the StepOne real-time PCR system (Applied Biosystems, Waltham, MA, USA). Primer sequences are described in Table S3. 3D chondrogenic differentiated pellets were homogenized by Multi-Beads Shocker (Yasui Kikai Corporation, Osaka, Japan) according to the manufacturer's instructions.

Microarray experiments. To show the general activation of BMP downstream genes in FOP-iMSCs by Activin-A treatment, FOP- and resFOP-iMSCs were stimulated with 100 ng/mL Activin-A, 100 ng/mL BMP-7 or 10 ng/mL TGF-β3, and after 16 hours incubation, mRNA was extracted. To analyze the enhanced chondrogenesis in FOP-iMSCs by Activin-A treatment at the molecular level, 2D chondrogenic induction was performed in FOP- and resFOP-iMSCs stimulated with or without 100 ng/mL Activin-A. After 7 days incubation, mRNA was extracted. RNA was reverse transcribed, biotin-labeled and hybridized to Human Genome U133 Plus 2.0 Array (Fig. 1F-I) or GeneChip Human Gene 1.0 ST Expression Array (Fig. 3D), which were subsequently washed and scanned according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA, USA). Raw CEL files were imported into GeneSpring GX 12.6.1 software (Agilent Technologies, Santa Clara, CA, USA), and expression values were calculated with the MAS 5.0 algorithm (Fig. 1F-I) or RMA16 algorithm (Fig. 3D). PCA and hierarchical clustering were analyzed by GeneSpring GX. Pathway analysis and upstream analysis were performed by Ingenuity pathway analysis (Qiagen).

Binding and Affinity Cross-linking. A SNAP-tag (New England Biolabs, Ipswich, MA, USA) or V5-tag was fused in-frame to the N terminus of hACVR2A and hACVR2B or C terminus of hACVR1, respectively, and inserted into a pLentiTO vector (Thermo Fisher Scientific). Recombinant human Activin-A was iodinated using Pierce Pre-Coated

Iodination Tubes (Thermo Fisher Scientific) according to the manufacturer's instructions. Receptors were transiently transfected into LentiX293T cells by TransIT®-293 Transfection Reagent (Mirus Bio LLC., Madison, WI, USA). After 48 h incubation, cells were washed with PBS and incubated with ^{125}I-Activin-A in PBS for 1 h at room temperature. After washes with PBS, cells were incubated with 0.5 mM disuccinimidyl suberate in HBSS for 1 h at room temperature, with TBS for 30 min at 4 °C and solubilized in 1% (v/v) TritonX100 in TBS. Protein bands were visualized using Typhoon FLA 7000 (GE Healthcare).

2D chondrogenic induction. 2D chondrogenic induction was performed using a previously described protocol with modification (8). Briefly, iMSCs (1.5 x 10^5) were suspended in 5 µL of chondrogenic basal medium (DMEM: F12 (Invitrogen), 1% (v/v) ITS + Premix (BD Biosciences), 0.1 µM dexamethasone (WAKO), 0.17 mM AA2P (Sigma-Aldrich), 0.35 mM Proline (Sigma-Aldrich), 0.15% (v/v) glucose (Sigma-Aldrich), 1 mM Na-pyruvate, 2 mM GlutaMax-I (Invitrogen) and 1% (v/v) FBS) and subsequently transferred to fibronectin-coated 24-well plates (BD Biosciences). After 1 h, a total of 1 mL of the chondrogenic basal medium supplemented with several ligands or inhibitors was added. Micromass cultures were maintained at 37 °C under 5% CO_2 for 7 days. Differentiation properties were assayed by qPCR analysis, GAG quantification and Alcian Blue staining (9). Briefly, induced cells were fixed for 30 minutes with 4% paraformaldehyde (WAKO) and rinsed with PBS. These cells were then stained overnight with Alcian Blue solution (1% Alcian Blue, pH 1 (MUTO PURE CHEMICAL CO., LTD, Tokyo, Japan) and destained with acetic acid solution.

3D chondrogenic induction. 3D chondrogenic induction (3DCI) was performed using a previously described protocol with modification (8). Briefly, iMSCs (2.5×10^5) were suspended in 0.5 mL of chondrogenic basal medium supplemented with 100 ng/mL Activin-A, 100 ng/mL BMP-7 (BMP) or 10 ng/mL TGF-β3 (TGF), and subsequently transferred to PrimeSurface 96U (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) (Fig. 4A) or 15 mL tubes (Corning Inc., Corning, NY, USA). Cells were centrifuged to form pellets and maintained at 37 °C under 5% CO_2. The culture medium was changed every 2-3 days. Fixation and staining of the 3DCI pellet cultures were performed as previously described (8).

GAG value. The GAG content was quantified in pellets with the Blyscan Glycosaminoglycan Assay Kit (Biocolor Ltd., Belfast, UK). The DNA content was quantified using the PicoGreen dsDNA Quantitation Kit (Invitrogen).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized, and for human specific Anti-Nuclei Antibody, antigen retrieval was performed by microwave (300 W, 20 min). Samples were blocked with Blocking One (Nacalai Tesque, Inc.) for 60 min and then incubated with human specific Anti-Nuclei Antibody (EMD Millipore Corporation, Billerica, MA, USA) or Collagen I Antibody (Novus Biologicals, Littleton, CO, USA) diluted in Can Get Signal ® immunostain solution B (TOYOBO) for 16-18 h at 4 °C. Next, samples were washed several times in 0.2% tween20 (Sigma-Aldrich) in PBS and incubated with Goat anti-Mouse IgG1 Secondary Antibody Alexa Fluor® 568 conjugate (Invitrogen) or Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate (Invitrogen) diluted in Can Get Signal ® immunostain solution B for 1 h at room temperature. DAPI (10 μg/mL) was used to counterstain nuclei. Samples

were observed by BZ-9000E (KEYENCE CORPORATION, Osaka, Japan).

In vivo calcification of 3DCI pellets. 3DCI pellets cultured with 100 ng/mL Activin-A for 21 days in vitro were wrapped in 0.5 cm x 1 cm Gelfoam® (Pfizer Inc., New York, NY, USA) and transplanted beneath the dorsal skin of immunodeficient NOD/ShiJic-scid Jcl (NOD/SCID) mice (CLEA Japan, Inc., Tokyo, Japan) (10). Four weeks later, transplanted 3DCI pellets were harvested, fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, sectioned and stained with HE, von Kossa, Alcian blue, as previously reported (8), and human specific Anti-Nuclei Antibody. For X-ray images, mice were anesthetized with isoflurane (Abbvie Limited, Berkshire, UK), immobilized and X-rayed using μFX-1000 (Fujifilm Corporation, Tokyo, Japan) at 50 kV, 100 μA. Transplanted 3DCI pellets were scanned using X-ray CT systems (Fig. 4E: Latheta LCT-200, Hitachi Aloka Medical, Ltd., Tokyo, Japan; SI Appendix, Fig. S12B: inspeXio SMX-100CT, Shimadzu Corporation, Kyoto, Japan) and analyzed by VGStudio MAX 2.0 (Volume Graphics GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

iMSCs transplantation with Activin-A producing cells. FOP- (right leg) and resFOP-iMSCs (left leg) (4×10^6 respectively) were transplanted into the gastrocnemius muscle of NOD/SCID mice with C3H-DoxOn-hINHBA (5×10^5), which can achieve continuous exposure of Activin-A on transplanted iMSCs in vivo. In the Dox-induced group, 1 mg/mL Dox (Sigma-Aldrich) was administered via drinking water with 10 mg/mL sucrose (Nacalai Tesque, Inc.) for two weeks following transplantation. Six weeks after transplantation, transplanted cells were analyzed. μCT images were scanned

using inspeXio SMX-100CT, and bone volume was analyzed by TRI/3D-BON (Ratoc System Engineering Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Transplanted cells were harvested, fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned and stained with HE, von Kossa, Safranin O, human specific Anti-Nuclei Antibody and Collagen I Antibody. Safranin O staining was performed using the Safranin O Stain Kit (IHC WORLD, LLC., Ellicott, MD, USA) according to the manufacturer's instructions.

Statistics. The statistical significance of all experiments was calculated by Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P values less than 0.05 were considered statistically significant.

SI References

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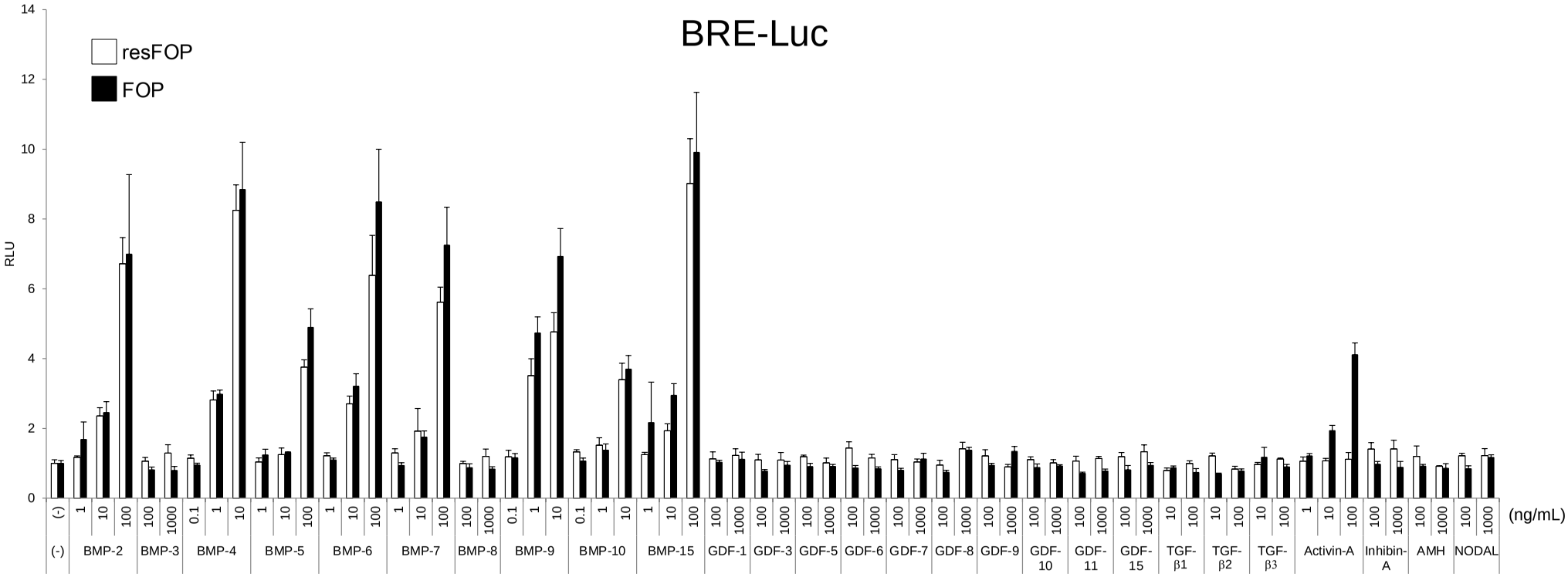


Fig. S1. Detailed data of FOP-ACVR1 specific ligand screening. FOP- and resFOP-iMSCs transiently transfected with BRE-Luc and CMV-Renilla were seeded into 384 well plates, and stimulated by ligands. After 16 hours incubation, luciferase activity was measured. Results are the mean ± standard error (SE). N = 3-4.

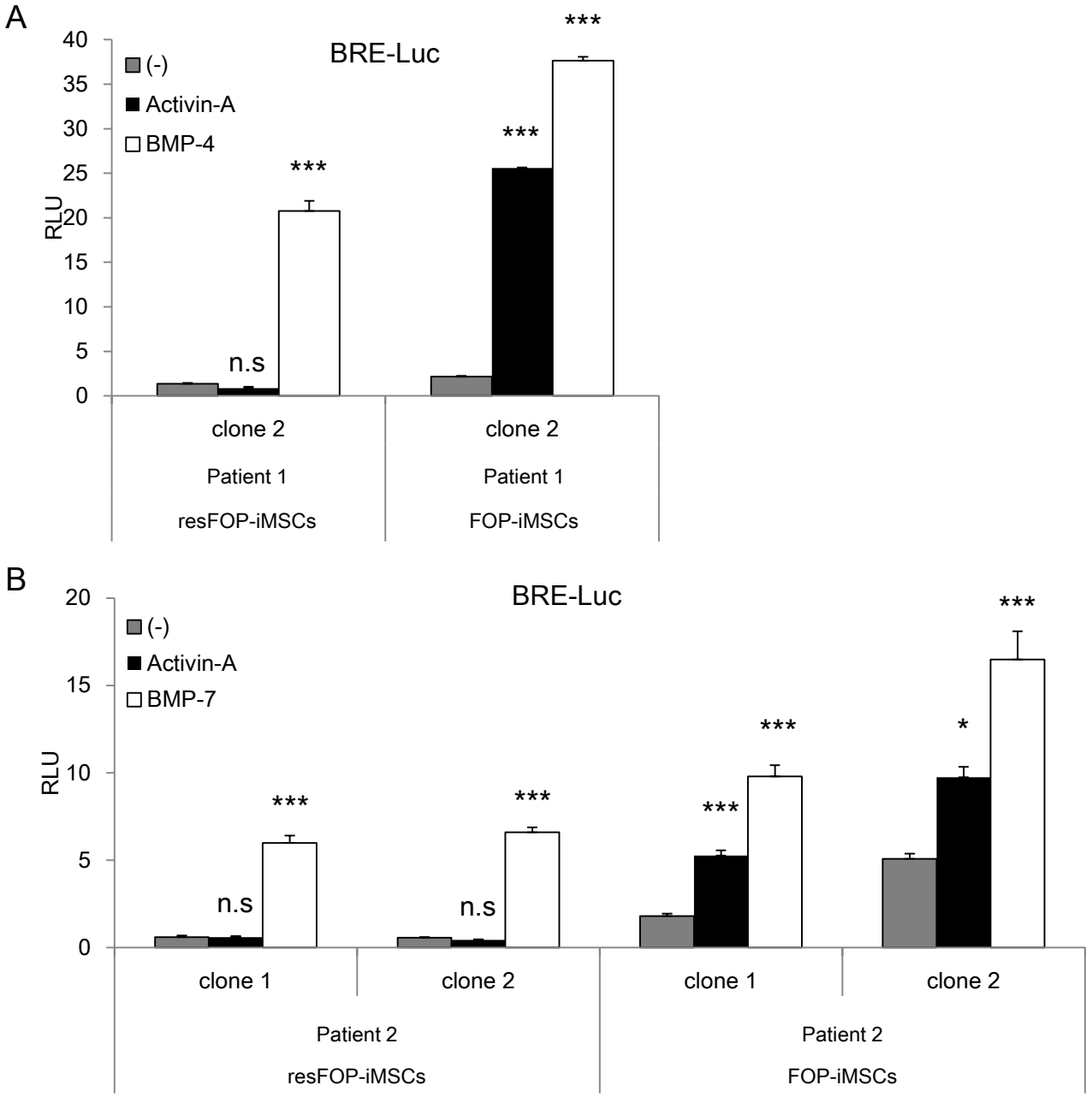


Fig. S2. Clone 2 of patient 1- and clones 1 and 2 of patient 2-derived FOP-iMSCs transduced abnormal BMP signaling, but resFOP-iMSCs did not. (A) BRE-Luc activity in FOP- and resFOP-iMSCs from clone 2 of patient 1 stimulated by Activin-A or 10 ng/mL BMP-4. (B) BRE-Luc activity in FOP- and resFOP-iMSCs from patient 2 stimulated by Activin-A or BMP-7. Results are the mean ± standard error (SE). N = 4. n.s., no significant difference; *, $P < 0.05$; ***, $P < 0.001$ by Dunnett's multiple comparisons *t*-test compared to the no ligand treatment control. Note all experiments in Fig. 1-5 were performed using FOP- and resFOP clone 1 from patient 1.

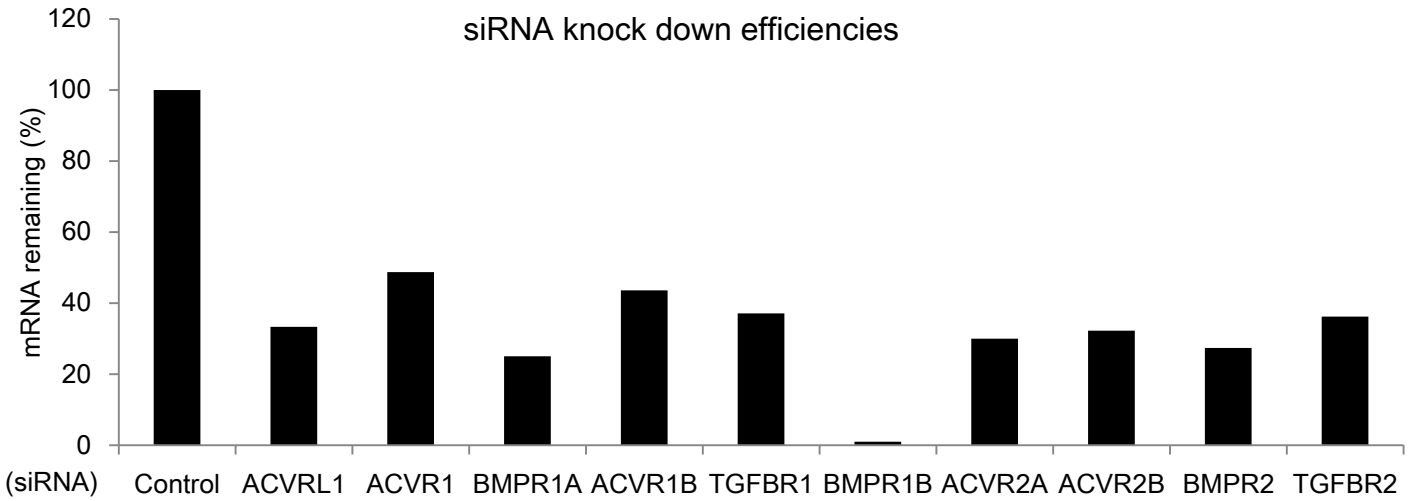
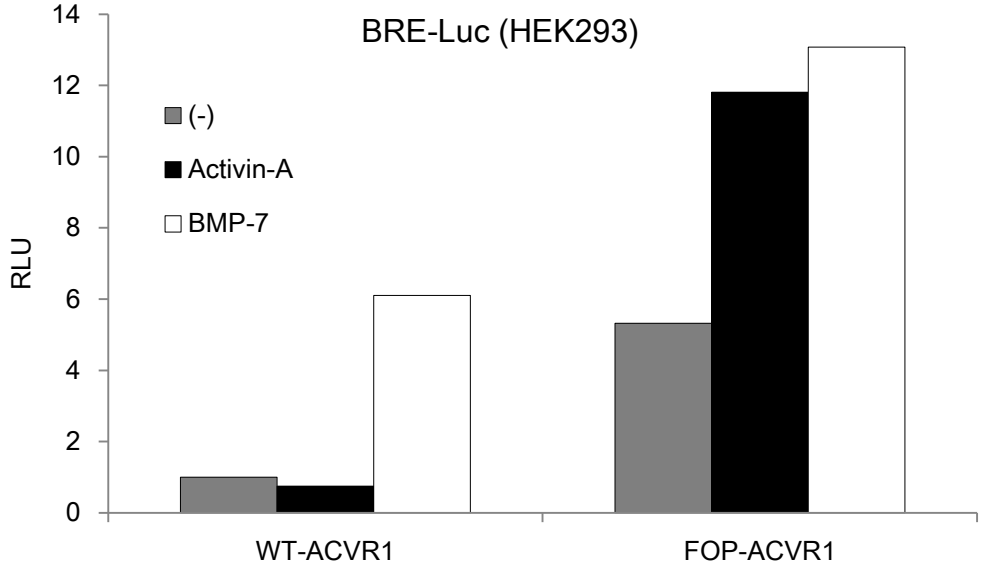


Fig. S3. Knock-down efficiencies of siRNAs. FOP-iMSCs transiently transfected with siRNAs specific for type I receptors or type II receptors were incubated for 16 hours, and RNA was extracted. N = 1.

A



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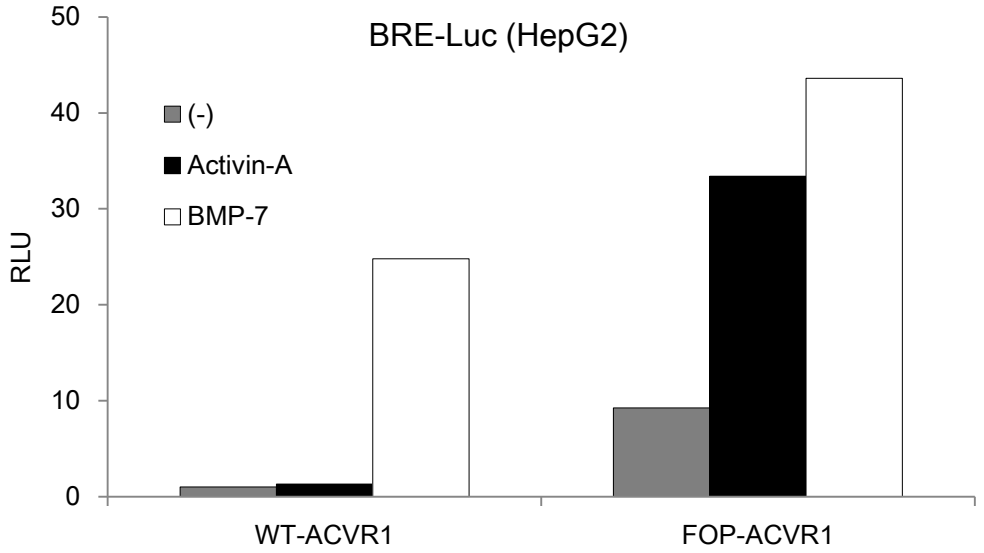


Fig. S4. Overexpression of the FOP-ACVR1 (R206H) conferred Activin-A responsiveness in HEK293 and HepG2. Cells transiently transfected with BRE-Luc, CMV-Renilla and WT- or FOP-ACVR1 were stimulated with 50 ng/mL Activin-A or 100 ng/mL BMP-7 in HEK293 (A) or 50 ng/mL BMP-7 in HepG2 (B). Results are the mean ± standard error (SE). N = 2.

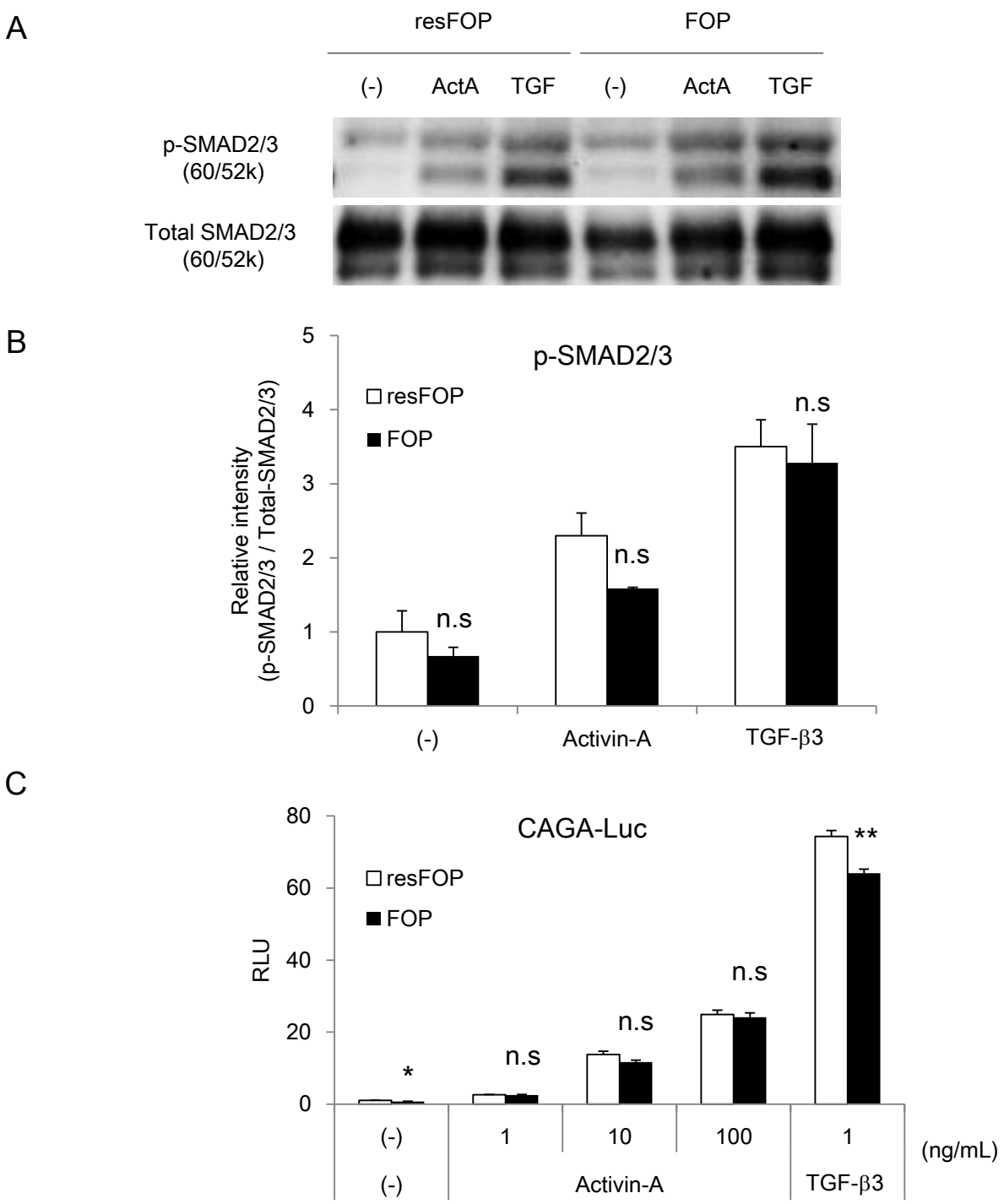


Fig. S5. Activin-A transduced TGF-β-SMAD2/3 signaling similarly in FOP- and resFOP-iMSCs. (A) Representative image of western blot analysis. Activin-A induced phosphorylation of SMAD2/3 similarly in FOP- and resFOP-iMSCs. ActA, 100 ng/mL Activin-A; TGF, TGF-β3 10 ng/mL. (B) Quantification of phosphorylation levels. Activin-A, 100 ng/mL; TGF-β3, 10 ng/mL. (C) Activin-A increased CAGA-Luc activity similarly in FOP- and resFOP-iMSCs. Cells were stimulated with ligands for 3 hours at 37 °C under 5% CO_2. Results are the mean ± standard error (SE). N = 3 (Western blot) or N = 4 (CAGA-Luc). n.s., no significant difference; *, $P < 0.05$; **, $P < 0.01$ by Student's *t*-test compared to the value of resFOP treated with the same condition.

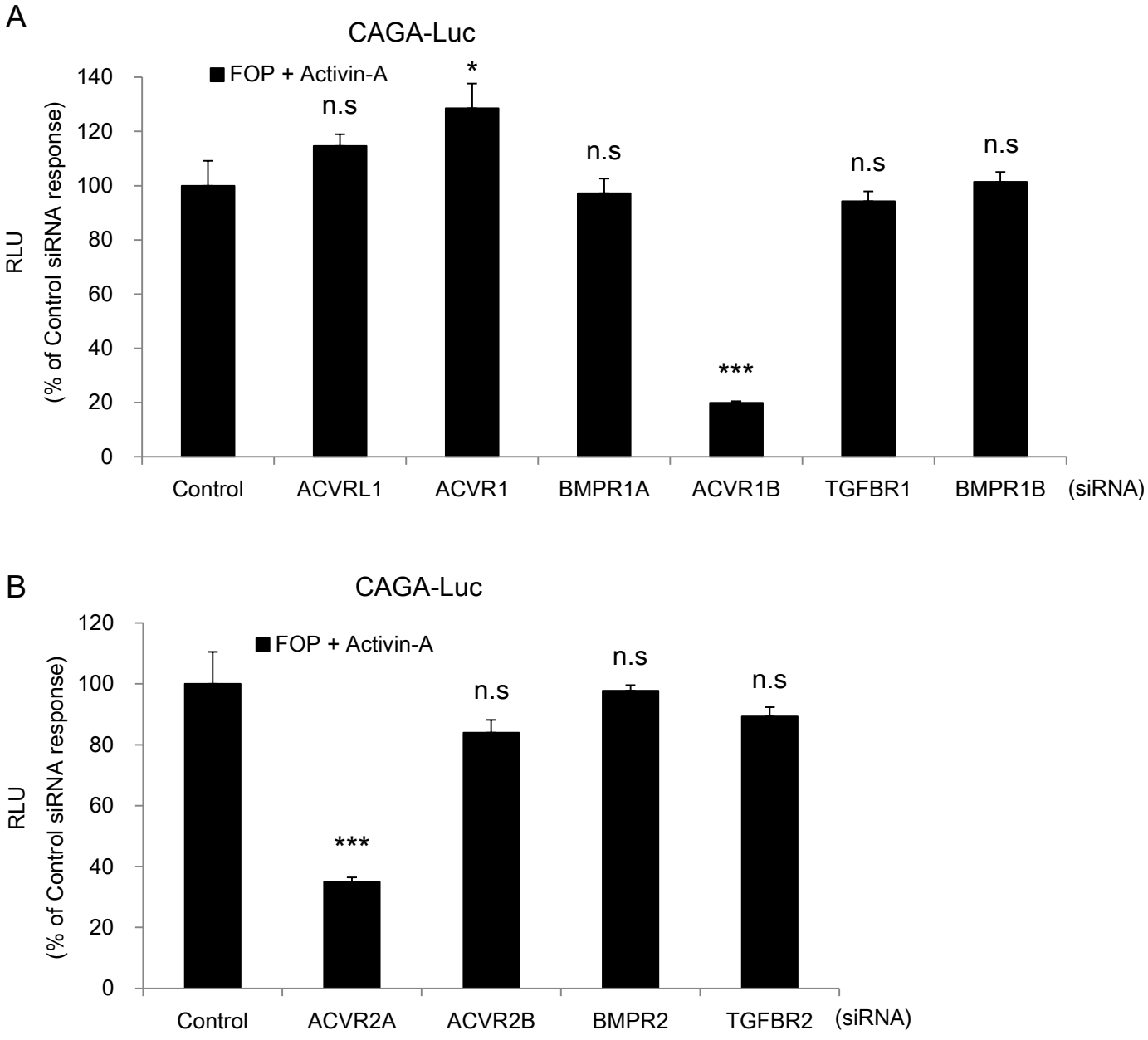


Fig. S6. Activin-A transduces TGF-β-SMAD2/3 signaling through ACVR1B and ACVR2A in FOP-iMSCs. FOP-iMSCs transiently transfected with CAGA-Luc, CMV-Renilla and siRNAs specific for type I receptors (A) or type II receptors (B) were stimulated with Activin-A for 3 hours at 37 °C under 5% CO_2. Results are the mean ± standard error (SE). N = 4, n.s., no significant difference; *, $P < 0.05$; ***, $P < 0.001$ by Dunnett's multiple comparisons *t*-test compared to control siRNA transfected FOP-iMSCs.

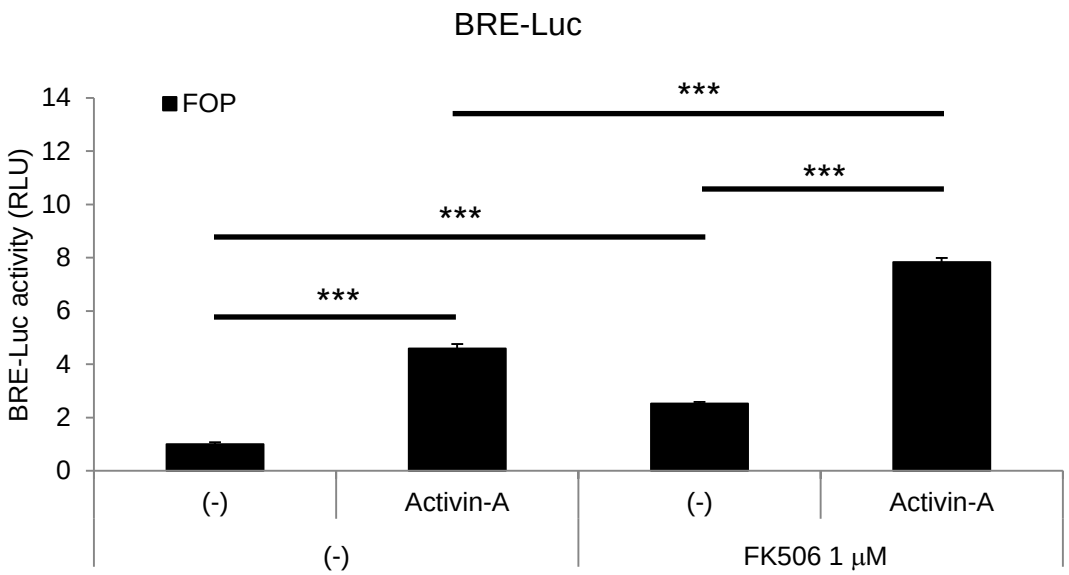


Fig. S7. FK506 enhanced the constitutive activity of FOP-iMSCs. FOP-iMSCs transiently transfected with BRE-Luc and CMV-Renilla were treated with 1 μM FK506 and/or Activin-A for 16 hours. Results are the mean ± standard error (SE). N = 4, ***, P < 0.001 by Turkey's multiple comparisons *t*-test.

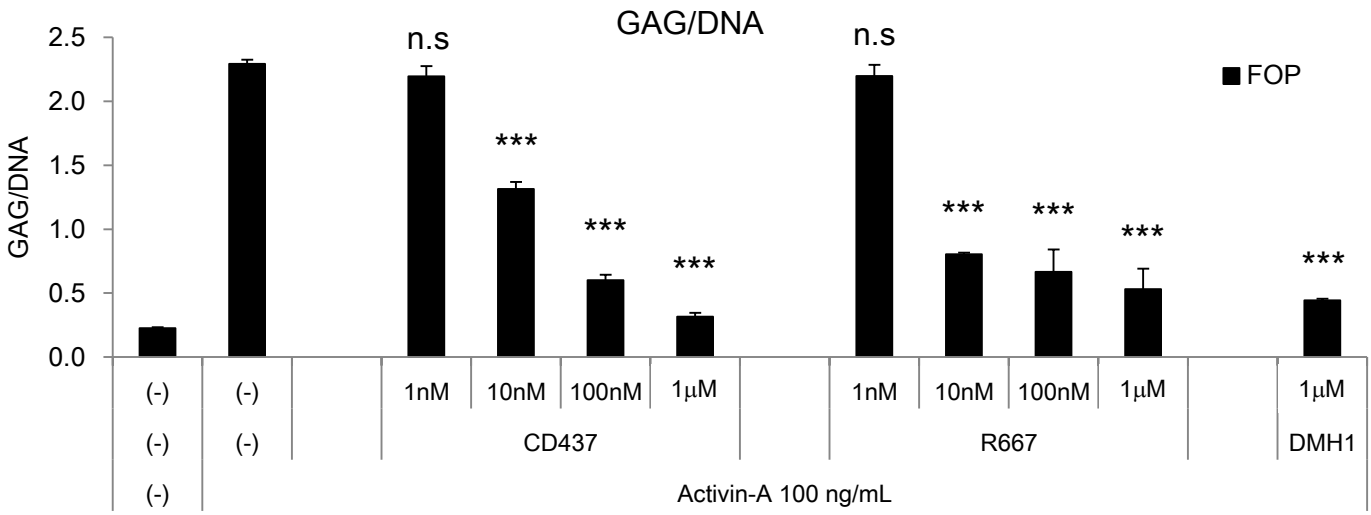


Fig. S8. GAG/DNA in the micromass of FOP-iMSCs cultured with Activin-A was inhibited by RARγ agonists. Results are the mean ± standard error (SE). N = 3. n.s., no significant difference; ***, $P < 0.001$ by Dunnett's multiple comparisons *t*-test compared to Activin-A-treated micromass without RARγ agonists.

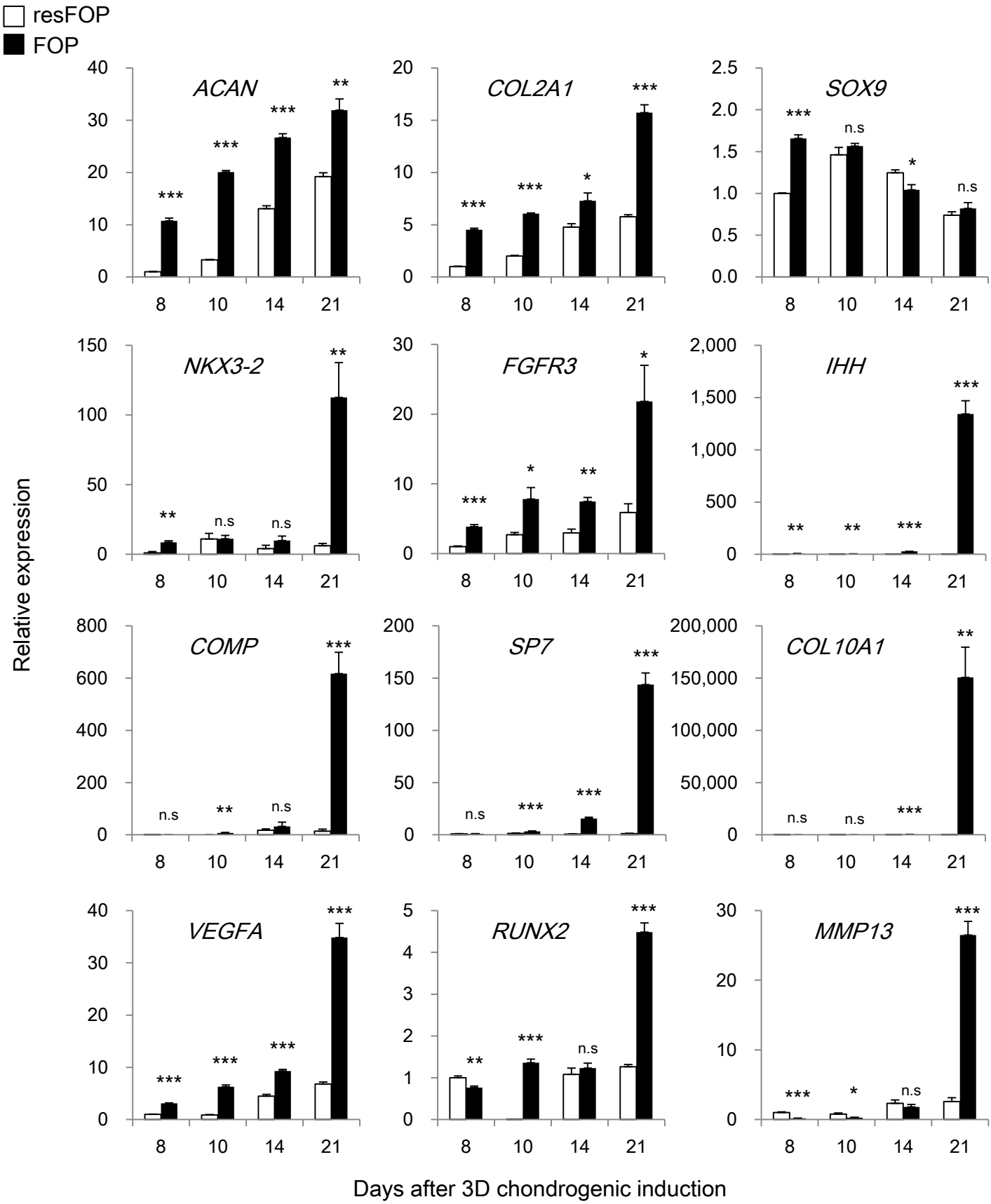


Fig. S9. Expression levels of chondrogenic markers in FOP and resFOP-3DCI pellets cultured with Activin-A. Results are the mean ± standard error (SE). N =4. n.s., no significant difference; *, P < 0.05; **, P < 0.01; ***, P < 0.001 by Student's *t*-test compared to resFOP. resFOP (day8) was normalized to1.

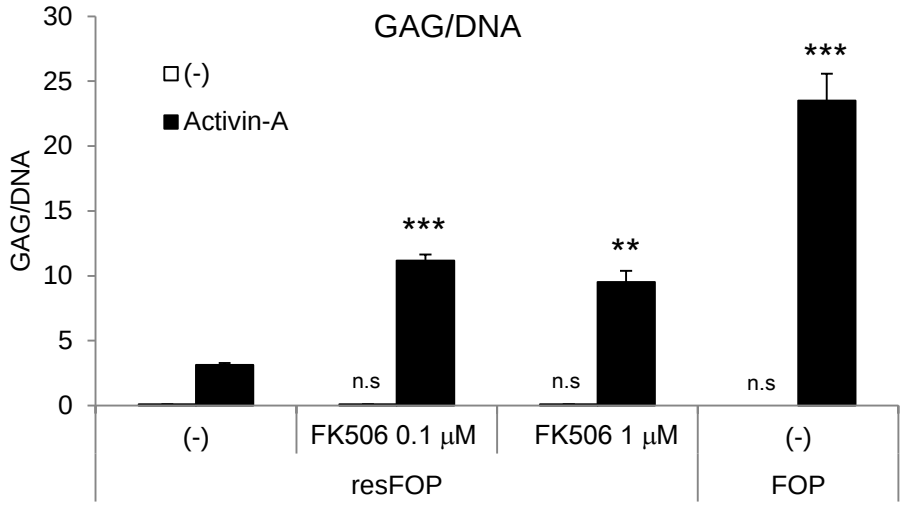


Fig. S10. FK506 treatment enhanced 3D chondrogenesis of resFOP-iMSCs treated with Activin-A. resFOP-3DCI pellets were cultured with FK506 and/or Activin-A for 21 days . Results are the mean ± standard error (SE). N = 4, n.s., no significant difference; **, $P < 0.01$; ***, $P < 0.001$ by Dunnett's multiple comparisons compared to the value of no FK506 treatment control of resFOP with the same Activin-A treatment.

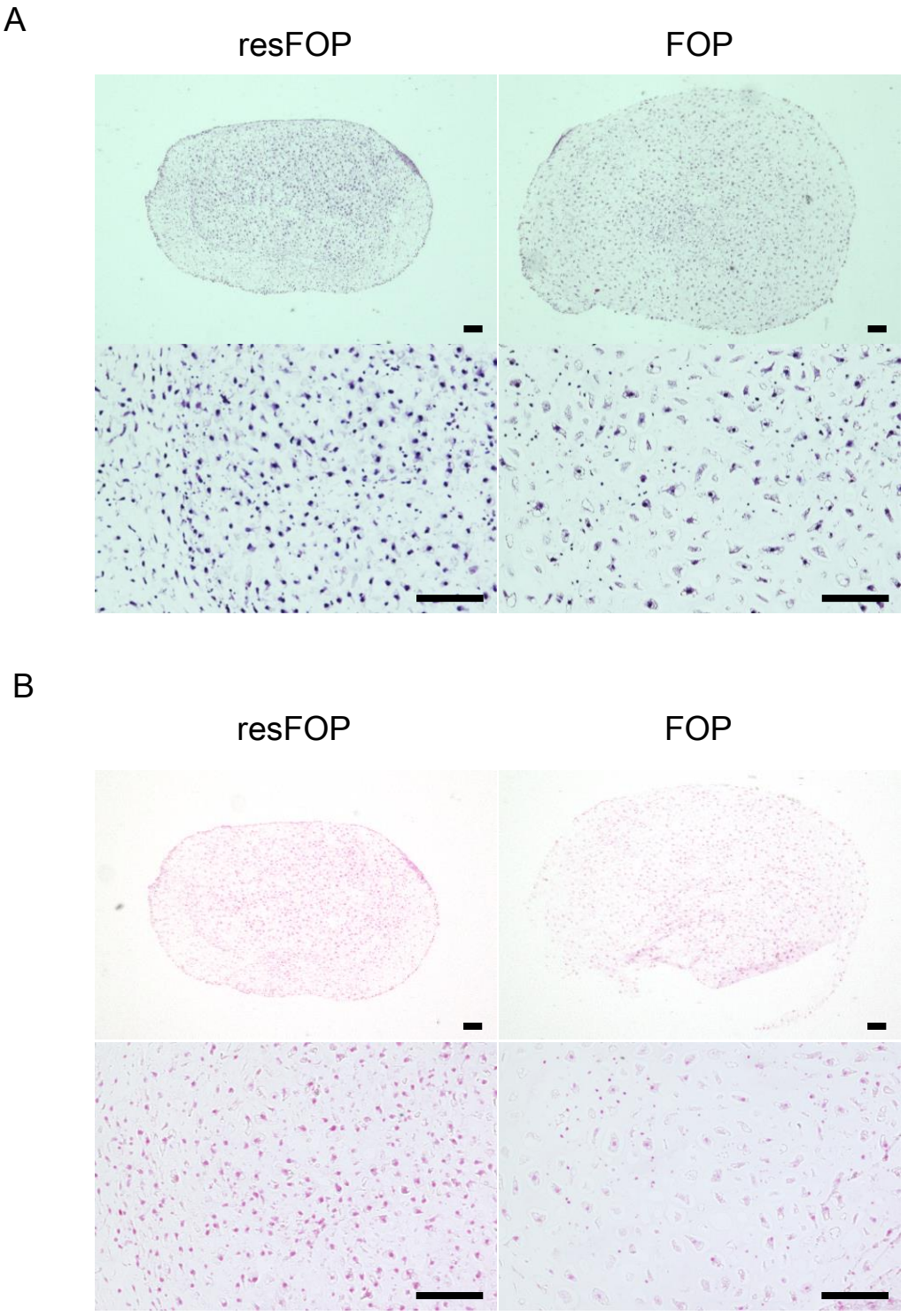
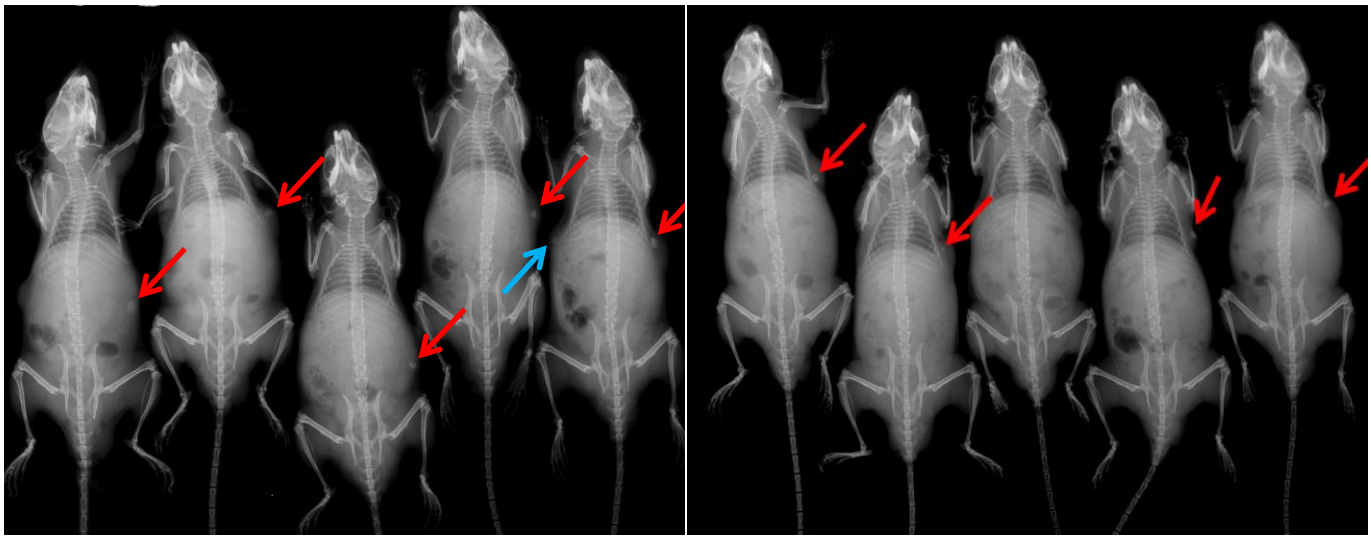


Fig. S11. Histology of 3DCI pellets from FOP- and resFOP-iMSCs cultured with Activin-A (100 ng/ml). Each pellet was cultured for 21 days and examined with HE (A) or von Kossa (B) staining. Scale bars, 100 μm.

A



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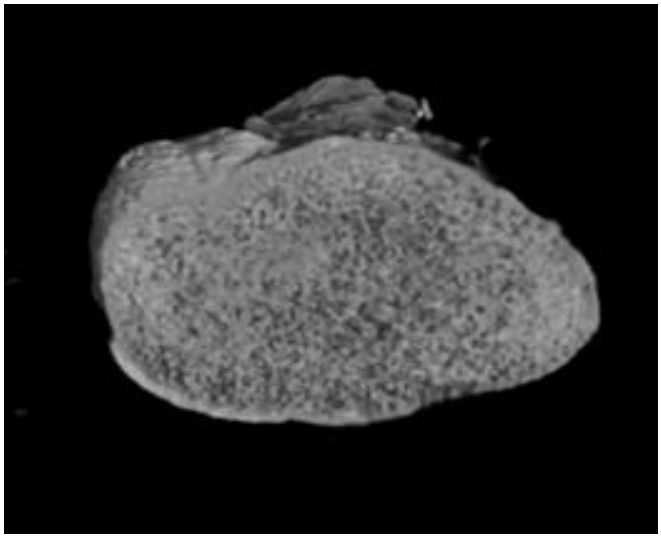
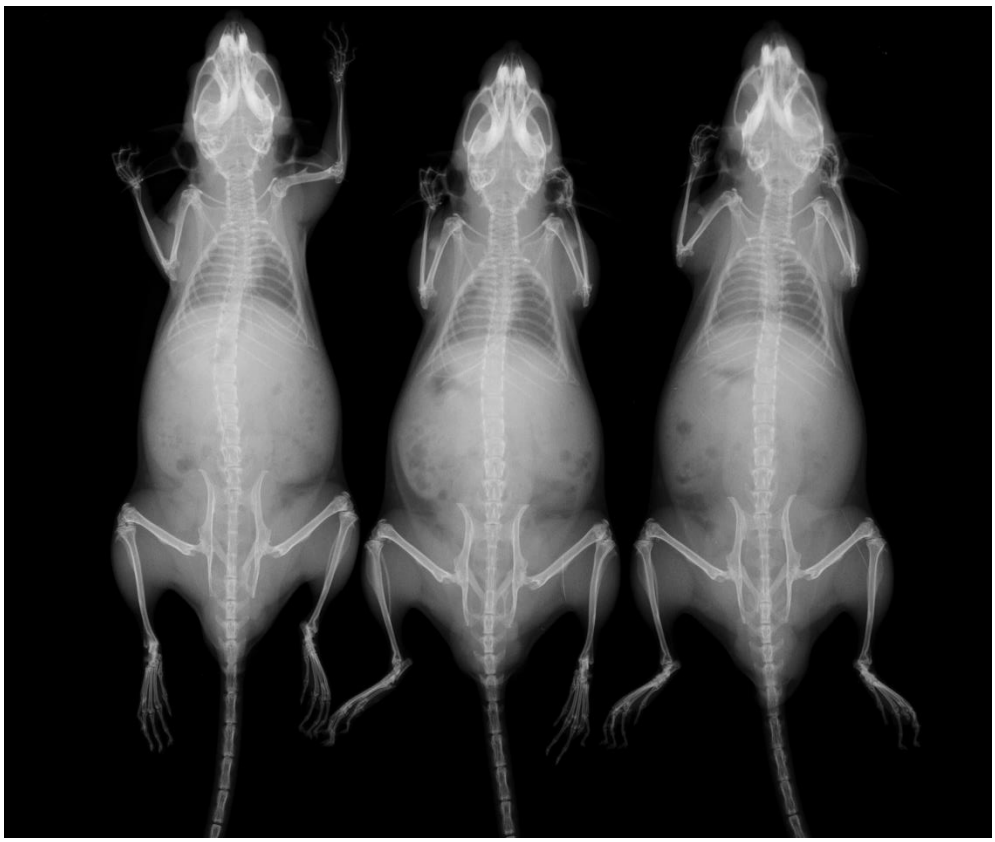


Fig. S12 . X-ray images and sectioned μCT image of FOP-3DCI pellets spontaneously calcified in vivo. (A) X-ray image of mice transplanted with FOP- and resFOP-3DCI pellets 4 weeks after transplantation. Red arrows show transplanted FOP-3DCI pellets, and the blue arrow shows transplanted resFOP-3DCI pellets. (B) The sectioned μCT image shows a transplanted FOP-3DCI pellet.

Dox (-)



Dox (+)

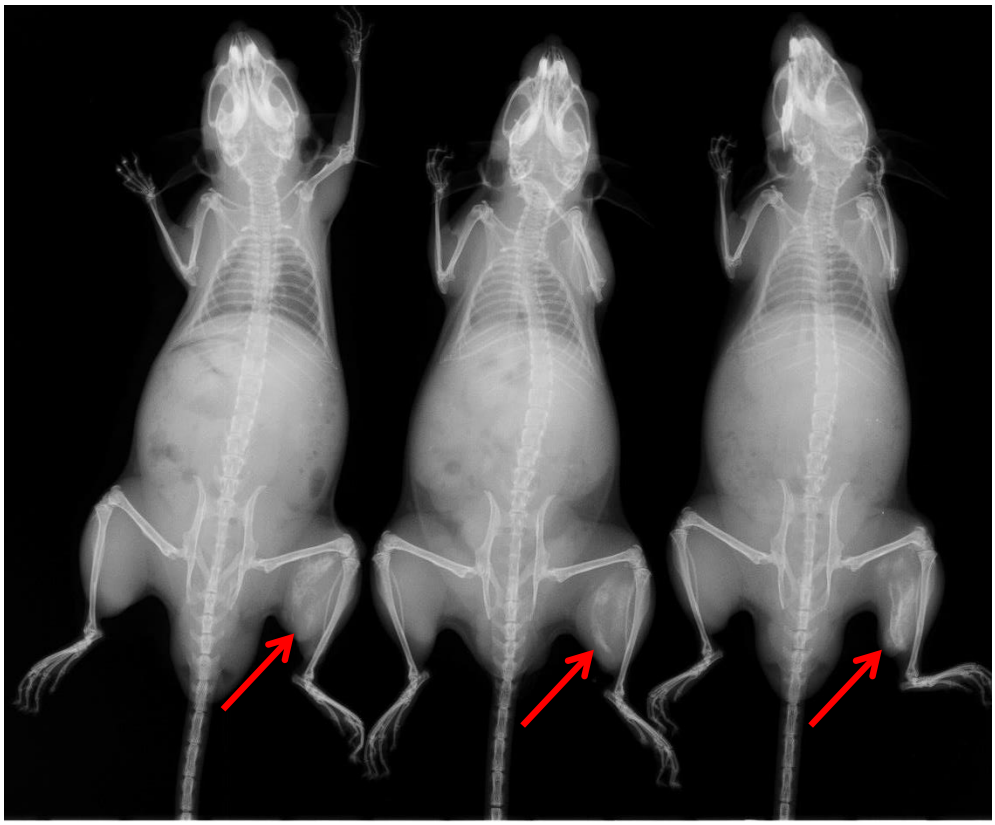
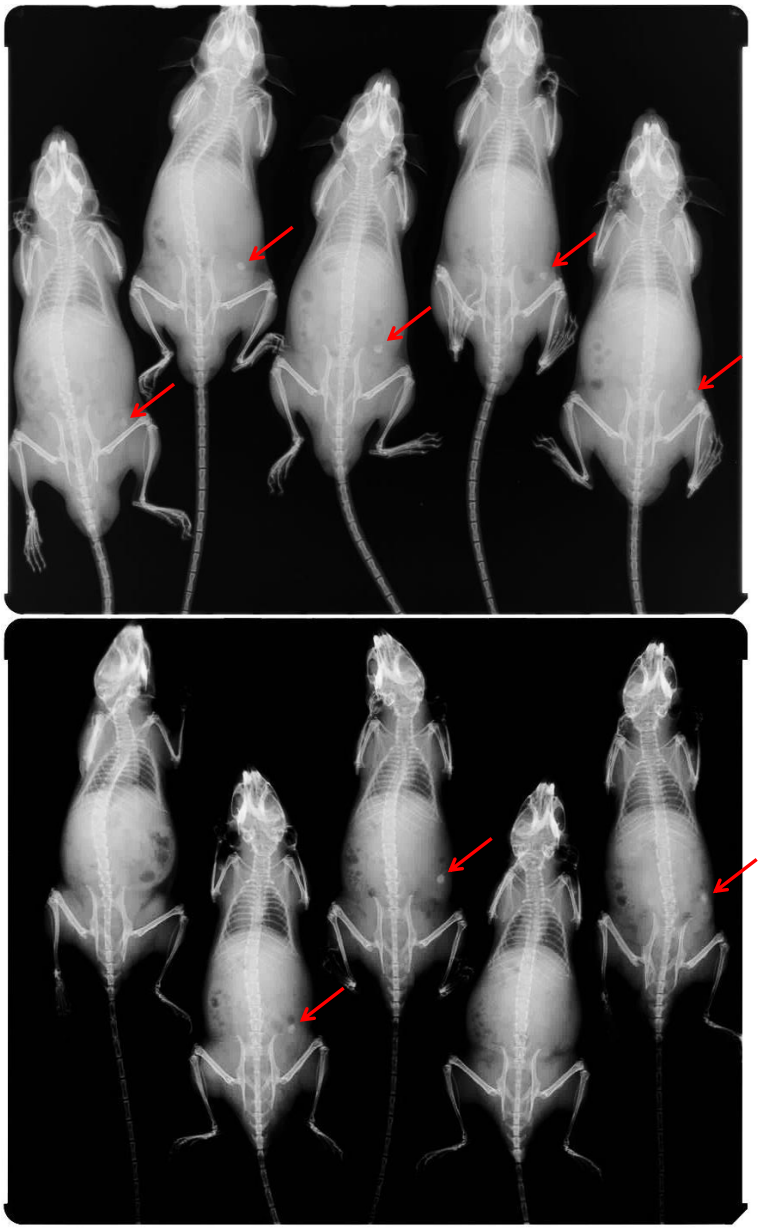


Fig. S13. X-ray images of transplanted FOP- and resFOP-iMSCs with Dox-inducible Activin-A expressing cells 6 weeks after transplantation. Red arrows show FOP-iMSCs derived bone. N = 3.

A

PBS-soaked Gelfoam®

Activin-A-soaked Gelfoam®



B

5 week after transplantation	PBS-soaked Gelfoam®	Activin-A-soaked Gelfoam®
# of FOP-3DCI pellets calcified	5/5	3/5
# of resFOP-3DCI pellets calcified	0/5	0/5

Fig. S14. Administration of Activin-A with transplants did not accelerate the calcification. FOP- or resFOP-3DCI pellets cultured for 21 days with Activin-A (100 ng/ml) were mixed with PBS- or Activin-A (10 μg/mL)-soaked Gelform® and then transplanted subcutaneously in the same NOD/ShiJic-scid Jcl (NOD/SCID) mice (N=5) at the right and left sides, respectively. (A) X-ray images of mice transplanted with FOP- and resFOP-3DCI pellets. Red arrows show transplanted FOP-3DCI pellets. (B) Number of calcified samples in FOP- or resFOP-3DCI pellets with either PBS- or Activin-A-soaked Gelform®. Calcification of pellets was assessed by X-ray imaging.

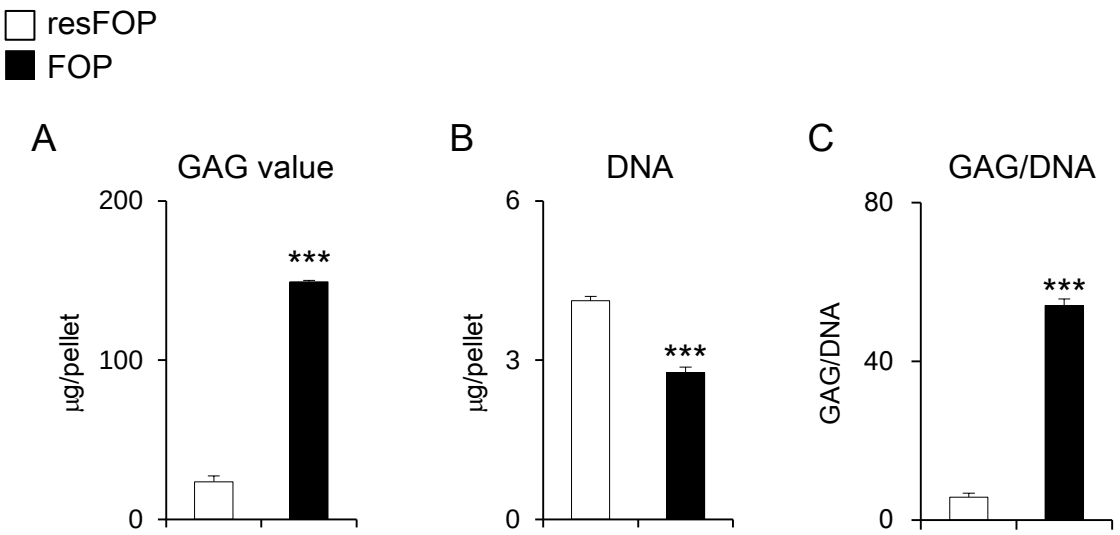


Fig. S15. GAG values (A) and GAG/DNA (C), but not DNA content (B) were enhanced in FOP-3DCI pellets cultured with Activin-A for 21 days. Results are the mean ± standard error (SE). N = 3. ***, P < 0.001 by Dunnett's multiple comparisons *t*-test compared to resFOP.

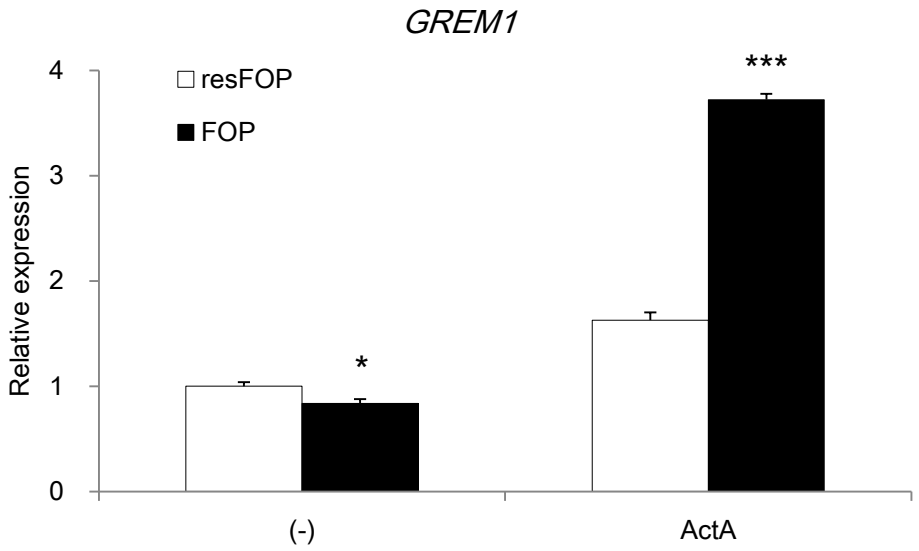


Fig. S16. Activin-A stimulation induced the expression of *GREM1* in FOP-iMSCs higher than in resFOP-iMSCs. FOP- and resFOP-iMSCs were stimulated with 100 ng/mL Activin-A. After 16 hours incubation, mRNA was extracted and qPCR analysis was performed. Results are the mean ± standard error (SE). N =3. *, $P < 0.05$; ***, $P < 0.001$ by Student's t-test compared to the value of resFOP treated with the same condition.

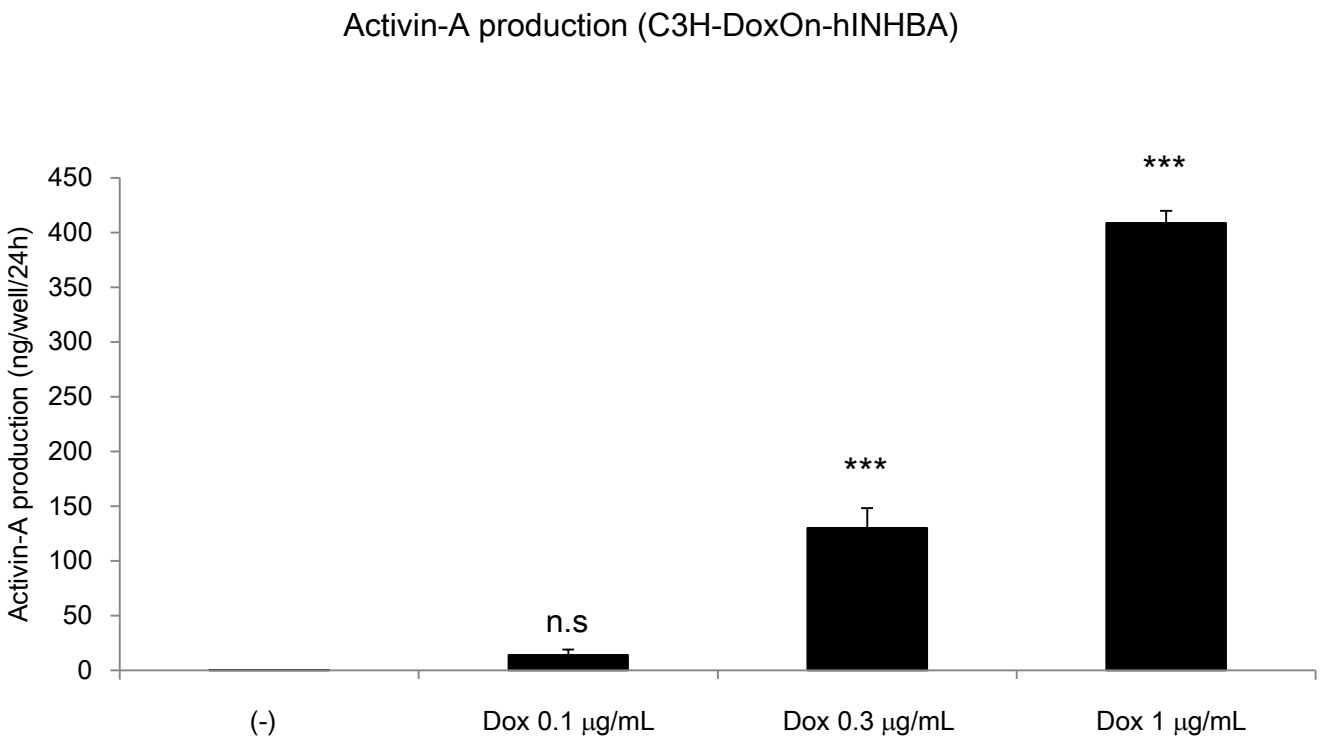


Fig. S17. Dox concentration-dependent Activin-A production by C3H-DoxOn-hINHBA cells. Cells (5x10^5 cells in 12-well plates) were stimulated with Dox for 24 hours, and cultured supernatants were collected. The amount of Activin-A was analyzed by ELISA. Results are the mean ± standard error (SE). N =3. n.s., no significant difference; ***, $P < 0.001$ by Dunnett's multiple comparisons *t*-test compared to Dox (-).

Table S1. siRNA sequences.

Gene	siRNA ID	Sense	Antisense
ACVRL1	s987	GGAUCAAGAAGACACUACAtt	UGUAGUGUCUUCUUGAUCCgc
ACVR1	s976	GUUGCUCUCCGAAAAUUUAtt	UAAAUUUUCGGAGAGCAACtc
BMPR1A	s282	GAAUCUGGAUAGUAUGCUUtt	AAGCAUACUAUCCAGAUUCtg
ACVR1B	s977	AGUACUUGAUGAAACCAUUtt	AAUGGUUUCAUCAAGUACUtc
TGFBR1	s229438	GGUCUGUGACUACAACAUAtt	UAUGUUGUAGUCACAGACCca
BMPR1B	s2041	GGACGAGAGCUUGAACAGAtt	UCUGUUCAAGCUCUCGUCCaa
ACVR2A	s981	GGAUGAUAUCAACUGCUAUtt	AUAGCAGUUGAUAUCAUCCag
ACVR2B	s985	GCUCCAACCUCGAAGUAGAtt	UCUACUUCGAGGUUGGAGCct
BMPR2	s2046	GGACAAUAUUAUGCUCGAAtt	UUCGAGCAUAAUAUUGUCCca
TGFBR2	s14078	GGAGAAAGAAUGACGAGAAtt	UUCUCGUCAUUCUUUCUCCat

Table S2. Antibodies for western blotting and immunostaining.

	Name	Company	Cat. No	Concentration
1st antibody	Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465) / Smad8 (Ser426/428) Antibody	Cell Signaling Technology	#9511L	1:1000
	Smad1/5/8 Antibody (N-18)-R	Santa Cruz Biotechnology	sc-6031-R	1:1000
	Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (D27F4) Rabbit mAb	Cell Signaling Technology	#8828	1:1000
	Smad2/3 (D7G7) XP® Rabbit mAb	Cell Signaling Technology	#8685	1:1000
	Anti-Nuclei Antibody, clone 235-1	EMD Millipore	MAB1281	1:30
	Collagen I Antibody	Novus Biologicals	NB600-408	1:100
2nd antibody	Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	#7074	1:100000
	Goat anti-Mouse IgG1 Secondary Antibody, Alexa Fluor® 568 conjugate	Invitrogen	A-21124	1:500
	Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen	A-11008	1:500

Table S3. Primers for RT-qPCR.

Name	Forward	Reverse
ACTB	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
ACAN	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
COL2	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT
SOX9	GACTTCCGCGACGTGGAC	GTTGGGCGGCAGGTACTG
NKX3-2	GCCGCTTCCAAAGACCTAGAG	CCAACACCGTCGTCCTCG
FGFR3	AGTACCTGGACCTGTCGGC	CCTCACATTGTTGGGGACCA
IHH	CGGTGGACATCACCACATCA	CGTGGGCCTTTGACTCGTAA
COMP	CAACTGTCCCCAGAAGAGCAA	TGGTAGCCAAAGATGAAGCCC
SP7	ATCCAGCCCCCTTTACAAGC	TAGCATAGCCTGAGGTGGGT
COL10A1	CCCAGCACGCAGAATCCATC	AGTGGGCCTTTTATGCCTGT
VEGFA	CAATCGAGACCCTGGTGGAC	TCTCTCCTATGTGCTGGCCT
RUNX2	TTACTTACACCCCGCCAGTC	TATGGAGTGCTGCTGGTCTG
MMP13	CATGAGTTCGGCCACTCCTT	CCTGGACCATAGAGAGACTGGA
ID1	CCAACGCGCCTCGCCGGATC	CTCCTCGCCAGTGCCTCAG
ID3	CTGGACGACATGAACCACTG	GTAGTCGATGACGCGCTGTA
GREM1	CTCCTTCTGCAAGCCCAAGA	CAACGACACTGCTTCACACG
ACVRL1	CACGGACTGCTTTGAGTCCT	TCTGCTGATCCACACACACC
ACVR1	GCGGTAATGAGGACCACTGT	CCCTGCTCATAAACCTG
BMPR1A	AGCTACGCCGGACAATAGAA	AATGAGCAAAACCAGCCATC
ACVR1B	TGCTCGAAGATGCAATTCTG	AACCTTTCGCATTTCCTCAA
TGFBR1	TGTAAAGTCATCACCTGGCCT	ATGGTGAATGACAGTGCGGT
BMPR1B	GTTCCCTTTATGATTATCTGAAGTCC	TTAGACACTCATCACTGCTCCAC
ACVR1C	GGTCGACAGTGCTCCTACAG	TCTTCCATGCCACACCTCAC
ACVR2A	ACACAGCCCACTTCAAATCC	AGGAGGGTSGGCCATCTTGT
ACVR2B	GATCTTCCCACTCCAGGACA	CTCGGCAGCAATGAACTGTA
BMPR2	GCTGTTGGGACCAGGATG	TTGCGTTCATTCTGCATAGC
TGFBR2	AGACGTTGACTGAGTGCTGG	TTAGGGAGCCGTCTTCAGGA
AMHR2	GGCCTATGAGGCAGAACTGG	CTCTTGAGGATGGGCCAAGG