

SUPPLEMENTAL FIGURE AND TABLE LEGENDS

Supplemental Figure 1. Schematic and detailed results of 1st screening. (A and B)

Schematic of the cell preparation and HTS assay (5xA-Luc assay). First, FOP-5xA-Luc-iPSCs were induced to iNCCs and expanded, and frozen stocks were produced. Next, iMSCs were induced, and transiently expanded (A). Finally, luciferase assay was performed 4 d after chondrogenic induction with Activin-A and compounds (B). (C and D) Accuracy of the HTS as assessed by Zormed 4 d (C) and S/B ratio (D). Detailed information is described in Methods.

Supplemental Figure 2. Dose-dependent assay results of 76 hit compounds. 5xA-Luc

assay was performed using same protocol as HTS. Results are the mean of n = 2. 10, 100 and 1000 nM of test compounds were assessed. Listed compounds show IC₅₀ < 1 μM. 100 ng/mL Activin-A was used to accelerate chondrogenesis.

Supplemental Figure 3. AlamarBlue assay results of 76 hit compounds. AlamarBlue

assay was performed to remove cytotoxic compounds. Results are the mean of n = 2. 10, 100 and 1000 nM of test compounds were assessed. Listed compounds show inhibition < 20% at any dose in the same culture condition of 5xA-Luc assay.

Supplemental Figure 4. mTOR inhibitors decreased expression of chondrogenic

markers. (A and B) qPCR analysis of chondrogenic markers in 2D (A) and 3D (B) chondrogenesis of FOP-iMSCs stimulated with Activin-A for 7 d or 21 d, respectively. Results are the mean ± standard error (SE). n = 3. n.s., no significant difference; *, P < 0.05; **, P < 0.01; ***, P < 0.001 by Dunnett' u multiple comparisons *t*-test compared to

the DMSO treatment control with Activin-A. E, 100 nM everolimus; R, 100 nM rapamycin; T, 100 nM temsirolimus treatment groups.

Supplemental Figure 5. Rapamycin suppressed HO in FOP-ACVR1 conditional transgenic mice triggered by Cardiotoxin. (A) Genotyping PCR of FOP-ACVR1 (R206H) transgenic mice. *Coll1a1::FOP-ACVR1* allele (551bp) and WT allele (331bp) or *Rosa26::M2rtTA* allele (340bp) and WT allele (650bp) are shown. Heterozygous *Rosa26::M2rtTA* mice with heterozygous *Coll1a1::FOP-ACVR1* allele were used to induce the *FOP-ACVR1* gene. (B-E) Muscle injury by Cardiotoxin injection and oral administration of Dox induced HO, which was suppressed by intraperitoneal administration of 5 mg/kg rapamycin (once daily, five times a week). Four weeks after Cardiotoxin injection and rapamycin administration, mice were analyzed. (B) X-rays (upper panels) and μ CT (lower panels) observations. (C) Average heterotopic bone volume. (D) Histological analysis of the Cardiotoxin-injected region. Haematoxylin and eosin (HE) staining, Safranin O staining (acidic proteoglycan), von Kossa staining (calcium) and anti-COL1 (bone) staining are shown. Scale bars, 10 mm (B) or 100 μ m (D). (E and F) Body weight of Cardiotoxin-injected (E) or Activin-A-injected (F) FOP-ACVR1 conditional transgenic mice. Results are the mean \pm SD of 11 transgenic mice. Staining are shown. Scale bars, (B-E), n = 11 (Vehicle or Rapa) (F). n.s., no significant difference; *, P < 0.05 by Student's *t*-test compared to vehicle treatment group. Representative of 2 independent experiments (Cardiotoxin experiment).

Supplemental Figure 6. mTOR inhibitors suppressed BMP-7-induced HO. BMP-7 was injected into the right gastrocnemius muscle of male C57BL/6 mice (6-8w), who were administered drugs once daily intraperitoneally (A and B) or once daily, 5 times a

week orally (**C** and **D**) for 2 weeks thereafter. (**A** and **C**) X-rays (upper panels) and μ CT (lower panels) observations. (**B** and **D**) Average heterotopic bone volume. Scale bars, 5 mm (**A**) or 10 mm (**C**). Results are the mean \pm standard error (SE). N = 5 (**A-D**). ***, P < 0.001 by Dunnett' u multiple comparisons *t*-test compared to the vehicle treatment group. Representative of 3 independent experiments (rapamycin experiments). Eve 5, everolimus 5 mg/kg; Rapa 5, rapamycin 5 mg/kg.

Supplemental Figure 7. Oral administration of rapamycin and delayed rapamycin treatment after transplantation also suppressed HO derived from FOP-iPSCs in vivo. (**A** and **B**) Oral administration of 3, 10 or 30 mg/kg rapamycin (once daily, five times a week) suppressed the HO derived from FOP-iMSCs triggered by Activin-A. Six weeks after transplantation and rapamycin administration, mice were analyzed. (**A**) X-rays (upper panels) and μ CT (lower panels) observations. (**B**) Average heterotopic bone volume. (**C** and **D**) Intraperitoneal administration of 5 mg/kg rapamycin (once daily, five times a week) was started at 0w, 2w and 4w after transplantation. Seven to eight weeks after transplantation, mice were analyzed. (**C**) X-rays (upper panels) and μ CT (lower panels) observations. (**D**) Average heterotopic bone volume. Scale bars, 10 mm (**A** and **C**). Results are the mean \pm standard error (SE). n = 4 (Vehicle, Rapa 10 or 30) or n=5 (Rapa 3) (**A** and **B**), n = 4 (Vehicle), n = 3 (4w- Rapa) or n = 5 (2w- or 0w- Rapa) (**C** and **D**). n.s., no significant difference; *, P < 0.05; ***, P < 0.001 by Dunnett' u multiple comparisons *t*-test compared to the vehicle treatment group.

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S6 (p-S6) was enhanced in FOP-iMSCs compared with resFOP-iMSCs in 2DCI assays triggered by Activin-A. The 2DCI assay was performed with or without Activin-A or 100 nM rapamycin, and 24 h after induction, the cells were harvested. The strength of the DMSO treated resFOP was set as one. Results are the mean \pm standard error (SE). n = 3. n.s., no significant difference; **, P < 0.01 by Student's *t*-test compared to resFOP treated with the same ligand/compound.

Supplemental Figure 9. mTOR signaling pathway related genes were significantly changed in FOP-iMSCs compared with resFOP-iMSCs stimulated by Activin-A , but not without stimulation or with BMP-7 or TGF- β 3 stimulation in the 2DCI assay. 24 h after 2D chondroinduction and stimulation, cells were harvested, and microarray analysis was performed. Using differentially expressed genes (1.5 fold change), mTOR signaling pathway genes (preset genes related to mTOR signaling by Ingenuity Pathway Analysis) were analyzed by Ingenuity Pathway Analysis. $-\log$ (p-value) > 1.3 (p = 0.05) was considered significant.

Supplemental Figure 10. ENPP2 inhibitors suppressed chondrogenesis of FOP-iMSCs stimulated by Activin-A. The 2DCI assay was performed with or without Activin-A or ENPP2 inhibitors. 7 days after induction and treatment, the cells were harvested, and GAG/DNA was quantified. Results are the mean \pm standard error (SE). n = 3 or n=6 (DMSO treatment control with Activin-A). n.s., no significant difference; **, P < 0.01; ***, P < 0.001 by Dunnett's multiple comparisons *t*-test compared to the DMSO treatment control with Activin-A.

Supplemental Figure 11. The expression of HIF1 α and related downstream genes was not up-regulated in FOP-iMSCs compared with resFOP-iMSCs during 2D chondrogenesis. 2D chondrogenic induction was performed in FOP- and resFOP-iMSCs stimulated with 100 ng/mL Activin-A, 100 ng/mL BMP-7 or 10 ng/mL TGF- β 3. After 6, 24, 48 hours or 7 days incubation, mRNA was extracted, and qPCR analysis (**A**) or microarray analysis (**B**) was performed. Results are the mean \pm SD. n = 3 (**A**) or n=1 (**B**). The expression level of resFOP (0 h) was set as one (**A**).

Supplemental Figure 12. Knock-down (KD) efficiencies of siRNA. FOP-iMSCs transiently transfected with siRNAs were incubated for 16 hours, and RNA was extracted. n = 1.

Supplemental Table 1. The number of mice harboring HO (> 20 mm³ bone volume) in FOP-ACVR1 conditional transgenic mice triggered by Cardiotoxin, related to Supplemental Figure 5.

Supplemental Table 2. The number of mice harboring HO (> 15 mm³ bone volume) in C57BL/6 triggered by BMP-7, related to Supplemental Figure 6, A and B. BMP-7 was injected into the right gastrocnemius muscle of male C57BL/6 mice (6-8w), who were administered drugs once daily intraperitoneally for 2 weeks thereafter.

Supplemental Table 3. The number of mice harboring HO (> 15 mm³ bone volume) in C57BL/6 triggered by BMP-7, related to Supplemental Figure 6, C and D. BMP-7

was injected into the right gastrocnemius muscle of male C57BL/6 mice (6-8w), who were administered drugs once daily, 5 times a week orally for 2 weeks thereafter.

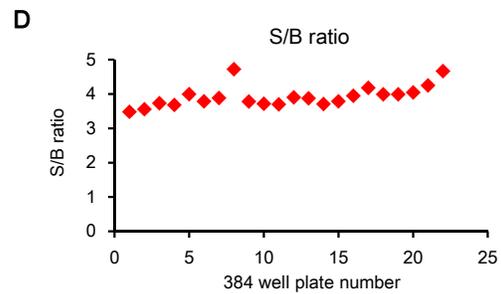
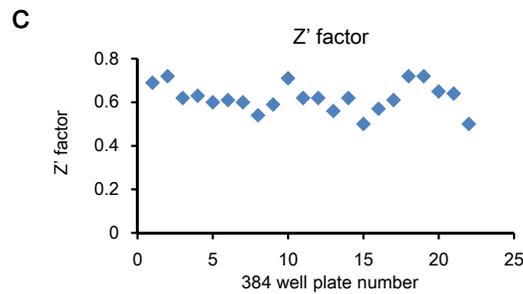
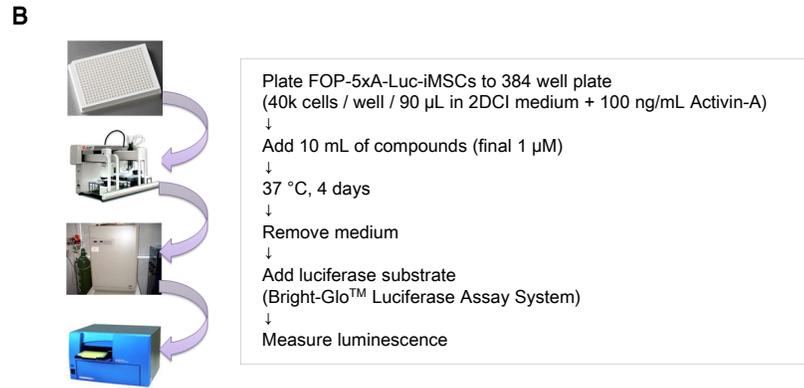
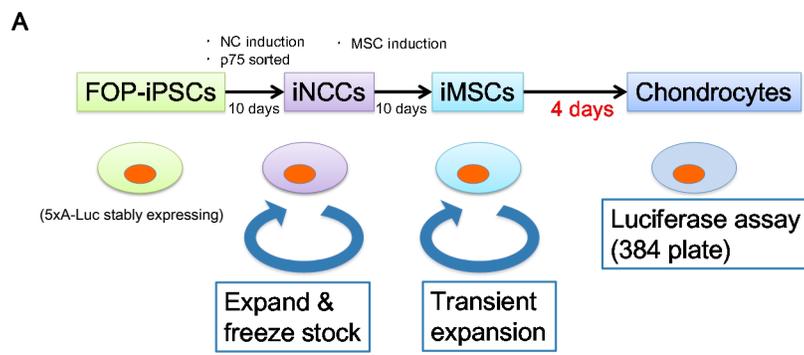
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Supplemental Table 5. The number of mice harboring (HO > 15 mm³ bone volume) derived from FOP-iPSCs in vivo, related to Supplemental Figure 7, C and D. Intraperitoneal administration of 5 mg/kg rapamycin (once daily, five times a week) was started at 0w, 2w and 4w after transplantation.

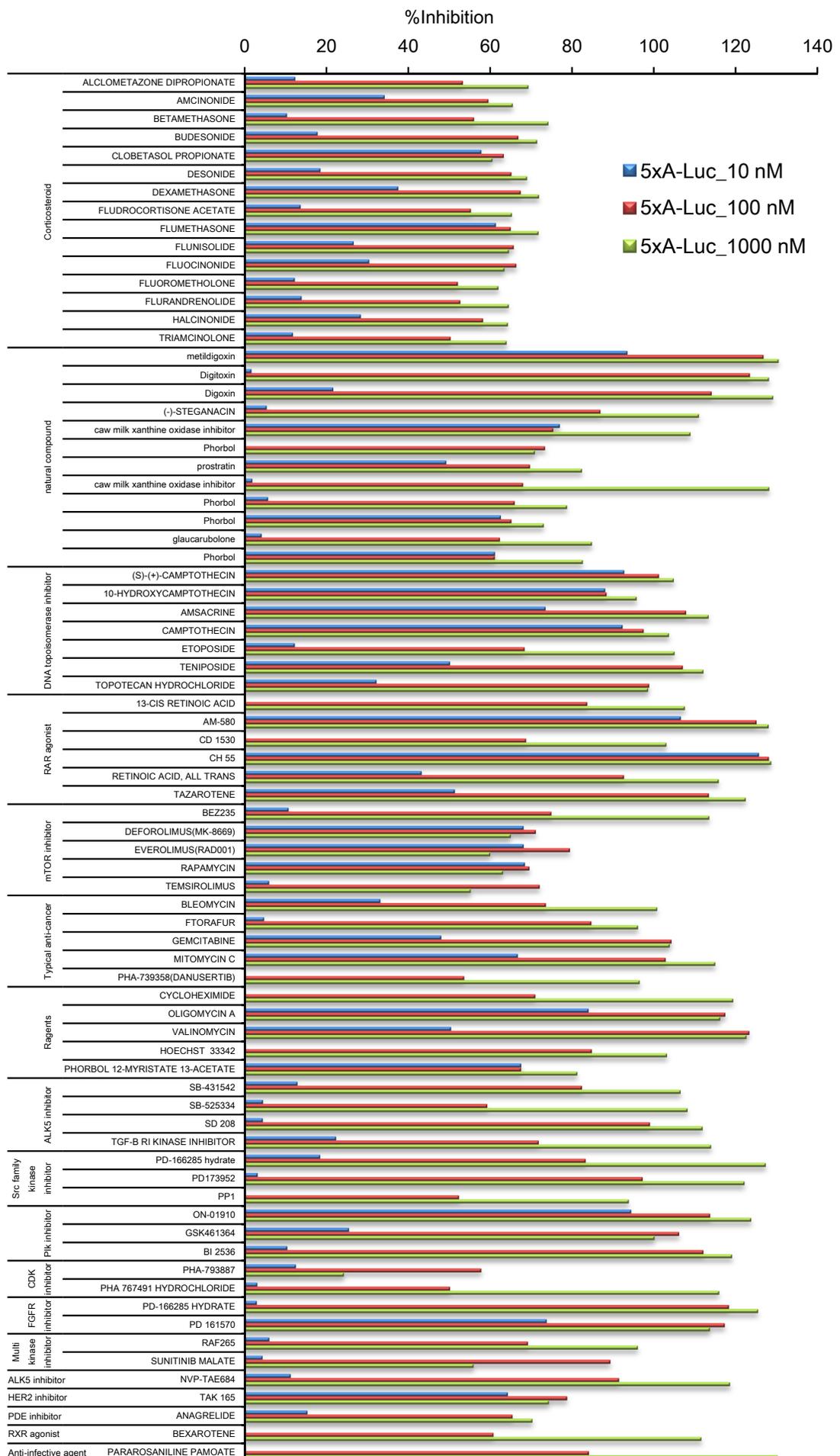
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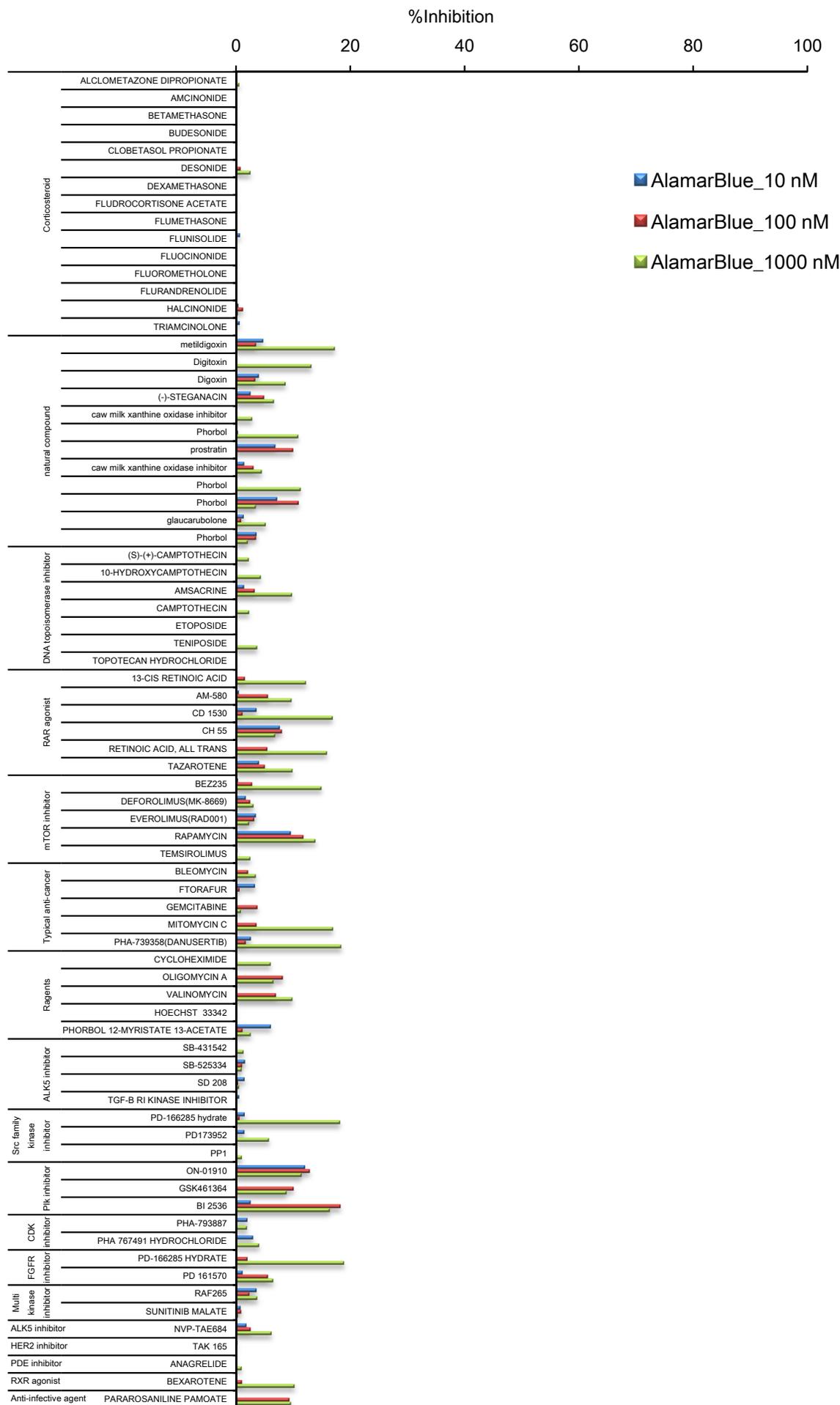
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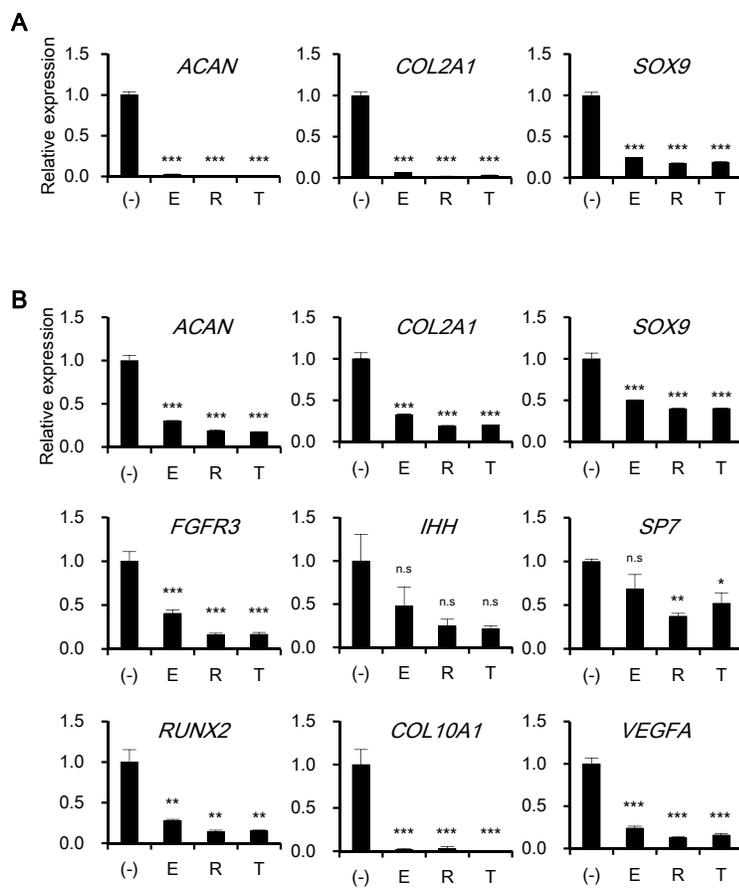
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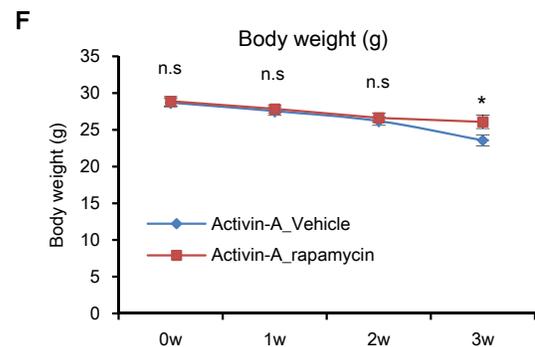
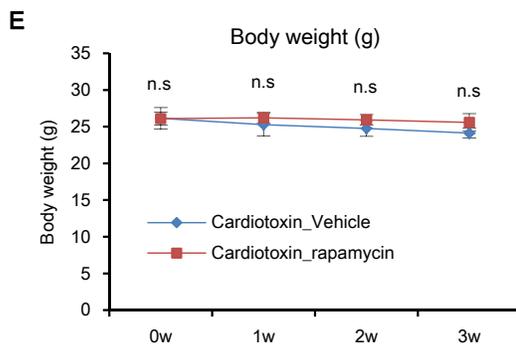
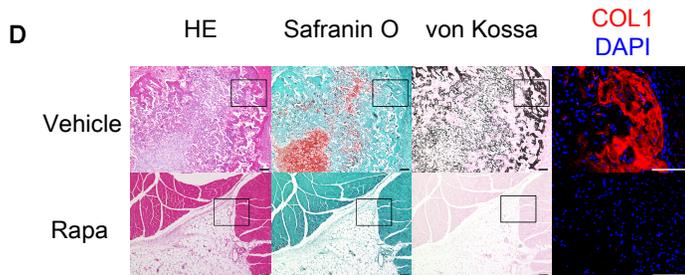
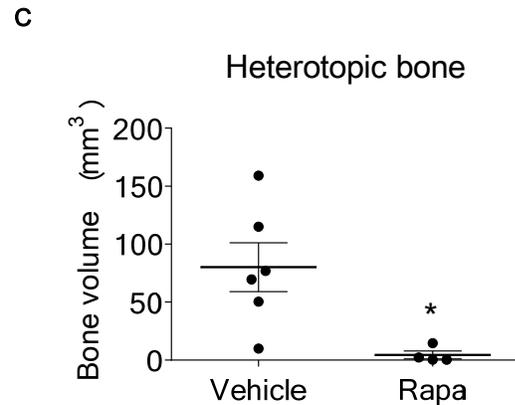
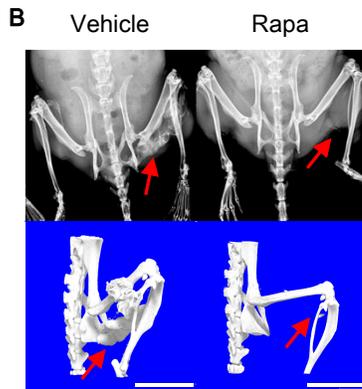
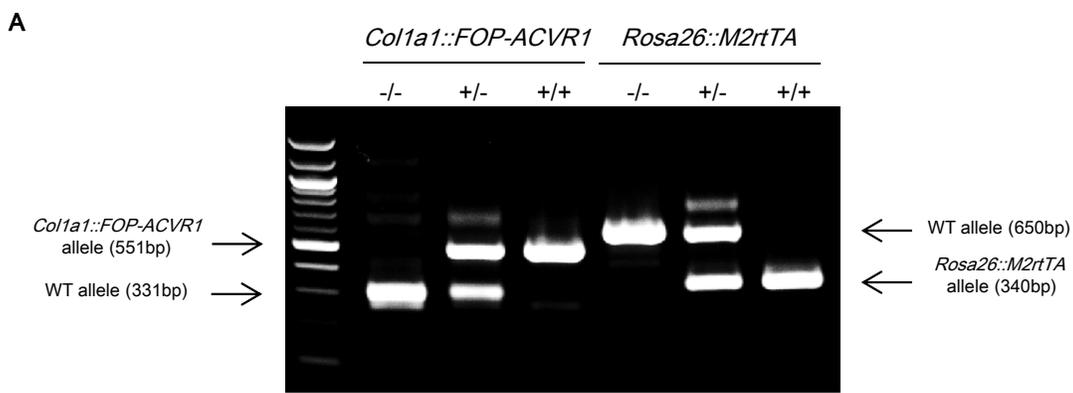
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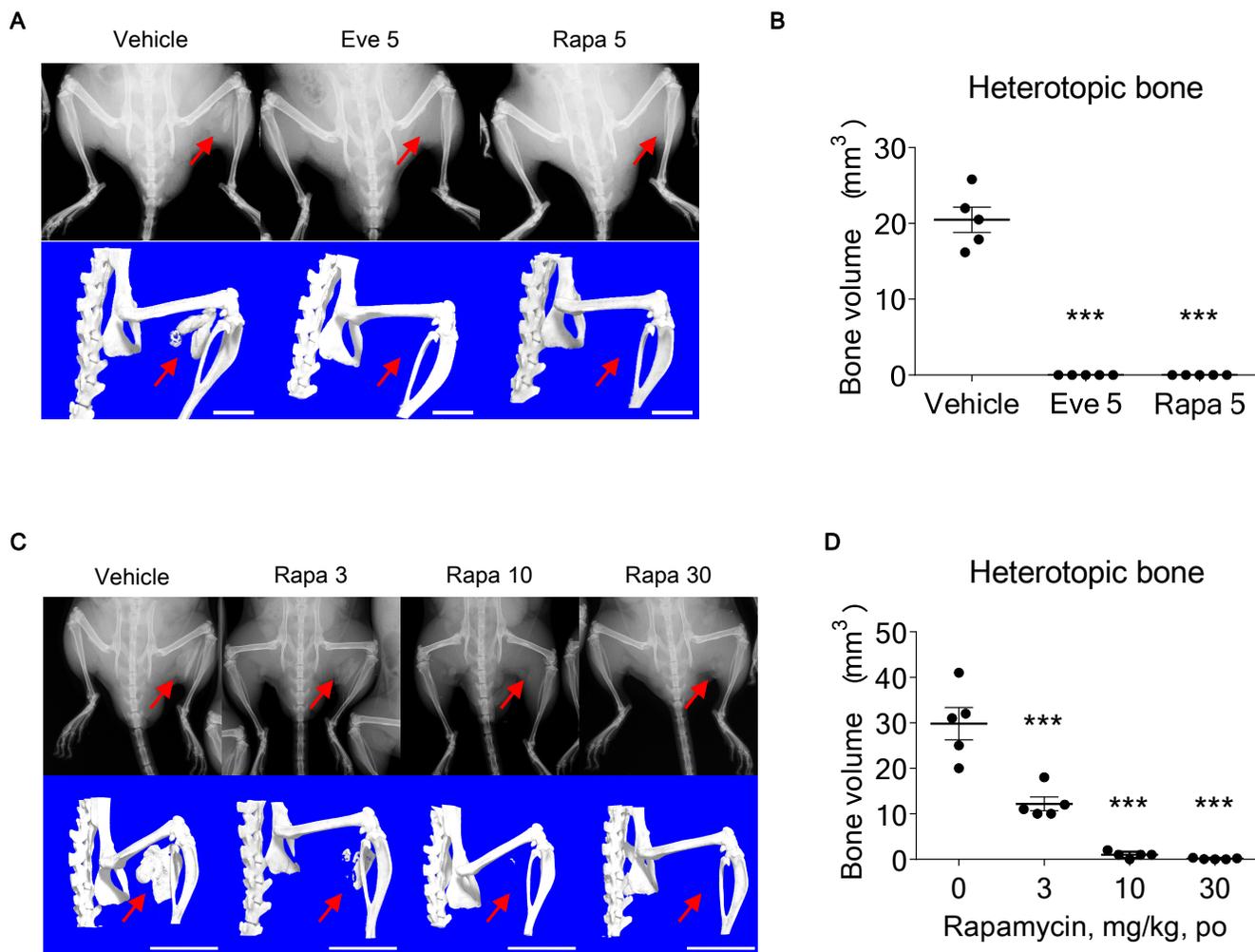
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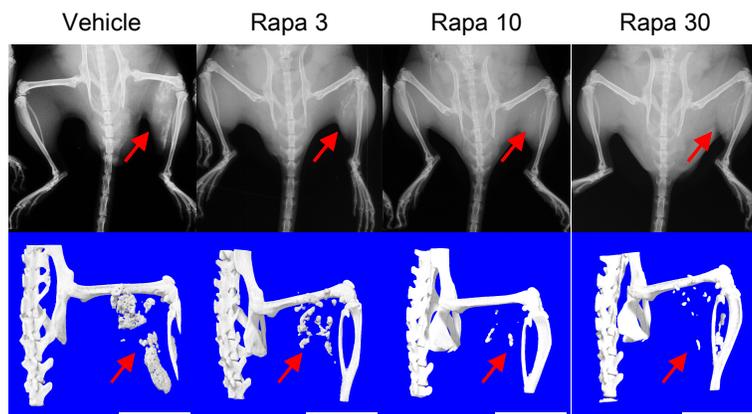


Supplemental Figure 5. Rapamycin suppressed HO in FOP-ACVR1 conditional transgenic mice triggered by Cardiotoxin. (A) Genotyping PCR of FOP-ACVR1 (R206H) transgenic mice. *Col1a1::FOP-ACVR1* allele (551bp) and WT allele (331bp) or *Rosa26::M2rtTA* allele (340bp) and WT allele (650bp) are shown. Heterozygous *Rosa26::M2rtTA* mice with heterozygous *Col1a1::FOP-ACVR1* allele were used to induce the *FOP-ACVR1* gene. (B-E) Muscle injury by Cardiotoxin injection and oral administration of Dox induced HO, which was suppressed by intraperitoneal administration of 5 mg/kg rapamycin (once daily, five times a week). Four weeks after Cardiotoxin injection and rapamycin administration, mice were analyzed. (B) X-rays (upper panels) and μ CT (lower panels) observations. (C) Average heterotopic bone volume. (D) Histological analysis of the Cardiotoxin-injected region. Haematoxylin and eosin (HE) staining, Safranin O staining (acidic proteoglycan), von Kossa staining (calcium) and anti-COL1 (bone) staining are shown. Scale bars, 10 mm (B) or 100 μ m (D). (E and F) Body weight of Cardiotoxin-injected (E) or Activin-A-injected (F) FOP-ACVR1 conditional transgenic mice. Results are the mean \pm standard error (SE). n = 6 (Vehicle) or 4 (Rapa) (B-E), n = 11 (Vehicle or Rapa) (F). n.s., no significant difference; *, P < 0.05 by Student's *t*-test compared to vehicle treatment group. Representative of 2 independent experiments (Cardiotoxin experiment).

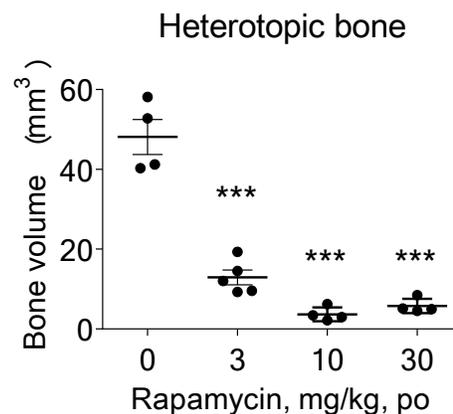


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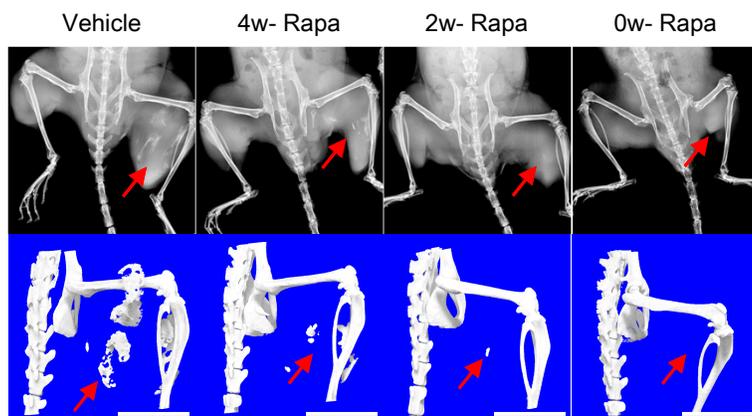
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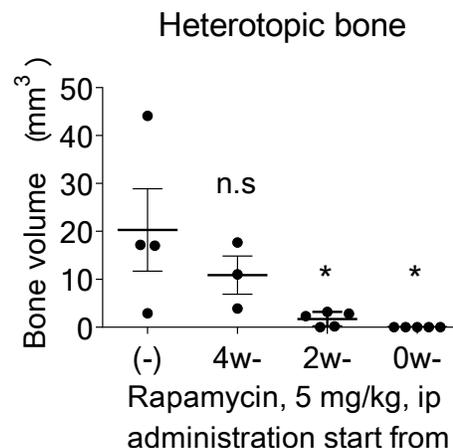
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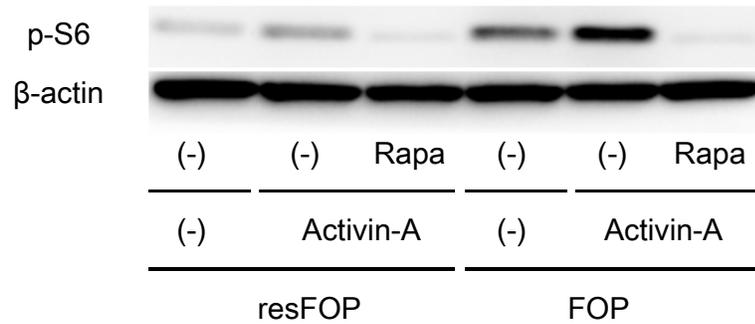
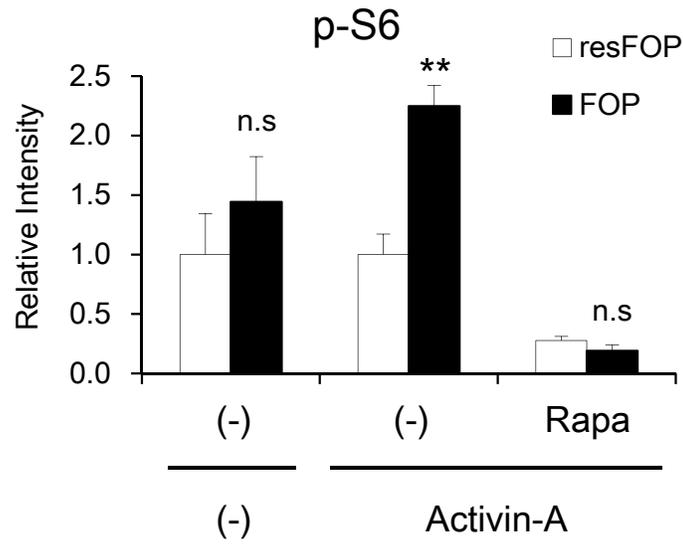
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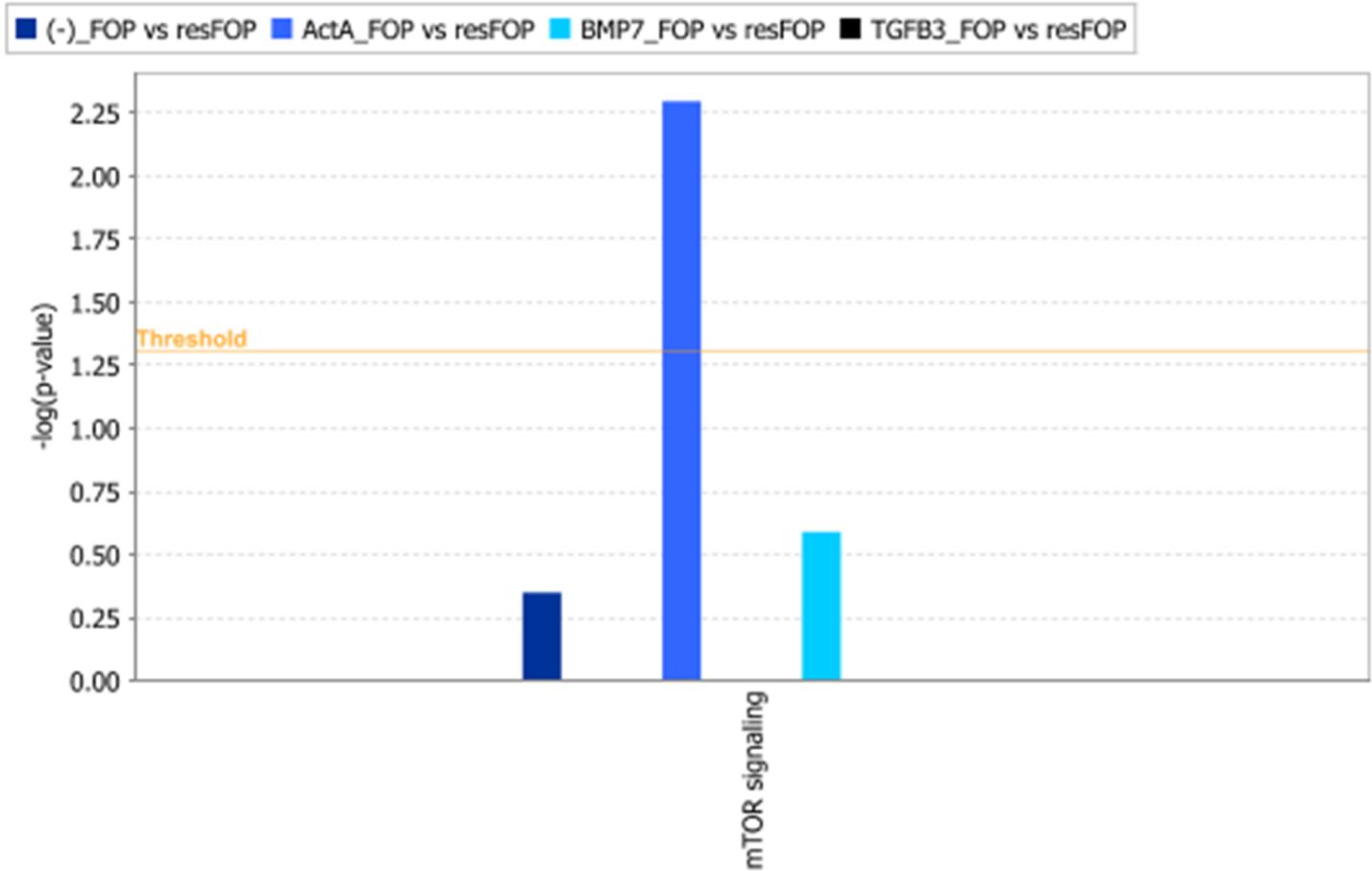
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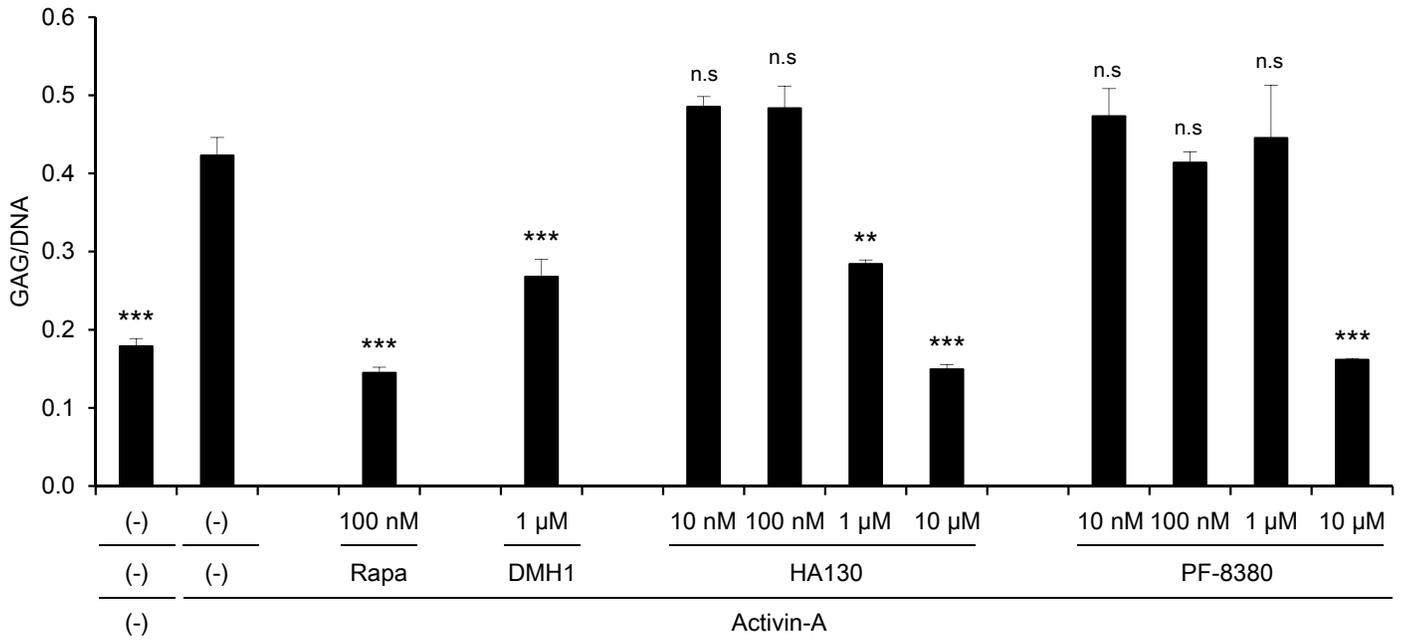


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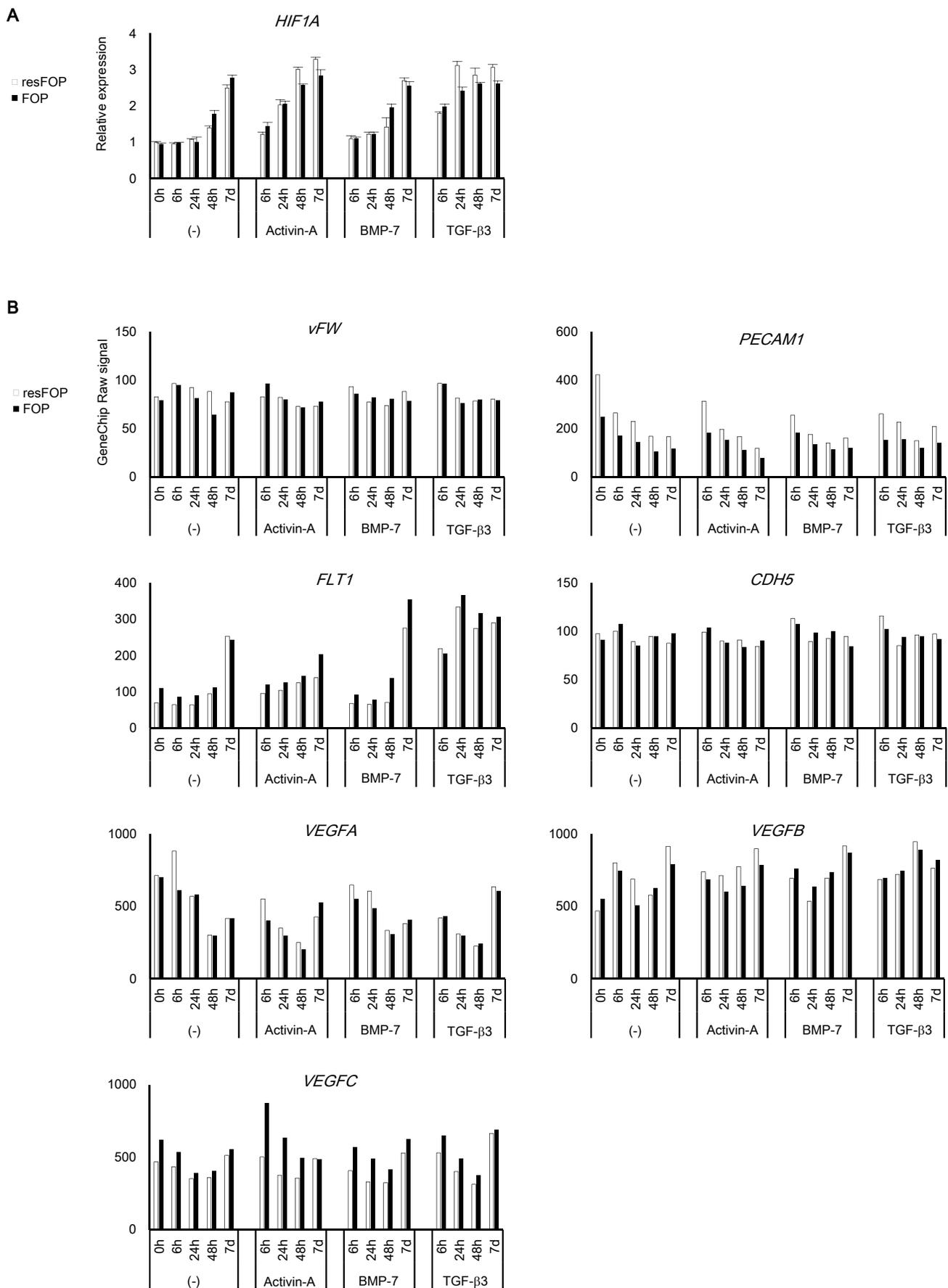


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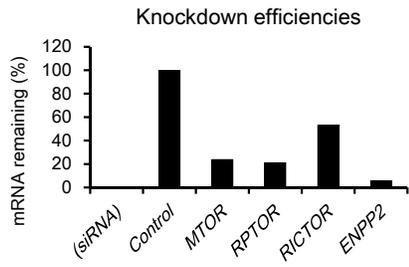
GAG/DNA



Supplemental Figure 10. ENPP2 inhibitors suppressed chondrogenesis of FOP-iMSCs stimulated by Activin-A. The 2DCI assay was performed with or without Activin-A or ENPP2 inhibitors. 7 days after induction and treatment, the cells were harvested, and GAG/DNA was quantified. Results are the mean \pm standard error (SE). $n = 3$ or $n=6$ (DMSO treatment control with Activin-A). n.s., no significant difference; **, $P < 0.01$; ***, $P < 0.001$ by Dunnett's multiple comparisons t -test compared to the DMSO treatment control with Activin-A.



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Supplemental Figure 12. Knock-down (KD) efficiencies of siRNA. FOP-iMSCs transiently transfected with siRNAs were incubated for 16 hours, and RNA was extracted. n = 1.

Supplemental Table 1. The number of mice harboring HO (> 20 mm³ bone volume) in FOP-ACVR1 conditional transgenic mice triggered by Cardiotoxin, related to Supplemental Figure 5.

Compound	# of HO (> 20 mm ³)
Vehicle	5/6
Rapa	0/4

Supplemental Table 2. The number of mice harboring HO (> 15 mm³ bone volume) in C57BL/6 triggered by BMP-7, related to Supplemental Figure 6, A and B. BMP-7 was injected into the right gastrocnemius muscle of male C57BL/6 mice (6-8w), who were administered drugs once daily intraperitoneally for 2 weeks thereafter.

Compound	# of HO (> 15 mm ³)
Vehicle	5/5
Eve 5	0/5
Rapa 5	0/5

Supplemental Table 3. The number of mice harboring HO (> 15 mm³ bone volume) in C57BL/6 triggered by BMP-7, related to Supplemental Figure 6, C and D. BMP-7 was injected into the right gastrocnemius muscle of male C57BL/6 mice (6-8w), who were administered drugs once daily, 5 times a week orally for 2 weeks thereafter.

Compound	# of HO (> 15 mm ³)
Vehicle	5/5
Rapa 3	1/5
Rapa 10	0/5
Rapa 30	0/5

Supplemental Table 4. The number of mice harboring HO (> 20 mm³ bone volume) derived from FOP-iPSCs in vivo, related to Supplemental Figure 7, A and B. Oral administration of 3, 10 or 30 mg/kg rapamycin (once daily, five times a week).

Compound	# of HO (> 20 mm ³)
Vehicle	4/4
Rapa 3	0/5
Rapa 10	0/4
Rapa 30	0/4

Supplemental Table 5. The number of mice harboring (HO > 15 mm³ bone volume) derived from FOP-iPSCs in vivo, related to Supplemental Figure 7, C and D. Intraperitoneal administration of 5 mg/kg rapamycin (once daily, five times a week) was started at 0w, 2w and 4w after transplantation.

Compound	# of HO (> 15 mm ³)
Vehicle	3/4
4w- Rapa	1/3
2w- Rapa	0/5
0w- Rapa	0/5

Supplemental Table 6. siRNA sequences, related to Supplemental Figure 12.

Gene	siRNA ID	Sense	Antisense
<i>MTOR</i>	s604	GGAGCCUUGUUGAUCCUAtt	UAAGGAUCAACAAGGCUCat
<i>RPTOR</i>	s33215	GCAUUGGACUUGCUUGGAAtt	UCCAAGCAAGUCCAUGCtc
<i>RICTOR</i>	s48409	CACUUACUACUACCGGAAtt	UCCGGUAAGUAGUAAGUGct
<i>ENPP2</i>	s10267	CCAACUCACUACACAGCAAtt	UGCUGUAGUAGUGAGUUGGaa

Supplemental Table 7. Primers for RT-qPCR.

Gene	Forward	Reverse
<i>ACTB</i>	CACCATTGGCAATGAGCGGTTT	AGGTCTTTGCGGATGTCCACGT
<i>ACAN</i>	TCGAGGACAGCGAGGCC	TCGAGGGGTAGCGGTAGAGA
<i>COL2</i>	GGCAATAGCAGGTTACGTACA	CGATAACAGTCTTGCCCCACTT
<i>SOX9</i>	GACTTCCGCGACGTGGAC	GTTGGGCGGCAGGTA
<i>FGFR3</i>	AGTACCTGGACCTGTCGGC	CCTCACATTGTTGGGGACCA
<i>COL10A1</i>	CCCAGCACGCAGAATCCATC	AGTGGGCCTTTTATGCCTGT
<i>VEGFA</i>	CAATCGAGACCCTGGTGGAC	TCTCTCCTATGTGCTGGCCT
<i>MTOR</i>	GACGAGAGATCATCCGCCAG	ACAAGGGACCGACCATAAG
<i>RPTOR</i>	CGGCTGACCTATTCACCTCC	CAGGCACCAGACTGACACAT
<i>RICTOR</i>	GGGGTGTCTCAAGAAGGCTC	GCGAAGGAGTATACGGCACA
<i>ENPP2</i>	CCAACCATGCCAGAGGAAGT	ATCATCACAAGTGCAGCCCA
<i>HIF1A</i>	TGTAATGCTCCCTCACCCA	TGCAGGGTCAGCACTACTTC

Supplemental Table 8. Antibodies for western blotting and immunostaining.

	Name	Company	Cat. No	Concentration
1st antibody	Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465) / Smad9 (Ser465/467) (D5B10) Rabbit mAb	Cell Signaling Technology	#13820	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
	Phospho-S6 Ribosomal Protein (Ser235/236) Antibody	Cell Signaling Technology	#2211	1:1000
	Monoclonal Anti-b-Actin-Peroxidase clone AC-15	SIGMA-ALDRICH	A3854	1:200000
	Anti-human Vimentin antibody	Abcam	ab16700	1:40
	Collagen I Antibody	Novus Biologicals	NB600-408	1:100
2nd antibody	Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	#7074	1:10000
	Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen	A-11008	1:100
	Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 555 conjugate	Invitrogen	A-21428	1:500