

# Combined rapamycin and mesenchymal stem/stromal cells derived from induced pluripotent stem cells-mediated delivery of ACVR2B-Fc fusion protein reduces heterotopic ossification in a mouse model of fibrodysplasia ossificans progressiva

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## Abstract

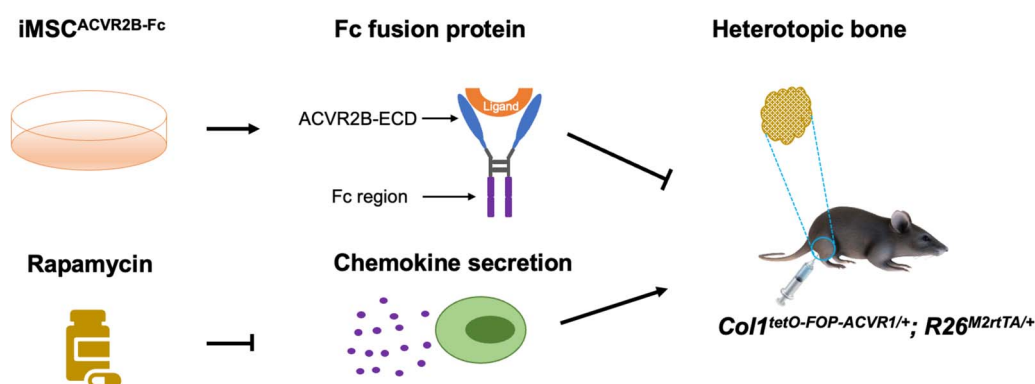
Fibrodysplasia ossificans progressiva (FOP) is a genetic disease characterized by extraskeletal heterotopic ossification (HO). The underlying mechanism is the aberrant activation of bone morphogenetic protein (BMP) signaling caused by a point mutation in the *ACVR1* gene. Although FOP is an ultra-rare disease, its symptoms severely impair patients' daily activities and quality of life. Furthermore, the HO is also observed in broader clinical contexts, including major surgeries and trauma, highlighting the significance of its study. We have previously reported that rapamycin suppressed Activin-A-triggered progression of cartilage formation in FOP mice. We recently found that the ACVR2B-Fc fusion protein produced by mesenchymal stem/stromal cells derived from induced pluripotent stem cells (iMSCs) attenuates BMP signaling activated by Activin-A and BMP-9 in FOP patient-derived iMSCs. Transplantation of ACVR2B-Fc-producing iMSCs (iMSC<sup>2B-Fc/Luci</sup>) reduces primary HO in FOP mice. In this study, we found that intraperitoneal administration of rapamycin reduced primary HO in a dose-dependent manner in FOP-ACVR1<sup>R206H</sup> transgenic mice. A low concentration of rapamycin (0.3 mg/kg, 5 times/wk) efficiently suppressed chondrogenesis. The combination of iMSC<sup>2B-Fc/Luci</sup> transplantation with low concentration of rapamycin significantly reduced primary and recurrent HO. Rapamycin improved cell survival in transplanted iMSC<sup>2B-Fc/Luci</sup> cells by attenuating chemokine secretion, likely resulting in improved ACVR2B-Fc fusion protein production. Our results suggest that combining stem cell therapy and rapamycin can reduce primary HO and surgery-induced HO.

**Keywords:** fibrodysplasia ossificans progressiva, heterotopic ossification, mesenchymal stem/stromal cells, ACVR2B-Fc fusion protein, rapamycin

## Lay Summary

Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disorder characterized by progressive formation of extraskeletal bone in muscles, tendons, ligaments, and other connective tissues, namely heterotopic ossification (HO). This study investigated combining therapeutics to reduce primary and surgery-induced HO in the mouse model of FOP. Through immunosuppression with low concentration of rapamycin, the cell survival and function of transplanted iMSC<sup>ACVR2B-Fc</sup> were improved, reducing primary and recurrent HO more efficiently. Therefore, this study suggests a promising approach for treating FOP through stem cell therapy.

## Graphical Abstract



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## Introduction

Fibrodysplasia ossificans progressiva (FOP) is caused by a gain-of-function mutation in the glycine- and serine-rich (GS) domain of the *ACVR1* gene, which results in extraskeletal heterotopic ossification (HO).<sup>1</sup> FOP flare-ups arise spontaneously or are triggered by soft tissue injuries caused by accidental trauma, muscular stretching, fatigue, influenza-like illness, intramuscular vaccination, biopsies, and surgeries.<sup>2</sup> Thus, surgical resection of HO lesions in patients with FOP is prohibited as the resulting injury causes local and systematic aggravation of HO. Drug candidates of FOP can be classified into three categories based on their blocking sites: (1) extracellular antibodies or receptobodies targeting activins, bone morphogenetic proteins (BMPs), or the extracellular domain of *ACVR1*; (2) intracellular kinase inhibitors targeting the kinase domain of *ACVR1*; and (3) intracellular agonists or inhibitors targeting downstream effectors.

As a molecular inhibitor of downstream, rapamycin reportedly suppressed trauma-induced and genetic (*ACVR1*<sup>Q207D</sup>) de novo HO by inhibiting *Hif1a*.<sup>3</sup> The same group also found that rapamycin reduces fibrosis and mesenchymal condensation at the muscle injury site, which results in heterotopic osseous lesions.<sup>4</sup> We also identified the mammalian target of rapamycin (mTOR) signaling as a crucial pathway for the abnormal chondrogenesis of mesenchymal stromal/stem cells derived from FOP-patient induced pluripotent stem cells (FOP-iMSCs) using high-throughput screening.<sup>5</sup> Oxidative phosphorylation is a downstream mediator in mTORC1-induced chondrogenesis.<sup>6</sup> Oral administration of rapamycin (3, 10, and 30 mg/kg; 5 times/wk) dose-dependently suppresses BMP-7-induced HO in C57BL/6 mice and FOP-iMSCs-derived HO in NOD/SCID mice, wherein 10 mg/kg of rapamycin inhibits > 95% of HO compared with the control.<sup>5</sup> Intraperitoneal administration of rapamycin (5.0 mg/kg, 5 times/wk) suppresses primary HO and surgery-induced HO in FOP mice.<sup>7</sup> When converting the above-mentioned effective dose for mouse to human using the equation of human equivalent dose (HED) (mg/kg) = animal no observed adverse effect levels (NOAEL) (mg/kg) × (Weight<sub>animal</sub> [kg]/Weight<sub>human</sub> [kg])<sup>(1-0.67)</sup> or HED (mg/kg) = Animal dose (mg/kg) × (Animal *K<sub>m</sub>*/Human *K<sub>m</sub>*),<sup>8</sup> the effective rapamycin dose for human will be as much as 10 times higher than the maintenance dose for prophylaxis of renal transplant rejection (PO, 2 mg per day)<sup>9</sup> and the current clinical trial to reduce FOP-HO (PO, 1.0-4.0 mg per day, UMIN000028429). Thus, it is necessary to determine the minimal effective concentration of rapamycin required to suppress HO in a mouse model.

Besides, extracellular recombinant antibody-like *ACVR2A*-Fc and *ACVR2B*-Fc profoundly inhibit Activin-A-dependent BMP signaling and chondrogenesis in vitro<sup>10</sup> and prevent HO formation in vivo.<sup>11</sup> However, commercial *ACVR2A*-Fc and *ACVR2B*-Fc are extremely expensive. We have recently reported that iMSC-mediated delivery of the *ACVR2B*-Fc fusion protein reduced primary HO in FOP mice, suggesting a novel approach for treating FOP through stem cell therapy.<sup>12</sup> However, short-term survival after iMSC<sup>*ACVR2B*-Fc</sup> transplantation and the limited reduction of recurrent HO necessitate optimization of the iMSC<sup>*ACVR2B*-Fc</sup> delivery strategy. In this study, we investigated the immunomodulatory effects of rapamycin beyond its ability to suppress HO, enhancing the performance of transplanted iMSC<sup>*ACVR2B*-Fc</sup> in reducing primary and recurrent HO.

## Materials and methods

### Mouse model and in-vivo imaging

Animal procedures were approved by the Institutional Animal Committee of Kyoto University (#16-73, #22-154, #22-155, and #23-218). The FOP-*ACVR1*<sup>R206H</sup> transgenic mouse model has been described<sup>12</sup> with minor modifications. Sample size of mice was calculated based on the reported formula.<sup>13</sup> Four to five female mice aged 16-21 wk were randomly allocated into each group using a random-number table. Rapamycin (MedChemExpress, dissolved in 10% DMSO with 0.5% w/v methylcellulose 400 solution) at gradient concentrations (0, 0.1, 0.3, 0.6, 2.5, and 5.0 mg/kg; 5 times/wk) was intraperitoneally administered as the injury of gastrocnemius muscle was induced by cardiotoxin (CTX, 9.1 µg/mouse, MedChemExpress). Three injections of  $1.5 \times 10^6$  cells in 100 µL Alpha Modification of Eagle's Medium (αMEM) were locally administered every 6-7 d using a 27G syringe in the primary HO model and two injections for the recurrent HO model. X-ray and µCT imaging and calculation of the volume of heterotopic bone were performed as previously described. A 13-wk-old NOD-SCID IL2Rγ<sup>null</sup> (NSG) mice (Jackson Laboratory) was used for transplanting cells (iMSC<sup>EiP/Luci</sup>,  $3 \times 10^6$ ) through the right gastrocnemius muscle, IP, and tail vein injection. Luminescent signals were detected using an in-vivo imaging system (PerkinElmer). The mice that died before the endpoint of the experiments were excluded from the analysis.

### Cell culture

The induction and maintenance of iPSC-derived neural crest cells (iNCCs) and MSCs (iMSCs) were described previously.<sup>14</sup> The generation of iMSC<sup>2B-Fc/Luci</sup> and iMSC<sup>EiP/Luci</sup> cells were performed as previously described.<sup>12</sup> Briefly, 5 µg of pPV-EF1a-Luciferase-BleoR and 1 µg of PBase plasmids were respectively mixed with 5 µg of pPB-CAG-*ACVR2B*-Fc-His-Puro or 5 µg of pPV-EF1a-EGFP-IRES-Puro. A total of 100 µL of the DNA and iMSC mixture was obtained. After electroporation using a NEPA21 electroporator (Nepagene, Japan), stably expressing cells were screened using 1 µg/mL puromycin (Gibco) with 400 µg/mL zeocin (InvivoGen) for 7 d.

### Rotarod and treadmill test

Rotarod and treadmill tests were referred previously.<sup>12</sup> Briefly, mice were loaded onto a rotarod station (ENV-574M, Med Associates) and Rodent Treadmill (Ugo Basile SRL). The rotation speed was set from 2 to 20 rpm and maintained at 20 rpm until the mice fell. The riding times were recorded. The treadmill movement test (+5° inclination) started at a 5 m/min velocity, gradually increasing to a maximum of 20 m/min (shock: 1 Hz, shock intensity 0.2 mA). Exhaustion was defined as the mice being shocked 15 times or not returning to the treadmill and staying on the shock grid for 5 s. Running distances were recorded.

### ELISA

Mouse blood samples were left at room temperature (RT, 20-25 °C) for 30 min until clot formation. The serum was harvested after centrifugation for 15 min at 2000 × g. Activin-A and Fc fragment in serum were detected using Human/Mouse/Rat Activin-A Quantikine ELISA Kit (DAC00B, R&D Systems) and Human Fcγ (Fc Fragment of IgG) ELISA Kit

**Table 1.** Immunohistochemical antibodies.

Name	Company	Cat. No.	Concentration
Collagen I alpha 1 antibody (COL-1)	Novus Biologicals	NB600-450	1:100
Anti-human vimentin antibody	Abcam	ab16700	1:100
Donkey anti-rabbit IgG (H + L), Alexa Fluor 488	Invitrogen	A21206	1:100
Goat anti-mouse IgG (H + L), Alexa Fluor 555	Invitrogen	A21422	1:100

(E-EL-H1745, Elabscience). The absorbances at 450 nm were read using an EnVision Multilabel Reader (PerkinElmer).

### Cytokine array

The serum (150  $\mu$ L) of each mouse was then subjected to cytokine array analysis (Mouse Cytokine Array Panel A, R&D Systems, ARY006) according to the manufacturer's instructions. Spot pixel density was quantified using the Protein Array Analyzer of ImageJ software (<https://imagej.net/ij/>).

### Histochemical and IHC analyses

Fixed and decalcified samples (4% paraformaldehyde for 24 hr, 12% EDTA for 10 d) were embedded in paraffin, sectioned, deparaffinized and stained with H&E and Alcian blue. For IHC staining, antigen retrieval was performed via incubation with Liberate Antibody Binding Solution (Plysciences, USA) for 20 min at RT. Non-specific antigens were blocked using Blocking One (Nacalai Tesque) for 1 hr at RT. For antibodies generated from the mouse host, tissues were blocked with ReadyProbes Mouse on Mouse IgG Blocking Solution (Invitrogen) for 1 hr at RT. Antibodies (diluted in Can Get Signal Immunostain Solution B, TOYOBO) were incubated overnight at 4 °C. After rinsing in PBST buffer, samples were incubated with Alexa Fluor secondary antibodies diluted in Can Get Signal Immunostain Solution B for 1 hr at RT. A mounting medium with DAPI (Vector Laboratories) was used to counterstain the nuclei. Images were captured using a BZ-X810 microscopy (Keyence). The antibodies for IHC are listed in Table 1.

### Statistical analysis

The statistical significance of all experiments was calculated using one-way analysis of variance with Dunnett's or Tukey's multiple comparison test and two-way analysis of variance with Tukey's multiple comparisons test, as indicated in each figure legend. All statistical analyses were performed using GraphPad Prism version 9.5.1 (GraphPad Software). A *p*-value <.05 were considered statistically significant. Significance levels are: \**p* <.05; \*\**p* <.01; \*\*\**p* <.001; \*\*\*\**p* <.0001.

## Results

### Intraperitoneally administered rapamycin reduced primary HO in a dose-dependent manner

Rapamycin (0, 0.1, 0.3, 0.6, 2.5, and 5.0 mg/kg; i.p., 5 times/wk) was administered on the same day that the muscle injury was triggered by CTX injection (Figure 1A). The body weights of the mice in most of the groups remained relatively stable throughout the experiments (Figure 1B). At the end of the experiment, mice were subjected to X-ray and  $\mu$ CT scans under general anesthesia (Figure 1C). Quantitative bone volume analysis revealed that rapamycin reduced primary HO levels in a dose-dependent manner. Rapamycin (0.3 mg/kg)

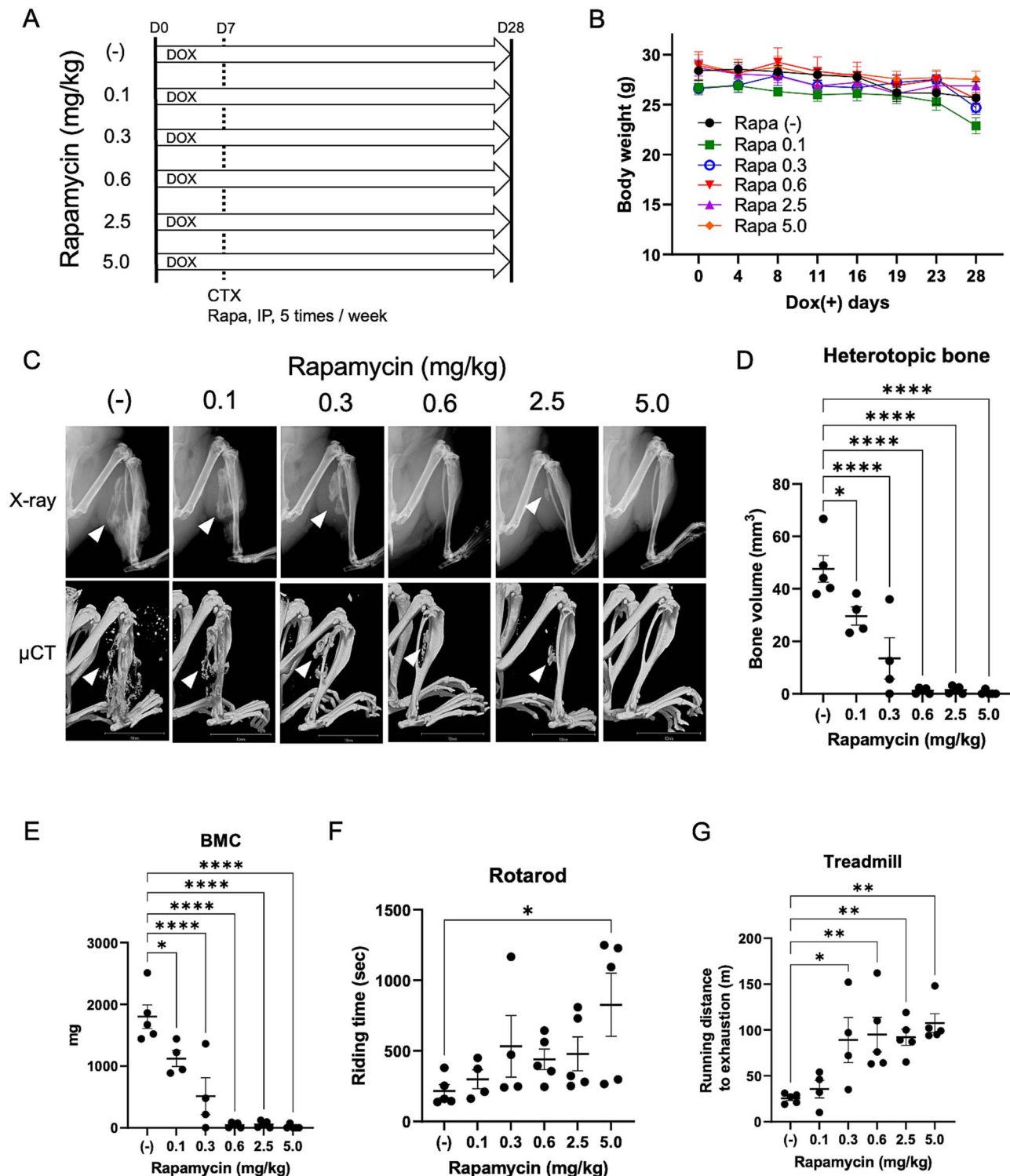
inhibited HO by ~60% (Figure 1D). The bone mineral content (BMC) of HO was consistent with the bone volume (Figure 1E). Rapamycin also dose-dependently improved the rotarod (Figure 1F) and treadmill (Figure 1G) performance. Positive staining for Alcian blue (cartilage) and COL1 (bone) indicated the occurrence of endochondral ossification (Figure S1).

### Low-dose rapamycin enhanced the effects of iMSC ACVR2B-Fc to reduced primary HO

Luciferase-expressing iMSC<sup>EiP/Luci</sup> and iMSC<sup>2B-Fc/Luci</sup> cells were generated as previously described.<sup>12</sup> In-vivo imaging was performed in the NSG and FOP mice to determine the survival time of the transplanted cells under immunodeficient and immunocompetent conditions. iMSC<sup>EiP/Luci</sup> cells ( $3 \times 10^6$ ) resuspended in 100  $\mu$ L aMEM medium were transplanted into NSG mice through the right gastrocnemius muscle, IP, and tail vein injection. Only a weak luciferase signal was detected in the systemically delivered (tail vein) mouse on day 1 after transplantation (Figure S2A). The luciferase signal rapidly decreased and was undetectable on day 7. As muscle injury was triggered by CTX, rapamycin (0.3 mg/kg, 5 times/wk) was administered intraperitoneally on day 7. iMSC<sup>EiP/Luci</sup> or iMSC<sup>2B-Fc/Luci</sup> cells ( $1.5 \times 10^6$ ) were locally injected into the injured muscle from day 8 (Figure 2A). The luciferase signals in the mice treated with rapamycin decreased more slowly over time than those in the control and were detectable for at least 5-6 d, although the differences were not statistically significant (Figure S2B). Thus, three injections of  $1.5 \times 10^6$  cells were locally administered every week (Figure 2A). The weights of the mice in all groups were comparable and slightly decreased over time (Figure 2B). The X-ray and  $\mu$ CT scans showed that low rapamycin concentrations combined with iMSC<sup>2B-Fc/Luci</sup> significantly suppressed primary HO, wherein no HO was formed in two out of four mice (Figure 2C-E). This combination improved the rotarod and treadmill performance to some extent, although no statistical difference was observed (Figure 2F and G). No significant difference was found in serum Activin-A levels among the groups (Figure 2H). Rapamycin increased the secretion of Fc fusion proteins in the serum (Figure 2I), suggesting that it increased the survival or function of transplanted iMSC<sup>2B-Fc/Luci</sup> cells. H&E staining of the whole section showed that the combined administration decreased the area of the HO lesions (Figure S3). The transplanted iMSCs were marked by vimentin, which did not co-stain with the mouse-derived COL1<sup>+</sup> bone tissue, suggesting that the transplanted iMSCs did not differentiate into bone tissue.

### Low-dose rapamycin enhanced the effects of iMSC 2B-Fc/Luci to reduced recurrent HO

On day 21, as many HO lesions as possible were surgically resected under general anesthesia (Figure 3A). Rapamycin (0.3 mg/kg, 5 times/wk) was intraperitoneally administered.

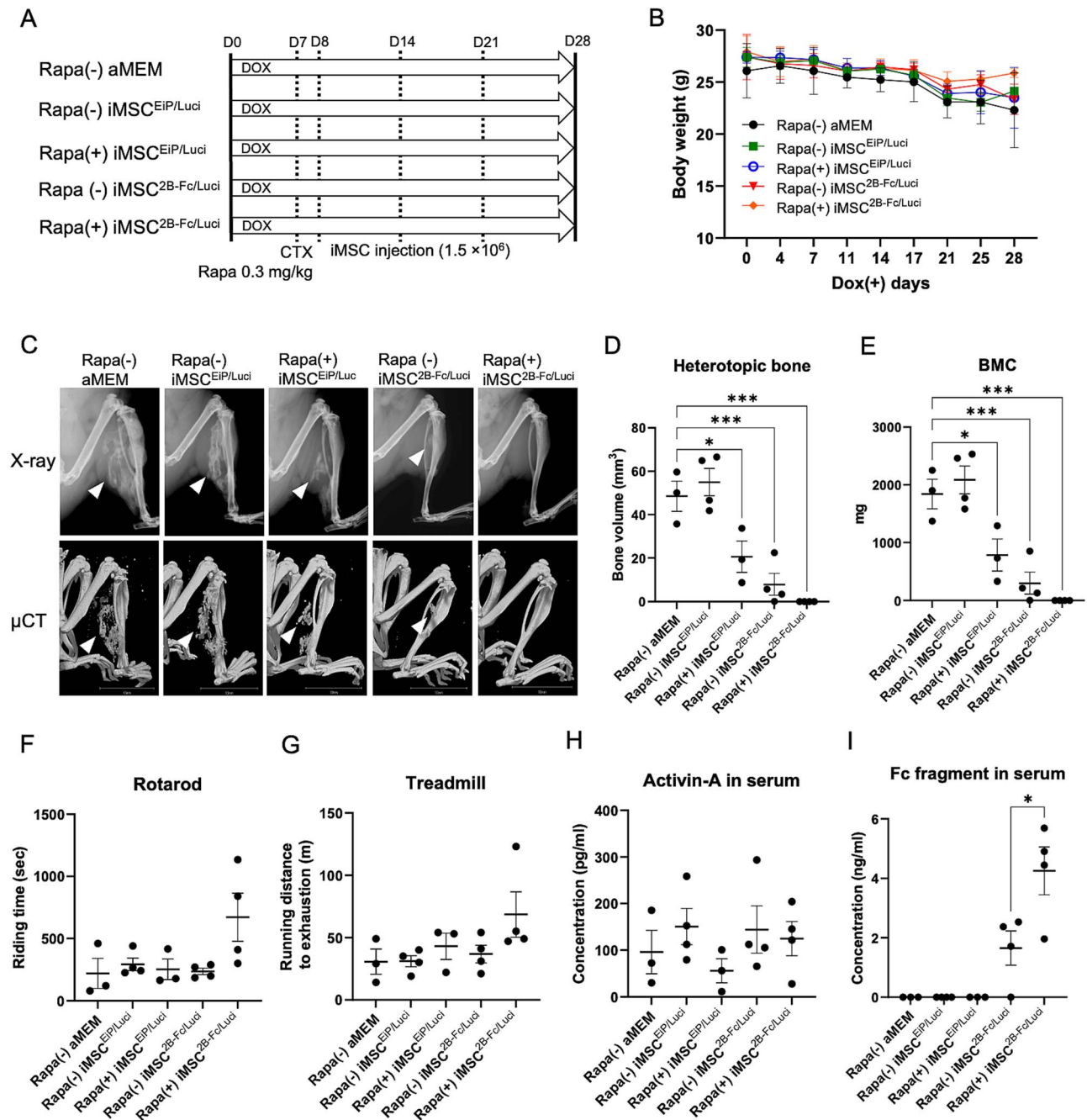


**Figure 1.** Intraperitoneally administered rapamycin reduces primary HO in a dose-dependent manner. (A) Schematic view of the experiments. Female mice aged 16 wk were administrated with drinking water supplemented with 2 mg/mL DOX and 10 mg/mL sucrose to initiate the FOP-ACVR1<sup>R206H</sup> expression. CTX (9.1  $\mu$ g/mouse) was injected in gastrocnemius on day 7. Rapamycin with indicated concentrations (0, 0.1, 0.3, 0.6, 2.5, 5.0 mg/kg) was intraperitoneally administrated from day 7 (5 times/wk). (B) The body weight of mice was monitored every 3-5 d. (C) Representative X-ray and  $\mu$ CT images from each group. Arrowheads indicate HO. Scale bar, 10 mm. (D) Average of HO volume on day 28. (E) BMC of HO lesions. (F) The exercise capacities were analyzed using the rotarod test. (G) Endurance tests were evaluated using a rodent treadmill. Results represent the mean  $\pm$  SEM. \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*\*,  $p < .0001$ . Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test. Abbreviations: HO, heterotopic ossification; DOX, doxycycline hyclate; CTX, cardiotoxin; BMC, bone mineral content.

iMSC<sup>EiP/Luci</sup> or iMSC<sup>2B-Fc/Luci</sup> cells ( $1.5 \times 10^6$ ) were locally transplanted every week from day 22. The weights of mice in all groups decreased after surgery but were comparable with time (Figure 3B). X-ray and  $\mu$ CT scans were performed

on days 21 (pre- and post-resection) and 35, respectively (Figure 3C). The volume of recurrent HO was the volume on day 35 subtracted from the remaining post-resection volume on day 21. Rapamycin in combination with iMSC<sup>2B-Fc/Luci</sup>



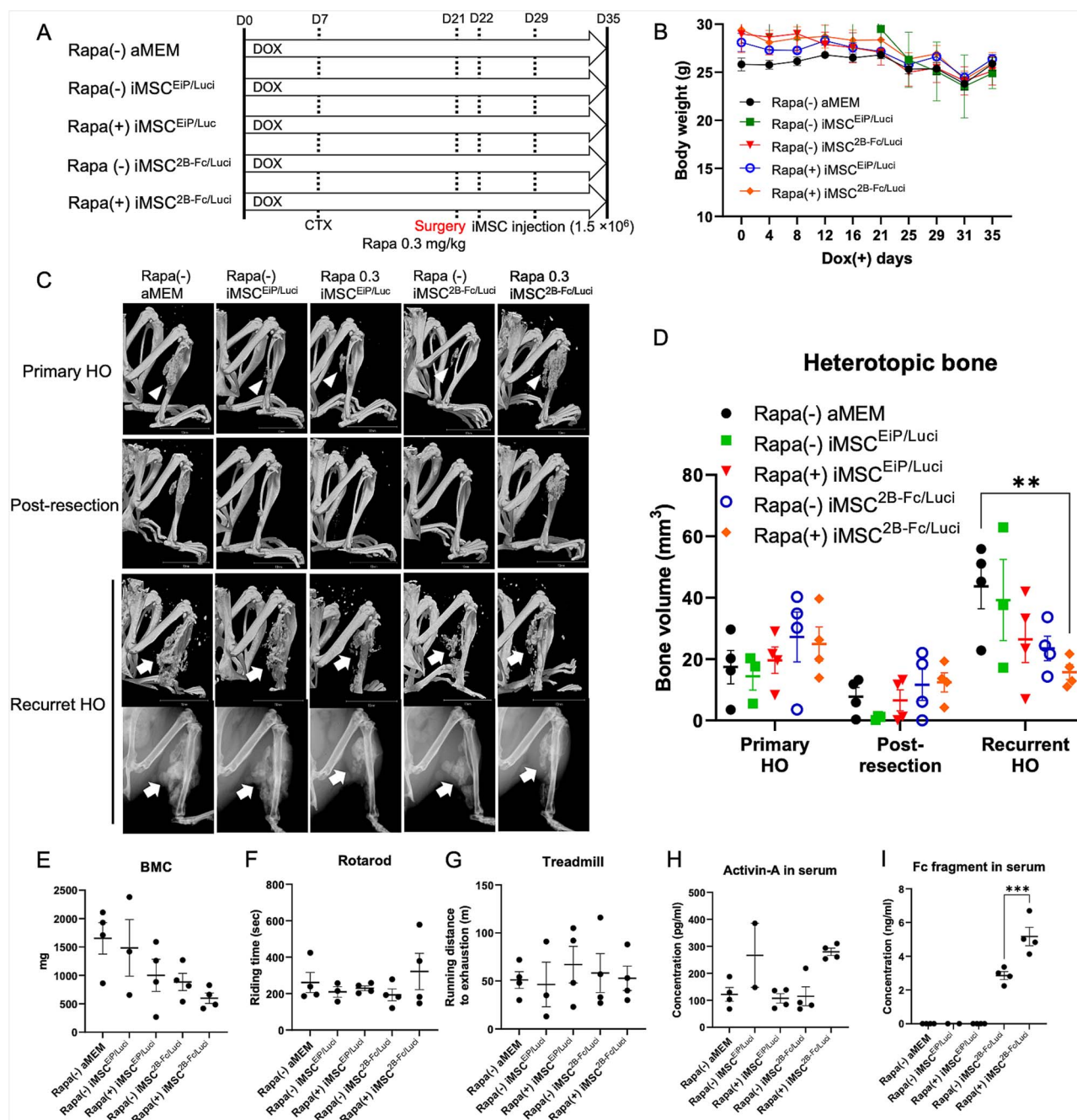


**Figure 2.** Low-dose rapamycin enhanced the effects of iMSC<sup>2B-Fc/Luci</sup> to reduce primary HO. (A) Schematic view of the experiments. Rapamycin (0.3 mg/kg, 5 times/wk) was intraperitoneally administrated after CTX injected on day 7. Cells in 100  $\mu$ L aMEM (iMSC<sup>EiP/Luci</sup> or iMSC<sup>2B-Fc/Luci</sup>,  $1.5 \times 10^6$ ) were locally administered every 7 d from day 8. (B) The body weight of mice was monitored every 3-5 d. (C) Representative X-ray and  $\mu$ CT images from each group. Arrowheads indicate HO. Scale bar, 10 mm. (D) Average of HO volume on day 28. (E) BMC of HO lesions. (F) The exercise capacities were analyzed using the rotarod test. (G) Endurance tests were evaluated using a rodent treadmill. (H, I) Activin-A and Fc-fragment in mouse serum were detected by ELISA. Results represent the mean  $\pm$  SEM. \*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ . Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test. Data in (I) were analyzed using one-way ANOVA with Tukey's multiple comparison test. Abbreviations: HO, heterotopic ossification; iMSC, mesenchymal stem/stromal cells derived from induced pluripotent stem cells; CTX, cardiotoxin; aMEM, Alpha Modification of Eagle's Medium; BMC, bone mineral content.

significantly reduced the recurrent HO (Figure 3D) and BMC (Figure 3E). No statistical differences were observed in rotarod and treadmill performance (Figure 3F and G) or Activin-A levels in mouse serum (Figure 3H). Increased secretion of the Fc fusion protein into the serum was also confirmed in the combination group (Figure 3I). Histological and immunohistochemical (IHC) analyses of HO lesions were similar to those described above (Figure S4).

### Rapamycin attenuated cytokine secretion initiated by iMSC transplantation

Cytokine array assays were conducted to examine cytokine secretion in FOP mice under different treatments. In the rapamycin dose-dependent model, the low concentration of rapamycin (0.3 mg/kg) was likely to reduce cytokines, including chemokines CCL2, CXCL1, CXCL9, and CXCL10, and pro-inflammatory IL-16 (Figure 4A and B). In the



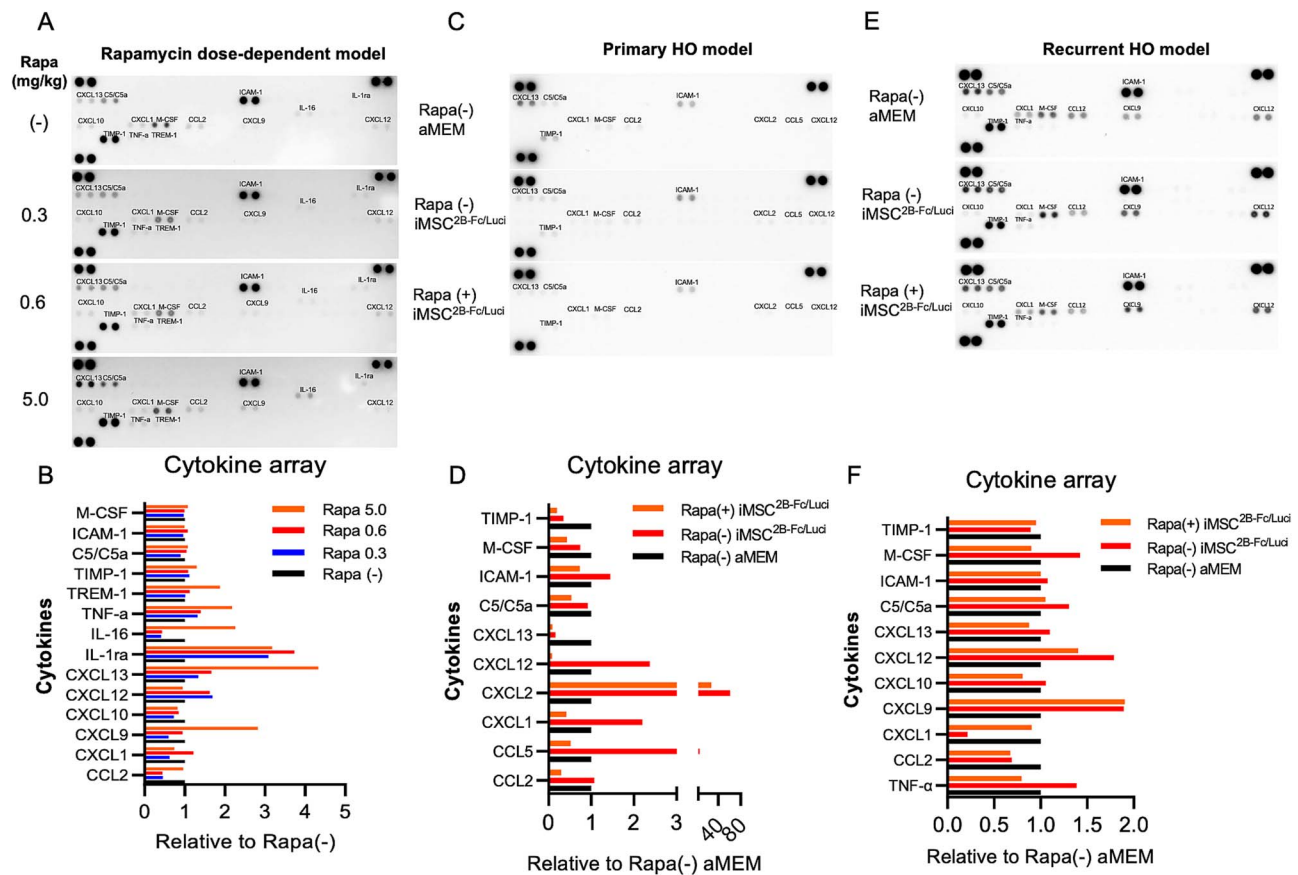
**Figure 3.** Low-dose rapamycin enhanced the effects of iMSC<sup>2B-Fc/Luc1</sup> to reduce recurrent HO. (A) Schematic view of the experiments. Rapamycin (0.3 mg/kg, 5 times/wk) was intraperitoneally administered after HO was surgically removed on day 21. Cells in 100  $\mu$ L aMEM (iMSC<sup>EIP/Luc1</sup> or iMSC<sup>2B-Fc/Luc1</sup>, 1.5  $\times$  10<sup>6</sup>) were locally administered every 7 d from day 22. (B) The body weight of mice was monitored every 3-5 d. (C) Representative X-ray and  $\mu$ CT images from each group. Arrowheads indicate primary HO, and arrows indicate recurrent HO. Scale bar, 10 mm. (D) Average of HO volume on day 21 (pre- and post-surgery) and recurrent HO (volume<sub>day35</sub> – volume<sub>post-surgery</sub>). (E) BMC of HO lesions. (F, G) The exercise capacity and endurance were evaluated using the rodent rotarod and treadmill tests, respectively. (H, I) Activin-A and Fc-fragment in mouse serum were detected by ELISA. Results represent the mean  $\pm$  SEM. \*\*,  $p$  < .01; \*\*\*,  $p$  < .001. Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test. Data in (D) were analyzed using two-way ANOVA with Tukey's multiple comparison test. Data in (I) were analyzed using one-way ANOVA with Tukey's multiple comparison test. Abbreviations: HO, heterotopic ossification; iMSC, mesenchymal stem/stromal cells derived from induced pluripotent stem cells.

primary HO model, rapamycin (0.3 mg/kg) was likely to decrease the increase in cytokines triggered by iMSC<sup>2B-Fc/Luc1</sup> transplantation, including the chemokines CCL2, CCL5, CXCL1, CXCL2, and CXCL12, and the soluble cell adhesion molecule ICAM-1 (Figure 4C and D). In the recurrent HO model, rapamycin decreased the enhancement of cytokines triggered by iMSC<sup>2B-Fc/Luc1</sup> transplantation, including pro-inflammatory TNF- $\alpha$ , chemokines CXCL10, CXCL12, and

CXCL13, anaphylatoxin C5/C5a, soluble cell adhesion molecule ICAM-1, and hematopoietic growth factor M-CSF (Figure 4E and F).

## Discussion

We recently developed xeno-free iMSCs derived from induced pluripotent stem cells (iPSCs), which resemble the



**Figure 4.** Rapamycin attenuated secretion of cytokines initiated by iMSC transplantation. (A) The mouse serum in the rapamycin dose-dependent model (0, 0.3, 0.6, 5.0 mg/kg) was subjected to cytokine array assay. The spots on the lower right corner of the membrane (invisible) represent negative control. The spots on the other three corners indicate positive control. (B) The pixel density of the targets was normalized to the pixel density of the positive control on the upper left corner of the membrane. Quantifications are relative to the group of rapamycin (-). (C, E) Mouse serum from the indicated groups in both primary (C) and recurrent (E) HO models was subjected to cytokine array assay. (D, F) Quantifications are relative to the group of rapamycin (-) and aMEM. Abbreviations: HO, heterotopic ossification; iMSC, mesenchymal stem/stromal cells derived from induced pluripotent stem cells.

tissue-derived MSCs and promote skeletal muscle regeneration via secretion of soluble factors.<sup>14</sup> Although the muscle injury was induced via CTX injection or surgery in the FOP, the transplantation of control iMSCs (iMSC<sup>EiP/Luci</sup>) did not inhibit primary or recurrent HO (Figures 2D and 3D). In contrast, transplantation of ACVR2B-Fc-producing iMSCs (iMSC<sup>2B-Fc/Luci</sup>) reduced primary HO and recurrent HO, suggesting that the aberrant activation of BMP signaling caused by the FOP-ACVR1 mutant is hypersensitive and rapidly responsive to injury, which cannot be mitigated by transplanted iMSC<sup>EiP/Luci</sup>.

MSCs express low levels of major histocompatibility complexes (MHC) and activate natural killer (NK) cell receptors on their surfaces upon interaction with immune cells.<sup>15</sup> However, interferon-gamma is well-documented to enhance MHC class I levels and triggers the expression of MHC class II in MSCs, potentially increasing their immunogenicity and leading to a detrimental effect on allograft survival.<sup>16,17</sup> Thus, MSCs are regarded as immune evasive rather than immune privileged.<sup>18</sup> Consequently, MSCs are susceptible to be attacked by cytotoxic CD8<sup>+</sup> T cells and NK cells.<sup>19</sup> Thus, multiple transplantations of MSCs must be conducted, which increases costs and hampers clinical application.<sup>20</sup> Priming MSCs with inflammatory cytokines typically results in the temporary upregulation of different immunosuppressive molecules and improves the survival of

transplanted cell grafts.<sup>21–23</sup> Additionally, the combination of rapamycin (an immunosuppressant) and MSCs fostered immunotolerance in animal models of pancreatic islet<sup>24</sup> and heart transplantation.<sup>25</sup>

In our previous study, the host immune rejection and a surgery-induced cytokine storm attenuated the survival and capability of xenogenic iMSCs to produce the ACVR2B-Fc fusion protein.<sup>12</sup> Thus, we hypothesized that rapamycin benefits the transplantation of iMSC<sup>2B-Fc/Luci</sup> by prolonging survival duration, reducing management frequency, and inhibiting HO efficiently. Even in the immunodeficient NSG mice, most transplanted iMSCs survived for under 7 d (Figure S2A). Rapamycin administration (0.3 mg/kg) was likely to extend the survival time of transplanted iMSCs to most 6 d (Figure S2B). Rapamycin (0.3 mg/kg) combined with iMSC<sup>ACVR2B-Fc</sup> significantly reduced primary (Figure 2D) and recurrent (Figure 3D) HO. Combined administration also improved rotarod and treadmill performances in the primary HO model, although the differences were not statistically significant (Figure 2F and G). In the recurrent HO, the HO volume data and the rotarod and treadmill performance data did not show consistent trends (Figure 3D–G), unlike in primary HO (Figure 2D–G), which showed more consistent trends. This may be due to the fact that in the FOP mice, to remove the primary HO lesion, a long muscle damage was formed very close to the knee joint, and therefore the recurrent



HO often formed near the knee, resulting in ankylosis of the joint in all groups. Given that symptom relief and functional recovery are critical for patients, further research is needed to find ways to prevent re-ossification, especially near joints. Notably, rapamycin increased the secretion of the ACVR2B-Fc fusion protein in the serum (Figures 2I and 3I), suggesting that it promoted cell survival or improved the production function of transplanted iMSCs. Chemokines are chemotactic cytokines that play a crucial role in regulating the migration and localization of immune cells within tissues, and are essential for the function of the innate immune system. Moreover, they facilitate the release of innate immune cells from the bone marrow and their recruitment from the bloodstream to affected tissues in response to infection and inflammation. Additionally, chemokines are vital for positioning innate immune sentinels in peripheral tissues, and they guide these activated cells to the nearest lymph nodes to initiate and shape an adaptive immune response upon activation of the innate immune system.<sup>26</sup> The cytokine array kit used in this current study (R&D Systems, ARY006) detected 40 cytokines, chemokines, and acute-phase proteins, including anti-inflammatory, pro-inflammatory, and immunomodulatory factors. A low rapamycin concentration mainly reduced immune activation by suppressing chemokine secretion upon local delivery of iMSC<sup>2B-Fc/Luci</sup> (Figure 4D and F).

The clinical application of rapamycin has several disadvantages, including poor water solubility, first-pass metabolism, low oral bioavailability, and specific binding to hemoglobin.<sup>27</sup> Recently, various formulation strategies (particularly the use of nanostructured carriers) were developed to overcome these disadvantages.<sup>28</sup> Injectable suspensions of rapamycin albumin-bound particles are approved by the U.S. FDA for adult patients with locally advanced, unresectable, or metastatic malignant perivascular epithelioid cell tumors ([www.fda.gov](http://www.fda.gov)). To overcome poor water solubility, rapamycin was dissolved in 10% dimethyl sulfoxide (DMSO) with a 0.5% w/v methylcellulose 400 solution. Intraperitoneal administration of rapamycin dose-dependently reduced primary HO and improved rotarod and treadmill performances (Figure 1C-G). Even a low rapamycin concentration (0.3 mg/kg) reduced ~60% HO (Figure 1E). Recently, hybrid spheroids consisting of rapamycin-releasing microparticles and MSCs markedly extended the survival of islets and facilitated the production of regional T<sub>reg</sub>.<sup>29</sup> This drug system realizes long-term immunosuppression via a single administration of low-dose rapamycin, which may reduce systemic immunodeficiency and toxic effects caused by long-term exposure to rapamycin.

In this study, human-derived iMSCs were used to deliver the therapeutic protein (ACVR2B-Fc) to the immunocompetent FOP mice. The human leukocyte antigen (HLA) carried by iMSCs may compromise their immunomodulation and tissue regeneration functions. iMSCs derived from HLA genome-edited iPSCs will further improve immune compatibility and may be an off-the-shelf product for clinical application.<sup>30</sup> The fact that a low rapamycin concentration promoted cell survival and production of the ACVR2B-Fc fusion protein and synergistically reduced primary and recurrent HO indicated that the combination of stem cell therapy and immunosuppressants may benefit patients with FOP by reducing recurrent HO caused by surgical removal of the primary lesion. Therefore, surgical procedures for the removal of HO may be feasible. Furthermore, the hybrid spheroids consisting of rapamycin-releasing microparticles and MSCs reported by

Nguyen et al. offer a promising strategy for optimizing this combination therapy for FOP.

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## Author contributions

Pan Gao (Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing—original draft), Yoshiko Inada (Data curation), Maria José López Iniesta (Supervision), Chengzhu Zhao (Conceptualization, Supervision), Megumi Goto (Resources, Supervision), Akitsu Hotta (Conceptualization, Resources, Supervision), Hidetoshi Sakurai (Conceptualization, Resources, Supervision), and Makoto Ikeya (Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing—original draft, Writing—review & editing)

## Supplementary material

Supplementary material is available at *JBMR Plus* online.

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## Conflicts of interest

The authors declare no competing interests.

## Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

## Ethics approval and consent to participate

All experimental protocols involving human subjects were approved by the Ethics Committee of the Department of Medicine and Graduate School of Medicine of Kyoto University (The Generation of Human Disease-Specific iPS Cells and the Use of Such iPS Cells for Disease Analysis (R0091, 06/04/2008)). Written informed consent was obtained from all patients. All the animal experiments were approved by the Institutional Animal Committee of Kyoto University (#16-73, #22-154, #22-155, and #23-218).

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