



Title	Iguratimod suppresses sclerostin and receptor activator of NF- κ B ligand production via the extracellular signal-regulated kinase/early growth response protein 1/tumor necrosis factor alpha pathway in osteocytes and ameliorates disuse osteoporosis in mice
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Highlights

- The anti-rheumatic drug iguratimod ameliorate osteoporosis of rheumatoid arthritis.
- The impact of iguratimod on osteocytes remained unclear.
- The effects of iguratimod was examined by disuse-induced osteoporosis in mice.
- Iguratimod mitigated hindlimb unloading-induced femur bone mass reduction.
- Iguratimod suppressed early growth response protein 1 expression in osteocytes.
- Inhibiting early growth response protein 1 decreased sclerostin and RANKL expression.
- Iguratimod may offer a novel treatment for disuse-induced osteoporosis.

1 **Original article**

2 **Title**

3 Iguratimod suppresses sclerostin and receptor activator of NF-κB ligand production via the
4 extracellular signal-regulated kinase/early growth response protein 1/tumor necrosis factor
5 alpha pathway in osteocytes and ameliorates disuse osteoporosis in mice

6

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30

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32

33 **Abstract**

34 Disuse osteoporosis is a prevalent complication among patients afflicted with rheumatoid
35 arthritis (RA). Although reports have shown that the antirheumatic drug iguratimod (IGU)
36 ameliorates osteoporosis in RA patients, details regarding its effects on osteocytes remain
37 unclear. The current study examined the effects of IGU on osteocytes using a mouse model of
38 disuse-induced osteoporosis, the pathology of which crucially involves osteocytes. A reduction
39 in distal femur bone mass was achieved after 3 weeks of hindlimb unloading in mice, which
40 was subsequently reversed by intraperitoneal IGU treatment (30 mg/kg; five times per week).
41 Histology revealed that hindlimb-unloaded (HLU) mice had significantly increased osteoclast
42 number and sclerostin-positive osteocyte rates, which were suppressed by IGU treatment.
43 Moreover, HLU mice exhibited a significant decrease in osteocalcin-positive cells, which was
44 attenuated by IGU treatment. In vitro, IGU suppressed the gene expression of receptor activator
45 of NF- κ B ligand (RANKL) and sclerostin in MLO-Y4 and Saos-2 cells, which inhibited
46 osteoclast differentiation of mouse bone marrow cells in cocultures. Although IGU did not
47 affect the nuclear translocation or transcriptional activity of NF- κ B, RNA sequencing revealed
48 that IGU downregulated the expression of early growth response protein 1 (EGR1) in
49 osteocytes. HLU mice showed significantly increased EGR1- and tumor necrosis factor alpha
50 (TNF α)-positive osteocyte rates, which ~~was~~were decreased by IGU treatment. EGR1

51 overexpression enhanced the gene expression of TNF α , RANKL, and sclerostin in osteocytes,
52 which was suppressed by IGU. Contrarily, small interfering RNA-mediated suppression of
53 EGR1 downregulated RANKL and sclerostin gene expression. These findings indicate that
54 IGU inhibits the expression of EGR1, which may downregulate TNF α and consequently
55 RANKL and sclerostin in osteocytes. These mechanisms suggest that IGU could potentially be
56 used as a treatment option for disuse osteoporosis by targeting osteocytes.

57

58 **Keywords**

59 disuse osteoporosis; early growth response protein 1; iguratimod; osteocytes; RANKL;
60 sclerostin

61

62 **1. Introduction**

63 Osteocytes play a crucial role in regulating the interplay between osteoblast-mediated
64 bone formation and osteoclast-driven bone resorption during bone metabolism driven by
65 mechanical loading [1-3]. Therefore, decreased mechanical loading has been found to promote
66 the development of disuse osteoporosis [3], the risk of which is quite high among individuals
67 with musculoskeletal disorders, such as rheumatoid arthritis (RA) [4-6]. Furthermore,
68 mechanical unloading diminishes the efficacy of existing osteoporosis treatments, such as
69 parathyroid hormone (PTH) analogs and bisphosphonates [7,8]. Hence, therapeutic strategies
70 distinct from conventional osteoporosis treatments are needed for improved prevention of
71 disuse osteoporosis.

72 Studies have identified various factors associated with disuse osteoporosis. First,
73 sclerostin, a glycoprotein mainly secreted by osteocytes and encoded by the SOST gene, has
74 been found to exert inhibitory effects on bone formation by inhibiting Wnt/ β -catenin signaling
75 [9]. Mechanical unloading stimuli ~~has~~have also been found to increase sclerostin expression,

76 thereby inhibiting bone formation [1,10]. Second, evidence has shown that the receptor
77 activator of NF- κ B ligand (RANKL), a type II membrane protein primarily expressed in
78 osteoblasts and osteocytes, induces osteoclastic differentiation and is upregulated by several
79 mechanical unloading-related factors, including sclerostin and tumor necrosis factor (TNF)- α
80 [11,12]. Moreover, one study found that osteoprotegerin (OPG), a decoy receptor for RANKL
81 that inhibits bone resorption, was one of the target genes involved in Wnt/ β -catenin signaling
82 [13]. Taken together, accumulated evidence suggests that mechanical unloading may promote
83 sclerostin and RANKL expression and decrease OPG expression, thereby suppressing bone
84 formation and increasing bone resorption. The third cause involves the NF- κ B pathway.
85 Reports have shown that mechanical unloading upregulates the NF- κ B pathway, which
86 consequently increases the expression of TNF α , RANKL, and SOST in osteocytes [14,15].

87 A recent meta-analysis reveals that Janus kinase inhibitors (JAKi) and biological
88 disease-modifying antirheumatic drugs (bDMARDs) showed some positive effects on bone
89 metabolism, although had no significant impact on BMD and fracture prevention
90 ~~A recent meta-analysis revealed that Janus Kinase inhibitors and biological disease-modifying antirheumatic~~
91 ~~drugs had no significant impact on bone mineral density and fracture prevention~~ [16,17,18].

92 However, other studies have shown that iguratimod (IGU), a synthetic small molecule disease-
93 modifying antirheumatic drug, prevented bone loss and improved bone metabolism in patients
94 with RA [19,20] while inhibiting RANKL-induced osteoclast differentiation [20,21]. Moreover,
95 we previously reported that IGU promoted bone morphogenetic protein-2 (BMP2)-induced
96 bone formation [21,22] and reduced glucocorticoid-induced disorder of bone metabolism in
97 vitro [22,23]. However, considering the lack of relevant studies, the effects of IGU on osteocytes
98 have remained unclear. Therefore, the current study aimed to investigate the effects of IGU
99 specifically on osteocytes utilizing a mouse model of disuse osteoporosis.

100

101 **2. Materials and methods**

102 **2.1 Experimental design and animal model**

103 Eight-week-old male C57BL/6J mice were purchased from Charles River
104 Laboratories (Osaka, Japan). All mice were kept in a temperature- and humidity-controlled
105 facility with a 12-h light/dark cycle and free access to food and water. After a week of
106 acclimatization, the mice were randomly assigned into three groups (Fig. 1A): (1) normal saline
107 (NS) mice (rest + NS injections; n = 8); (2) hindlimb-unloaded (HLU) + NS mice (HLU + NS
108 injections; n = 8); and (3) HLU + IGU mice (HLU + IGU injections; n = 8). NS and IGU (30
109 mg/kg per day) were injected intraperitoneally five times a week. The mice in the HLU + NS
110 and HLU + IGU groups were suspended by their tails for 21 days using tail suspension clips
111 (Yamashita Giken, Tokushima, Japan) to prevent their hindlimbs from weight-bearing loads
112 (Supplementary Fig. 1A). The tail suspension maintained a head-down tilt of 30°, ensuring that
113 their hindlimbs did not touch the cage floor, as previously described [2322]. The forelimbs
114 remained in contact with the cage bottom, enabling the mice to move freely within a 360° range
115 of motion. To assess the effects of tail suspension on their general body condition, the body
116 weight of the mice was measured daily after initiating tail suspension (Fig. 1B).

117 For euthanasia and bone sample collection, the mice were anesthetized with an
118 intraperitoneal injection of medetomidine (0.3 mg/kg), midazolam (4.0 mg/kg), and
119 butorphanol (5.0 mg/kg), as previously described [2423]. The left femurs were harvested and
120 used for microcomputed tomography (µCT) and histological analyses.

121

122 **2.2 Microcomputed tomography**

123 The distal femurs of the mice were scanned using a high-resolution µCT scanner
124 (SkyScan 1272; Bruker, Kontich, Belgium) with a voxel size of 8 µm. The region of interest
125 for analysis was defined as a region ranging from 500 to 1,500 µm proximal to the growth plate,

126 in which both condyles were no longer visible, and was imaged using 125 slices. Raw images
127 were reconstructed into three-dimensional cross-sectional datasets using a cone beam
128 algorithm with the SkyScan reconstruction software (NRecon, Bruker). Structural indices were
129 then calculated on the reconstructed images using the Skyscan CT Analyzer (CTAn) software
130 (Bruker). A custom processing algorithm was utilized with the CTAn to separate trabecular and
131 cortical bone based on the different thicknesses of the structures. Trabecular parameters
132 included the bone volume fraction (bone volume [BV]/total volume [TV]) and trabecular
133 number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp), whereas cortical parameters
134 included cortical thickness (Ct.Th).

135

136 **2.3 Histological analysis**

137 After μ CT, the femurs were fixed in formalin and decalcified for embedding. Tartrate-
138 resistant acid phosphatase (TRAP) staining was performed according to the manufacturer's
139 protocol (Cosmo Bio, Tokyo, Japan). The number of TRAP-positive cells per trabecular surface
140 was then counted as previously described [2423].

141

142 **2.4 Immunohistochemical analysis**

143 Samples were incubated with the following primary antibodies: antiosteocalcin
144 (Takara Bio, Shiga, Japan), antisclerostin (R&D Systems, Minneapolis, MN, United States), anti-
145 antiearly growth response protein 1 (EGR1) (Proteintech, Chicago, IL, United States), anti-
146 Heme Oxygenase 1 (HO-1) (Abcam, Cambridge, MA, United States) and anti-TNF α antibody
147 (LifeSpan BioSciences, Seattle, WA, United States). The sections were then incubated with a
148 secondary antibody (Histofine Simple Stain Mouse MAX PO; Nichirei Bioscience Inc., Tokyo,
149 Japan) and stained with 3,3'-Diaminobenzidine tetrahydrochloride (Dako, Tokyo, Japan). As
150 with μ CT, the region of interest for analysis was defined as a region ranging from 500 to 1,500

151 μm proximal to the growth plate [2423]. Within the region of interest, the number of
152 osteocalcin-positive cells was measured on five randomly selected trabecular bone surfaces,
153 while the percentage of sclerostin-positive osteocytes, EGR1-positive osteocytes, and TNF α -
154 positive osteocytes were evaluated for all osteocytes in the cortical bone. The number of HO-
155 1 positive cells within the trabecular bone region of interest was measured using the Image J
156 software.

157

158 **2.5 Histomorphometrical analysis**

159 To label active bone formation, all mice were subcutaneously injected with
160 tetracycline (20 mg/kg) and calcein (10 mg/kg) 5 and 2 days before being sacrificed,
161 respectively, as previously described [2524]. The left femurs were extracted, fixed in 70%
162 ethanol, treated with Villanueva bone stain, and embedded in methacrylate (Wako Pure
163 Chemical Industries, Osaka, Japan). Thereafter, the following histomorphometric parameters
164 were quantified: bone formation rate per total volume (BFR/TV) and eroded surface per bone
165 surface (ES/BS)

166

167 **2.6 Reagents and cell culture**

168 IGU was provided by Toyama Chemical Co. Ltd (Tokyo, Japan) and dissolved in
169 dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries). Approximately, the serum
170 concentration of IGU was reported to reach 3 $\mu\text{g}/\text{mL}$ in clinical dose for humans [2224]. We
171 used osteocyte-like cell line MLO-Y4, human osteosarcoma cell line Saos-2 cells, and
172 osteoblastic cell line MC3T3-E1 cells. MLO-Y4 cells were purchased from Kerafast (Boston,
173 MA, United States), whereas Saos-2 and MC3T3-E1 cells were acquired from Riken Cell Bank
174 (Tsukuba, Japan).

175 MLO-Y4 cells were cultured on type I collagen-coated dishes (Corning, Corning, New

176 York, United States) in α -minimum essential medium (α -MEM; Nacalai Tesque, Kyoto, Japan)
177 supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, United
178 States), 5% calf serum (Hyclone), and 1% antibiotic/antimycotic solution (A/A; Sigma-Aldrich,
179 St. Louis, MO, United States), as previously described [2224]. Adherent cells were seeded into
180 24-well plates at 1×10^5 cells/well. After 24 h, the cells were treated with/without 10 ng/mL
181 of TNF α (R&D Systems) and different concentrations of IGU for 5 days.

182 Saos-2 cells were cultured with Dulbecco's Modified Eagle Medium (Nacalai Tesque,
183 Kyoto, Japan) containing 10% FBS and 1% A/A in 24-well plates at 1×10^5 cells/well. After
184 24 h, the cells were treated with/without 100 ng/mL of BMP2 (Osteofarmer, Osaka, Japan) and
185 different concentrations of IGU in media containing 5 mM of β -glycerophosphate (Calbiochem,
186 San Diego, CA, United States), and 50 μ g/mL of ascorbic acid (Sigma-Aldrich) for 6 days, as
187 previously described [2625].

188 MC3T3-E1 cells were cultured with α -MEM containing 10% FBS and 1% A/A in 24-
189 well plates at 1×10^5 cells/well. After 24 h, the cells were treated with/without 10 ng/mL
190 BMP6 (R&D Systems), 100 ng/mL of recombinant mouse sclerostin protein (R&D Systems),
191 and 3 μ g/mL of IGU in media containing 10 mM β -glycerophosphate and 50 μ g/mL ascorbic
192 acid to induce osteoblast differentiation for 3 days, as previously described [2726].

193

194 **2.7 Coculture of MLO-Y4 cells and murine primary osteoclasts**

195 MLO-Y4 cells were seeded at 1,000 cells/cm² in a type I collagen-coated 96-well plate.
196 After a 24-h incubation period, the cells were treated with TNF α (10 ng/mL) and various
197 concentrations of IGU. Murine primary osteoclasts were obtained from bone marrow cells
198 flushed from the femurs and tibiae of 8-week-old male C57BL/6J mice. These cells were then
199 cultured overnight in α -MEM supplemented with 10% FBS, 1% A/A, and 10 ng/mL of
200 macrophage colony-stimulating factor (M-CSF; R&D Systems) at 37 °C in a humidified

201 atmosphere of 5% carbon dioxide, as previously described [2224]. Adherent cells were added
202 at 2,500 cells/cm² to the same 96-well plates seeded with MLO-Y4 cells and then cocultured
203 in α-MEM medium supplemented with 10% FBS and 1% A/A. The medium was replaced
204 every 2 days. On day 7, the cells were fixed and stained for TRAP.

205

206 **2.8 RNA extraction, first- strand complementary DNA synthesis, and reverse
207 transcription quantitative polymerase chain reaction (RT-qPCR)**

208 Total RNA was extracted from cells treated in a 24-well plate using the RNeasy Mini
209 kit (Qiagen, Düsseldorf, Germany). First-strand complementary DNA was synthesized from 1
210 µg of total RNA using the ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan)
211 following the manufacturer's protocol. RT-qPCR was performed using Fast SYBR Green
212 Master Mix (Life Technologies, Carlsbad, CA, United States) and a Step One Plus Real-Time
213 PCR System (Life Technologies). Gene expression levels were normalized to glyceraldehyde-
214 3-phosphate dehydrogenase, whereas fold changes were calculated relative to the control group
215 using the $2^{-\Delta\Delta Ct}$ method. The PCR primer sequences are described in the Supplementary Table.

216

217 **2.9 Alkaline phosphatase (ALP) activity assay**

218 ALP activity was assessed by measuring the release of p-nitrophenol from p-
219 nitrophenylphosphate at pH 9.8 using the ALP assay kit (FUJIFILM Wako Pure Chemical Co.,
220 Osaka, Japan) in accordance with the manufacturer's protocol. The optical density at 405 nm
221 was monitored to quantify the amount of p-nitrophenol released. The activity was normalized
222 to the protein content assessed using the Pierce™ Bicinchoninic acid (BCA) Protein Assay
223 Kit (Thermo Fisher, Waltham, MA, United States).

224

225 **2.10 Western blotting**

226 Western blotting was conducted as previously described [2224]. Cytoplasmic and
227 nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit
228 (Pierce, Rockford, IL, United States) according to the manufacturer's protocol.

229 The primary antibodies were as follows: phosphate anti-p38 antibody
230 (Thr180/Tyr182) (1:1,000), anti-p38 antibody (1:1,000), phosphate antistress-activated protein
231 kinase (SAPK)/Jun amino-terminal kinase (JNK) antibody (Thr183/Tyr185) (1:1,000), anti-
232 SAPK/JNK antibody (1:1,000), phosphate anti-NF- κ B p65 (1:1,000), anti-NF- κ B p65
233 (1:1,000), phosphate antiextracellular signal-regulated kinase 1/2 (ERK1/2; Thr202/Tyr204)
234 (1:2000), anti-ERK1/2 (p44/42) (1:1,000), β -actin (1:2000), and Lamin-B1 (1:1,000) antibody
235 purchased from Cell Signaling Technology (CST, Danvers, MA, United States). Antisclerostin
236 antibody (1:1,000) was acquired from R&D Systems.

237

238 **2.11 Luciferase assay**

239 For transient transfection, 1×10^6 Saos-2 cells were suspended in 100 μ L of Opti-
240 MEM medium (Thermo Fisher) and electroporated using NEPA21 Super Electroporator (Nepa
241 Gene, Chiba, Japan) at 175 V and a poring pulse length of 5 ms. The cells were then transfected
242 with κ B-Luc2P (pGL4.15; Promega, Madison, WI, United States). After electroporation, $3 \times$
243 10^4 cells were seeded into 96-well plates. Following a 24-h incubation period, the cells were
244 treated with or without TNF α , BMP2, IGU, and dexamethasone (Dex; Sigma-Aldrich).
245 Luciferase activity was measured 8 h after treatment using a Centro XS3 LB 960 Microplate
246 Luminometer (Berthold Technologies, Bad Wildbad, Germany) equipped with the Steady-
247 Glo® luciferase assay system (Promega) following the manufacturer's protocol. Luciferase
248 activity was normalized to the protein content measured by the PierceTM BCA Protein Assay
249 Kit (Thermo Fisher).

250

251 **2.12 Immunofluorescence staining**

252 Saos-2 cells were cultured in a 96-well plate at 5.0×10^3 cells/well. After 24 h of
253 incubation, the cells were treated with/without BMP2 and IGU. After 24 h of treatment, the
254 cells were fixed with paraformaldehyde for 30 min and blocked with 5% rabbit serum for 1 h.
255 Subsequently, the cells were incubated overnight at 4 °C with an anti-p65 antibody (CST,
256 1:1,000), incubated with antirabbit Alexa Flour 594 (CST, 1:1,000) at room temperature for 1
257 h in the dark, and counterstained with antifade mounting medium with Hoechst 33258 (Dojindo,
258 Kumamoto, Japan). Cell images were acquired using an IN Cell Analyzer 6000 (GE Healthcare,
259 Chicago, IL, United States), capturing 16 view fields per well. The Nuc/Cyto ratio of the NF-
260 kB signal was calculated using the mean signal intensity of nuclear areas and cytoplasmic areas
261 in cells of each field per well.

262

263 **2.13 RNA sequencing**

264 Cells underwent total RNA extraction using the RNeasy Mini kit (Qiagen) following
265 the manufacturer's protocol. RNA-Seq libraries were then generated using the TruSeq stranded
266 mRNA sample prep kit (Illumina, San Diego, CA, United States) according to the
267 manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq 6000
268 platform in the 100-bp single-end mode. The obtained reads were aligned to the human
269 reference genome sequences (hg19) using TopHat version 2.0.13 in combination with Bowtie2
270 version 2.2.3 and SAMtools version 0.1.19. The number of fragments per kilobase of exon per
271 million mapped fragments was calculated using Cufflinks version 2.2.1. Differential gene
272 expression analysis between groups was conducted using iDEP with a false discovery rate
273 threshold of <0.1 and a fold-change of >1.5. Volcano plot representation and Gene Ontology
274 enrichment analysis were performed using BioJupies, with adjusted p values of <0.05 and
275 absolute log2 fold-change of >1.

276

277 **2.14 Transient transfection**

278 Saos-2 cells were transfected with small interfering RNA (siRNA) targeting Egr1
279 (Thermo Fisher), siRNA control (Thermo Fisher), and the EGR1 overexpression vector,
280 pCMV6-EGR1 (pEGR1; OriGen, Rockville, MD, United States). Thereafter, the cells
281 underwent electroporation for transfection and were then seeded into 24-well plates. Following
282 a 24-h incubation period, RT-qPCR analyses were performed to ascertain the correct clone
283 targeting.

284

285 **2.15 Statistical analysis**

286 All numerical data were reported as mean \pm standard deviation and analyzed using
287 Prism software (GraphPad Prism for Windows, version 9.0, San Diego, CA, United States). An
288 unpaired Student's t-test was used for comparisons between two groups, whereas one-way
289 analysis of variance followed by Tukey's post-hoc test was used for comparisons between more
290 than three groups. A p value of <0.05 indicated statistical significance.

291

292 **2.16 Study approval**

293 All experimental protocols comply with the ARRIVE guidelines and were approved
294 by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate
295 School of Medicine (permission number 02-057-007).

296

297 **3. Results**

298 **3.1 Effects of IGU on disuse osteoporosis in HLU mice**

299 The mice were randomly assigned into three groups: NS, HLU + NS, and HLU + IGU
300 mice (Fig. 1A). All mice subjected to hindlimb unloading successfully completed the 21-day

301 unloading period. During this unloading period, the body weight of HLU + NS and HLU +
302 IGU mice remained consistently lower than that in the NS group. However, no significant
303 differences were observed among the three groups on day 21 (Fig. 1B).

304 μ CT was utilized to analyze the trabecular and cortical bone of the distal femurs.
305 Representative images of the trabecular bone of mice in each group are presented in Fig. 1C.
306 Analysis of the trabecular bone characteristics showed that the BV/TV, Tb.N, and Tb.Th were
307 significantly lower in HLU + NS mice than NS mice. In contrast, Tb.Sp was significantly
308 higher in HLU + NS mice than in NS mice. IGU treatment in HLU mice prevented BV/TV loss
309 and Tb.N reduction (Fig. 1D). Representative images of the cortical bone of mice in each group
310 are shown in Supplementary Fig. 1B. Analysis of the cortical bone characteristics in the
311 diaphyseal region showed that Ct.Th was also significantly lower in HLU + NS mice than in
312 NS mice. Although IGU treatment appeared to impede this cortical bone loss in HLU mice, it
313 did not promote a significant difference (Supplementary Fig. 1C).

314 TRAP staining of osteoclasts in the trabecular and cortical bone of the distal femur
315 (Fig. 2A) revealed significantly more TRAP-positive multinuclear cells in HLU + NS mice
316 than in NS mice, with IGU administration attenuating this increase. Subsequently, the effects
317 of HLU and IGU on osteoblastic differentiation ~~was~~were evaluated. Immunostaining of
318 osteocalcin showed significantly fewer osteocalcin-positive cells in HLU + NS mice than in
319 NS mice (Fig. 2B), with IGU treatment attenuating this reduction. Additionally, sclerostin
320 immunostaining was performed on osteocytes to investigate the effects of HLU and IGU on
321 osteocytes. The ~~percentate~~percentage of sclerostin-positive osteocytes was significantly higher
322 in HLU + NS mice than in NS mice (Fig. 2C). IGU administration in HLU mice attenuated the
323 increase in the percentage of sclerostin-positive osteocytes.

324 Figs. 2D, E, and G ~~presents~~ the histomorphometry of the distal femur. Compared to
325 NS mice, HLU + NS mice demonstrated short, thin trabeculae with scattered, small trabecula,

326 which were recovered by IGU administration (Figs. 2D, E).

327 In terms of bone formation, BFR/TV tended to be lower in HLU + NS mice than in
328 NS mice (Fig. 2F). IGU treatment in HLU mice tended to attenuate this reduction but not
329 significantly. Regarding bone resorption, HLU + NS mice exhibited significantly higher ES/BS
330 values than did NS mice. Conversely, HLU + IGU mice showed significantly lower ES/BS
331 values than did HLU+NS mice (Figs. 2G, H).

332

333 **3.2 Effects of IGU on osteocytes and osteoclastogenesis/osteoblastogenesis through
334 osteocytes in vitro**

335 Previous studies have demonstrated that mechanical unloading promotes TNF α ,
336 BMP2, RANKL, and sclerostin expression from bone tissues [1,10,[2827,2928](#)]. RT-qPCR
337 results exhibited that TNF α -treated osteocyte cell line MLO-Y4 showed markedly increased
338 RANKL expression and decreased OPG expression, which significantly increased the
339 RANKL/OPG ratio. IGU treatment significantly inhibited this upregulation of RANKL
340 expression, which significantly decreased the RANKL/OPG ratio (Fig. 3A). Next, to confirm
341 the indirect effects of IGU on osteoclast differentiation, we performed coculture experiments
342 utilizing bone marrow-derived macrophages (BMDMs) and MLO-Y4 cells treated with IGU
343 without adding RANKL. The coculture of BMDMs with MLO-Y4 cells promoted a notable
344 increase in the number of multinuclear osteoclasts. Alternatively, IGU treatment in MLOY4
345 cells significantly decreased the number of multinuclear osteoclasts (Fig. 3B).

346 Evidence indicates that bone morphogenetic proteins (BMP) are signaling molecules
347 that stimulate osteoblast differentiation and that sclerostin inhibits BMP-induced osteoblast
348 differentiation [[2726,3029](#)]. RT-qPCR ~~results~~ for the osteoblastic MC3T3-E1 cells
349 showed that regardless of the presence of sclerostin, IGU significantly enhanced gene
350 expression of ALP and osteocalcin (Fig. 3C). Furthermore, BMP6 administration promoted

351 ALP activity, whereas sclerostin inhibited ALP activity, with IGU administration restoring the
352 inhibitory effects of sclerostin (Fig. 3D).

353 Studies have shown that Saos-2 cells are capable of differentiating into osteocyte-like
354 cells and that BMP2 promotes the expression of sclerostin [2625,3130]. Our RT-qPCR ~~resutls~~
355 results revealed that BMP2 significantly increased the expression of osteocyte-related genes,
356 including SOST and Dmp-1 (Fig. 3E), ~~alhtough~~ although IGU administration suppressed
357 BMP2-induced expression of SOST and Dmp-1. However, BMP2 and IGU administration did
358 not significantly change the expression of Dkk-1, another inhibitory factor of bone formation.
359 Western blotting showed that IGU treatment dose-dependently suppressed sclerostin protein
360 expression (Fig. 3F), whereas no alterations were observed in Dkk-1 expression
361 (Supplementary Fig. 2).

362 Furthermore, to evaluate whether IGU influenced osteocyte apoptosis [3234,3332],
363 we investigated the mitogen-activated protein kinase signaling pathway, including SAPK/JNK
364 and p38, which regulates Saos-2 cell apoptosis. IGU treatment promoted the phosphorylation
365 of SAPK/JNK and p38 (Fig. 3G). However, no difference in the empty lacunae ratio was
366 observed among NS, HLU + NS, and HLU + IGU mice (Supplementary Fig. 3A). Moreover,
367 in the intrinsic apoptotic pathway, IGU treatment slightly suppressed Caspase-9 but did not
368 affect the expression of other genes in RT-qPCR (Supplementary Fig. 3B). In addition, the cell
369 proliferation assay showed no significant differences regardless of the presence or absence of
370 IGU (Supplementary Fig. 3C). Collectively, these findings suggest that IGU may not affect
371 osteocyte apoptosis.

372

373 **3.3 Effects of IGU on the NF-κB pathway in Saos-2 cells**

374 To assess whether IGU treatment suppresses the transcriptional activation of the
375 transcription factor NF-κB pathway, we investigated luciferase reporter assay using Saos-2

376 cells. Notably, TNF α administration significantly increased NF- κ B transcriptional activity.
377 Although IGU did not significantly affect NF- κ B activity, dexamethasone significantly
378 inhibited it (Fig. 4A).

379 Furthermore, we performed Western blotting with/without BMP2 and IGU to assess
380 the effects of IGU administration on NF- κ B translocation from the cytoplasm to the nucleus.
381 Examination of the cytoplasm and nuclear protein contents of NF- κ B through Western blotting
382 revealed no remarkable difference (Fig. 4B). Moreover, immunofluorescence staining found
383 that IGU administration did not significantly suppress NF- κ B translocation from the cytoplasm
384 to the nucleus (Fig. 4C). Collectively, these findings suggest that IGU may not affect the NF-
385 κ B pathway in osteocytes.

386

387 **3.4 The potential of IGU to regulate EGR1 expression identified through RNA sequencing
388 analysis in Saos-2 cells**

389 To further investigate the possible mechanisms explaining the effects of IGU on
390 osteocytes, we conducted RNA sequencing in Saos-2 cells from the untreated, BMP2-treated,
391 and BMP2 + IGU-treated groups (Supplementary Fig. 4). ~~Notably~~Notably, screening of
392 differentially expressed genes showed that IGU treatment effectively downregulated the
393 expression levels of several genes known osteocyte-related genes, including SOST and Dmp1
394 (Fig. 5A). Next, Gene Ontology enrichment analysis revealed that IGU treatment primarily
395 upregulated ossification regulation, skeletal system development, and collagen fibril
396 organization but primarily downregulated cellular response to type I interferon and the type
397 I interferon signaling pathway (Fig. 5B).

398 Figs. 5C and D ~~depicts~~depict the top 10 genes differentially regulated in ascending
399 order based on p values following IGU treatment. Among the top 10 genes, EGR1 is the only
400 one classified as a regulation factor of DNA-templated transcription and has been reported to

401 be involved in mechanical stress [3433]. In anticipation of its relevance to the effects of IGU
402 on osteocytes, we focused on this ~~particualr~~particular gene. In fact, immunostaining of EGR1
403 in the distal femur revealed that the percentage of EGR1-positive osteocytes was higher in
404 HLU + NS mice than in NS mice, with IGU treatment significantly attenuating this increase
405 (Fig. 5E). Moreover, RT-qPCR revealed that in Saos-2 cells, BMP2 treatment significantly
406 increased EGR1 gene expression, ~~whereas~~whereas IGU treatment effectively suppressed the
407 same EGR1 gene expression (Fig. 5F).

408

409 **3.5 IGU treatment improves bone metabolism by regulating the ERK/EGR1/TNF α
410 pathway**

411 Studies have shown that mechanical unloading triggers the production of reactive
412 oxygen species (ROS), which increase EGR1 transcriptional activity through the ERK pathway
413 and downstream TNF α expression [3433,3534]. Moreover, other reports have suggested that
414 heme oxygenase (HO)-1 was closely associated with ROS and that the expression of HO-1
415 increases by unloading [3635,3736]. In fact, immunostaining of HO-1 in the distal femur
416 revealed that the number of HO-1-positive cells was higher in HLU + NS mice than in NS mice,
417 with IGU treatment showing no significant effect (Fig. 6A). Therefore, we investigated the
418 downstream ERK pathway, which is the downstream of ROS. Western blotting in Saos-2 cells
419 showed that BMP2 promoted ERK1/2 phosphorylation, whereas IGU treatment inhibited this
420 promotion (Fig. 6B). Next, we investigated downstream signals of ERK1/2 in osteocytes.
421 Notably, immunostaining of TNF α in the distal femurs showed that the percentage of TNF α -
422 positive osteocytes was significantly higher in the HLU + NS mice than in NS mice. Strikingly,
423 IGU treatment significantly inhibited this increase (Fig. 6C). Thereafter, we further examined
424 whether EGR1 overexpression induced TNF α and its downstream factors, such as RANKL and
425 SOST expression, in osteocytes. Accordingly, we found that EGR1 overexpression

426 significantly increased not only TNF α expression but also RANKL and SOST expression (Fig.
427 6D). Furthermore, IGU treatment significantly inhibited all such increases. Moreover, RT-
428 qPCR analysis of EGR1-knockdown Saos-2 cells achieved through siEGR1 plasmid
429 transfection revealed that siEGR1 transfection significantly reduced the expressions of
430 RANKL and SOST by downregulating EGR1 expression (Fig. 6E).

431 A previous report suggested that an ion channel called Piezo1 was involved in the
432 sensing of mechanical signals by osteocytes [3837]. Immunostaining of Piezo1 showed that
433 HLU tended to downregulate the number of Piezo1-positive osteocytes, with IGU treatment
434 showing no significant effects (Supplementary Fig. 5A). We further investigated the
435 relationship between EGR1 and Piezo1. Accordingly, we found that EGR1 overexpression
436 significantly increased the expression of Piezo1 and Cyr61, a target gene of YAP/TAZ
437 downstream of Piezo1 (Supplementary Fig. 5B). In contrast, EGR1 knockdown showed no
438 difference in the expression of Piezo1 and Cyr61 (Supplementary Fig. 5C).

439 Taken together, these findings suggest that IGU may inhibit the ERK/EGR1 pathway
440 induced by mechanical unloading and consequent ROS production, thereby reducing
441 osteocyte-expressed RANKL and SOST via TNF α suppression (Fig. 7). Besides the direct
442 effect of IGU on osteoblasts and osteoclasts, these mechanisms may suppress
443 osteoclastogenesis and enhance osteoblastogenesis under unloading conditions, which may
444 ameliorate disuse osteoporosis.

445

446 **4. Discussion**

447 Previous reports have addressed the effects of IGU on postmenopausal osteoporosis
448 model mice and its effects on bone metabolic disorders caused by glucocorticoids [20, 22].
449 However, to the best of our knowledge, no previous reports have focused on the effects of IGU
450 on disuse osteoporosis. This study has been the first to demonstrate the detailed effects of IGU

451 in osteocytes and disuse osteoporosis. To the best of our knowledge, this study has been the first
452 to demonstrate the detailed effects of IGU in osteocytes and disuse osteoporosis. Our results
453 revealed that IGU improved disuse osteoporosis in mice by inhibiting sclerostin and RANKL
454 production through the ERK/EGR1/TNF α pathway in osteocytes.

455 Mechanical unloading on bone upregulates various cytokines, such as BMP2 and
456 TNF α [2827,2928,3938]. Furthermore, TNF α induces RANKL expression in osteocytes, which
457 play a major role in osteoclast differentiation [4039]. Sclerostin has been suggested to be
458 another primary cause of disuse osteoporosis. In fact, studies have shown that SOST-deficient
459 mice during hindlimb unloading were resistant to bone loss [3234] and that treating osteocytes
460 with unloading-related factors, such as BMP2 and TNF α , significantly upregulated the
461 transcriptional activity of the SOST [4140]. In our investigation, mechanical unloading
462 promoted an increase in TNF α and sclerostin expression in osteocytes, which was suppressed
463 by IGU treatment.

464 A previous study suggested that IGU improves bone metabolism potentailly
465 potentially through the inhibition of NF- κ B [4241]. Moreoever, reports have shown that IGU
466 may inhibit the activation of NF- κ B by interfering with its translocation into the nucleus while
467 not affecting the degradation of I κ B α in THP-1 cells, a human monocytic leukemia cell line,
468 and cultured human synovial cells [4342,4443]. However, these studies only evaluated the
469 nuclear translocation of NF- κ B and failed to show the transcriptional activity through luciferase
470 assay like in the eurrnet-current study. Given that the present study could not confirm the role
471 of IGU in inhibiting NF- κ B transcriptional activity in osteocytes, we conducted RNA
472 sequencing to investigate the transcription factor EGR1, one of the key proteins involved in
473 mechanical loading via the ROS/ERK pathway [3433]. Reports have suggest-suggested that
474 that unloading increases EGR1 expression in the tendon and muscles [3433,4544], although its
475 expression in osteocytes remained unclear. In the current study, hindlimb-unloaded mice

476 showed an increase in EGR1 expression in osteocytes. Our results also showed that HO-1 in
477 bone tissue ~~remained~~remained unaltered despite IGU administration, suggesting that IGU did
478 not affect ROS levels. Therefore, we focused on ERK, which is downstream of ROS, and found
479 that IGU inhibited the ERK pathway, consistent with the findings of a previous report [4645].
480 EGR1 activates TNF α [3635,4746], with cases of disuse osteoporosis showing elevated
481 expression levels of TNF α and downstream factors, such as RANKL and sclerostin, in cortical
482 bone osteocytes [4847]. This suggests a strong association between unloading conditions and
483 EGR1 within osteocytes. Finally, our results using EGR1 overexpression in osteocytes revealed
484 that EGR1 upregulates TNF α and downstream RANKL and SOST expression, which was
485 downregulated by IGU. Conversely, EGR1 downregulation by siEGR1 downregulated
486 RANKL and SOST expression. These results strongly indicate that EGR1 plays crucial roles
487 in the regulation of RANKL and SOST.

488 Recent studies have revealed that Piezo1, a mechanosensitive ion channel, is crucial
489 for the skeletal response to mechanical loading in osteoblasts and osteocytes [3837,4948].
490 Piezo1 functions as a catalyst for Ca $^{2+}$ influx in response to mechanical stimuli, subsequently
491 governing downstream signaling cascades. Despite a previous report suggesting that
492 extracellular Ca upregulated EGR1 [5049], the association between Piezo1 and EGR1 remains
493 unclear. Our study suggested that EGR1 overexpression upregulated the expression of Piezo1
494 and its downstream genes, with EGR1 downregulation having no effect on these expressions.
495 Taken together, our findings showed that enhanced EGR1 expression may induce Piezo1
496 compensation for the downregulation of mechanical loading-related signals by EGR1.
497 However, siEGR1-induced downregulation of EGR1 or IGU treatment had no effect on Piezo1
498 and downstream gene expression, suggesting that IGU treatment and consequently EGR1
499 suppression were not associated with Piezo1.

500 This study has certain limitations worth noting. First, isolating and culturing

501 osteocytes from IGU-treated mice proved challenging. Furthermore, incorporating EGR1
502 knockout or transgenic mice was difficult in this experiment. Nevertheless, the study's strength
503 lies in having been the first to elucidate the effects of IGUs on osteocytes using a mouse model
504 of disuse osteoporosis.

505

506 **5. Conclusion**

507 Our findings suggest that IGU inhibited sclerostin and RANKL production through
508 the ERK/EGR1/TNF α pathway in osteocytes, indicating its potential for becoming a unique
509 and effective treatment option for disuse osteoporosis by targeting osteocytes.

510

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515

516 **CRediT authorship**

517 **Declaration of conflicting interests**

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525 and S. Okada declare that they have no conflicts of interest.

526

527 **Contribution statement**

528 Taihei Miura: Conceptualization, Data curation, Methodology, Validation, Visualization,
529 Formal analysis, Investigation, Resources, Writing - original draft. Yuki Etani:
530 Conceptualization, Methodology, Supervision, Project administration. Takaaki Noguchi:
531 Conceptualization, Methodology, Supervision, Project administration. Makoto Hirao:
532 Conceptualization, Methodology, Supervision, Project administration. Kenji Takami:
533 Investigation, Resources. Atsushi Goshima: Investigation, Resources. Takuya Kurihara:
534 Investigation, Resources. Yuji Fukuda: Investigation, Resources. Nagahiro Ochiai:
535 Investigation, Resources. Takashi Kanamoto: Supervision. Ken Nakata: Supervision. Seiji
536 Okada: Supervision. Kosuke Ebina: Conceptualization, Methodology, Writing - review &
537 editing, Visualization, Supervision, Project administration.

538

539 **Availability of data and material**

540 The data set used or analyzed in this study is available from the corresponding author
541 upon reasonable request.

542

543 **References**

- 544 [1] J. Delgado-Calle, T. Bellido, The osteocyte as a signaling cell, *Physiol Rev* 102(1) (2022)
545 379-410. <https://doi.org/10.1152/physrev.00043.2020>
- 546 [2] L.F. Bonewald, The amazing osteocyte, *J Bone Miner Res* 26(2) (2011) 229-38.
547 <https://doi.org/10.1002/jbmr.320>
- 548 [3] L. Wang, X. You, L. Zhang, C. Zhang, W. Zou, Mechanical regulation of bone remodeling,
549 *Bone Res* 10(1) (2022) 16. <https://doi.org/10.1038/s41413-022-00190-4>
- 550 [4] M.P. Nagaraja, D. Risin, The current state of bone loss research: data from spaceflight and

551 microgravity simulators, J Cell Biochem 114(5) (2013) 1001-8.
552 <https://doi.org/10.1002/jcb.24454>

553 [5] E. Terpos, K. Fragiadaki, M. Konsta, C. Bratengeier, A. Papatheodorou, P.P. Sfikakis, Early
554 effects of IL-6 receptor inhibition on bone homeostasis: a pilot study in women with
555 rheumatoid arthritis, Clin Exp Rheumatol 29(6) (2011) 921-5.

556 [6] T. Rolvien, M. Amling, Disuse Osteoporosis: Clinical and Mechanistic Insights, Calcif
557 Tissue Int 110(5) (2022) 592-604. <https://doi.org/10.1007/s00223-021-00836-1>

558 [7] Y. Ma, W.S. Jee, Z. Yuan, W. Wei, H. Chen, S. Pun, H. Liang, C. Lin, Parathyroid hormone
559 and mechanical usage have a synergistic effect in rat tibial diaphyseal cortical bone, J Bone
560 Miner Res 14(3) (1999) 439-48. <https://doi.org/10.1359/jbmr.1999.14.3.439>

561 [8] K. Naruse, K. Uchida, M. Suto, K. Miyagawa, A. Kawata, K. Urabe, M. Takaso, M. Itoman,
562 Y. Mikuni-Takagaki, Alendronate does not prevent long bone fragility in an inactive rat model,
563 J Bone Miner Metab 34(6) (2016) 615-626. <https://doi.org/10.1007/s00774-015-0714-y>

564 [9] W. Balemans, M. Ebeling, N. Patel, E. Van Hul, P. Olson, M. Dioszegi, C. Lacza, W.
565 Wuyts, J.V. Den Ende, P. Willems, A.F. Paes-Alves, S. Hill, M. Bueno, F.J. Ramos, P.
566 Tacconi, F.G. Dikkers, C. Stratakis, K. Lindpaintner, B. Vickery, D. Foernzler, W. Van Hul,
567 Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein
568 (SOST), Hum Mol Genet 10(5) (2001) 537-43. <https://doi.org/10.1093/hmg/10.5.537>

569 [10] L. Qin, W. Liu, H. Cao, G. Xiao, Molecular mechanosensors in osteocytes, Bone Res 8
570 (2020) 23. <https://doi.org/10.1038/s41413-020-0099-y>

571 [11] A.E. Kearns, S. Khosla, P.J. Kostenuik, Receptor activator of nuclear factor kappaB ligand
572 and osteoprotegerin regulation of bone remodeling in health and disease, Endocr Rev 29(2)
573 (2008) 155-92. <https://doi.org/10.1210/er.2007-0014>

574 [12] J. Delgado-Calle, A.Y. Sato, T. Bellido, Role and mechanism of action of sclerostin in
575 bone, Bone 96 (2017) 29-37. <https://doi.org/10.1016/j.bone.2016.10.007>

- 576 [13] Y. Kobayashi, S. Uehara, N. Udagawa, N. Takahashi, Regulation of bone metabolism by
577 Wnt signals, *J Biochem* 159(4) (2016) 387-92. <https://doi.org/10.1093/jb/mvv124>
- 578 [14] G.R. Heiland, K. Zwerina, W. Baum, T. Kireva, J.H. Distler, M. Grisanti, F. Asuncion, X.
579 Li, M. Ominsky, W. Richards, G. Schett, J. Zwerina, Neutralisation of Dkk-1 protects from
580 systemic bone loss during inflammation and reduces sclerostin expression, *Ann Rheum Dis*
581 69(12) (2010) 2152-9. <https://doi.org/10.1136/ard.2010.132852>
- 582 [15] K. Baek, H.R. Hwang, H.J. Park, A. Kwon, A.S. Qadir, S.H. Ko, K.M. Woo, H.M. Ryoo,
583 G.S. Kim, J.H. Baek, TNF-alpha upregulates sclerostin expression in obese mice fed a high-fat
584 diet, *J Cell Physiol* 229(5) (2014) 640-50. <https://doi.org/10.1002/jcp.24487>
- 585 [16] A.M. Dubrovsky, M.J. Lim, N.E. Lane, Osteoporosis in Rheumatic Diseases: Anti-
586 rheumatic Drugs and the Skeleton, *Calcif Tissue Int* 102(5) (2018) 607-618.
587 <https://doi.org/10.1007/s00223-018-0401-9>
- 588 [17] S. Siu, B. Haraoui, R. Bissonnette, L. Bessette, C. Roubille, V. Richer, T. Starnino, C.
589 McCourt, A. McFarlane, P. Fleming, J. Kraft, C. Lynde, W. Gulliver, S. Keeling, J. Dutz, J.E.
590 Pope, Meta-analysis of tumor necrosis factor inhibitors and glucocorticoids on bone density in
591 rheumatoid arthritis and ankylosing spondylitis trials, *Arthritis Care Res (Hoboken)* 67(6)
592 (2015) 754-64. <https://doi.org/10.1002/acr.22519>
- 593 [18] A. Hamar, Z. Szekanecz, A. Pusztai, M. Czokolyova, E. Vegh, Z. Petho, N. Bodnar, K.
594 Gulyas, A. Horvath, B. Soos, L. Bodoki, H.P. Bhattoa, G. Nagy, G. Tajti, G. Panyi, E.
595 Szekanecz, A. Domjan, K. Hodosi, S. Szanto, G. Szucs, S. Szamosi, Effects of one-year
596 tofacitinib therapy on bone metabolism in rheumatoid arthritis, Osteoporos Int 32(8) (2021)
597 1621-1629. <https://doi.org/10.1007/s00198-021-05871-0>
- 598 [1948] L. Deng, F. Yao, F. Tian, X. Luo, S. Yu, Z. Wen, Influence of Iguratimod on Bone
599 Metabolism in Patients with Rheumatoid Arthritis: A Meta-analysis, *Int J Clin Pract* 2022
600 (2022) 5684293. <https://doi.org/10.1155/2022/5684293>

- 601 [2019] Y.X. Wu, Y. Sun, Y.P. Ye, P. Zhang, J.C. Guo, J.M. Huang, X.Z. Jing, W. Xiang, S.Y.
602 Yu, F.J. Guo, Iguratimod prevents ovariectomy- induced bone loss and suppresses
603 osteoclastogenesis via inhibition of peroxisome proliferator- activated receptor- γ , Mol Med
604 Rep 16(6) (2017) 8200-8208. <https://doi.org/10.3892/mmr.2017.7648>
- 605 [2120] K. Kuriyama, C. Higuchi, K. Tanaka, H. Yoshikawa, K. Itoh, A novel anti-rheumatic
606 drug, T-614, stimulates osteoblastic differentiation in vitro and bone morphogenetic protein-2-
607 induced bone formation in vivo, Biochem Biophys Res Commun 299(5) (2002) 903-9.
608 [https://doi.org/10.1016/s0006-291x\(02\)02754-7](https://doi.org/10.1016/s0006-291x(02)02754-7)
- 609 [2221] A. Miyama, K. Ebina, M. Hirao, G. Okamura, Y. Etani, K. Takami, A. Goshima, T.
610 Miura, S. Oyama, T. Kanamoto, H. Yoshikawa, K. Nakata, Effects of iguratimod on
611 glucocorticoid-induced disorder of bone metabolism in vitro, J Bone Miner Metab 39(4) (2021)
612 639-648. <https://doi.org/10.1007/s00774-021-01206-5>
- 613 [2322] T.J. Wronski, E.R. Morey-Holton, Skeletal response to simulated weightlessness: a
614 comparison of suspension techniques, Aviat Space Environ Med 58(1) (1987) 63-8.
- 615 [2423] T. Noguchi, K. Ebina, M. Hirao, T. Morimoto, K. Koizumi, K. Kitaguchi, H. Matsuoka,
616 T. Iwahashi, H. Yoshikawa, Oxygen ultra-fine bubbles water administration prevents bone loss
617 of glucocorticoid-induced osteoporosis in mice by suppressing osteoclast differentiation,
618 Osteoporos Int 28(3) (2017) 1063-1075. <https://doi.org/10.1007/s00198-016-3830-1>
- 619 [2524] Y. Etani, K. Ebina, M. Hirao, K. Kitaguchi, M. Kashii, T. Ishimoto, T. Nakano, G.
620 Okamura, A. Miyama, K. Takami, A. Goshima, T. Kanamoto, K. Nakata, H. Yoshikawa,
621 Combined effect of teriparatide and an anti-RANKL monoclonal antibody on bone defect
622 regeneration in mice with glucocorticoid-induced osteoporosis, Bone 139 (2020) 115525.
623 <https://doi.org/10.1016/j.bone.2020.115525>
- 624 [2625] L. Yu, M. van der Valk, J. Cao, C.Y. Han, T. Juan, M.B. Bass, C. Deshpande, M.A.
625 Damore, R. Stanton, P. Babij, Sclerostin expression is induced by BMPs in human Saos-2

626 osteosarcoma cells but not via direct effects on the sclerostin gene promoter or ECR5 element,
627 Bone 49(6) (2011) 1131-40. <https://doi.org/10.1016/j.bone.2011.08.016>

628 [2726] N. Kusu, J. Laurikkala, M. Imanishi, H. Usui, M. Konishi, A. Miyake, I. Thesleff, N.
629 Itoh, Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist
630 with unique ligand specificity, J Biol Chem 278(26) (2003) 24113-7.
631 <https://doi.org/10.1074/jbc.m301716200>

632 [2827] G. Aleshcheva, M. Wehland, J. Sahana, J. Bauer, T.J. Corydon, R. Hemmersbach, T.
633 Frett, M. Egli, M. Infanger, J. Grosse, D. Grimm, Moderate alterations of the cytoskeleton in
634 human chondrocytes after short-term microgravity produced by parabolic flight maneuvers
635 could be prevented by up-regulation of BMP-2 and SOX-9, FASEB J 29(6) (2015) 2303-14.
636 <https://doi.org/10.1096/fj.14-268151>

637 [2928] X. Jin, H. Wang, X. Liang, K. Ru, X. Deng, S. Gao, W. Qiu, Y. Huai, J. Zhang, L. Lai,
638 F. Li, Z. Miao, W. Zhang, A. Qian, Calycoxin prevents bone loss induced by hindlimb
639 unloading, NPJ Microgravity 8(1) (2022) 23. <https://doi.org/10.1038/s41526-022-00210-x>

640 [3029] M. Wu, G. Chen, Y.P. Li, TGF-beta and BMP signaling in osteoblast, skeletal
641 development, and bone formation, homeostasis and disease, Bone Res 4 (2016) 16009.
642 <https://doi.org/10.1038/boneres.2016.9>

643 [3130] M. Prideaux, A.R. Wijenayaka, D.D. Kumarasinghe, R.T. Ormsby, A. Evdokiou, D.M.
644 Findlay, G.J. Atkins, SaOS2 Osteosarcoma cells as an in vitro model for studying the transition
645 of human osteoblasts to osteocytes, Calcif Tissue Int 95(2) (2014) 183-93.
646 <https://doi.org/10.1007/s00223-014-9879-y>

647 [3234] C. Lin, X. Jiang, Z. Dai, X. Guo, T. Weng, J. Wang, Y. Li, G. Feng, X. Gao, L. He,
648 Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-
649 catenin signaling, J Bone Miner Res 24(10) (2009) 1651-61.
650 <https://doi.org/10.1359/jbmr.090411>

- 651 [3332] K. Tanaka, T. Yamaguchi, I. Kanazawa, T. Sugimoto, Effects of high glucose and
652 advanced glycation end products on the expressions of sclerostin and RANKL as well as
653 apoptosis in osteocyte-like MLO-Y4-A2 cells, Biochem Biophys Res Commun 461(2) (2015)
654 193-9. <https://doi.org/10.1016/j.bbrc.2015.02.091>
- 655 [3433] T. Uchida, Y. Sakashita, K. Kitahata, Y. Yamashita, C. Tomida, Y. Kimori, A. Komatsu,
656 K. Hirasaka, A. Ohno, R. Nakao, A. Higashitani, A. Higashibata, N. Ishioka, T. Shimazu, T.
657 Kobayashi, Y. Okumura, I. Choi, M. Oarada, E.M. Mills, S. Teshima-Kondo, S. Takeda, E.
658 Tanaka, K. Tanaka, M. Sokabe, T. Nikawa, Reactive oxygen species upregulate expression of
659 muscle atrophy-associated ubiquitin ligase Cbl-b in rat L6 skeletal muscle cells, Am J Physiol
660 Cell Physiol 314(6) (2018) C721-c731. <https://doi.org/10.1152/ajpcell.00184.2017>
- 661 [3534] J. Zhang, S. Xie, W. Ma, Y. Teng, Y. Tian, X. Huang, Y. Zhang, A newly identified
662 microRNA, mmu-miR-7578, functions as a negative regulator on inflammatory cytokines
663 tumor necrosis factor-alpha and interleukin-6 via targeting Egr1 in vivo, J Biol Chem 288(6)
664 (2013) 4310-20. <https://doi.org/10.1074/jbc.m112.351197>
- 665 [3635] Y. Fang, C. Xing, X. Wang, H. Cao, C. Zhang, X. Guo, Y. Zhuang, R. Hu, G. Hu, F.
666 Yang, Activation of the ROS/HO-1/NQO1 signaling pathway contributes to the copper-induced
667 oxidative stress and autophagy in duck renal tubular epithelial cells, Sci Total Environ 757
668 (2021) 143753. <https://doi.org/10.1016/j.scitotenv.2020.143753>
- 669 [3736] R.B. Hunter, H. Mitchell-Felton, D.A. Essig, S.C. Kandarian, Expression of
670 endoplasmic reticulum stress proteins during skeletal muscle disuse atrophy, Am J Physiol Cell
671 Physiol 281(4) (2001) C1285-90. <https://doi.org/10.1152/ajpcell.2001.281.4.c1285>
- 672 [3837] X. Li, L. Han, I. Nookaew, E. Mannen, M.J. Silva, M. Almeida, J. Xiong, Stimulation
673 of Piezo1 by mechanical signals promotes bone anabolism, Elife 8 (2019).
674 <https://doi.org/10.7554/elife.49631>
- 675 [3938] Y. Yan, L. Wang, L. Ge, J.L. Pathak, Osteocyte-Mediated Translation of Mechanical

676 Stimuli to Cellular Signaling and Its Role in Bone and Non-bone-Related Clinical
677 Complications, Curr Osteoporos Rep 18(1) (2020) 67-80. <https://doi.org/10.1007/s11914-020-00564-9>

679 [4039] J. Xiong, M. Onal, R.L. Jilka, R.S. Weinstein, S.C. Manolagas, C.A. O'Brien, Matrix-
680 embedded cells control osteoclast formation, Nat Med 17(10) (2011) 1235-41.
681 <https://doi.org/10.1038/nm.2448>

682 [4140] A. Sebastian, G.G. Loots, Transcriptional control of Sost in bone, Bone 96 (2017) 76-
683 84. <https://doi.org/10.1016/j.bone.2016.10.009>

684 [4241] C.H. Li, Z.Z. Ma, L.L. Jian, X.Y. Wang, L. Sun, X.Y. Liu, Z.Q. Yao, J.X. Zhao,
685 Iguratimod inhibits osteoclastogenesis by modulating the RANKL and TNF-alpha signaling
686 pathways, Int Immunopharmacol 90 (2021) 107219.
687 <https://doi.org/10.1016/j.intimp.2020.107219>

688 [4342] Y. Aikawa, M. Yamamoto, T. Yamamoto, K. Morimoto, K. Tanaka, An anti-rheumatic
689 agent T-614 inhibits NF- κ B activation in LPS- and TNF- α -stimulated THP-1 cells without
690 interfering with I κ B α degradation, Inflamm Res 51(4) (2002) 188-94.
691 <https://doi.org/10.1007/pl00000291>

692 [4443] M. Kohno, Y. Aikawa, Y. Tsubouchi, A. Hashiramoto, R. Yamada, Y. Kawahito, K.
693 Inoue, Y. Kusaka, M. Kondo, H. Sano, Inhibitory effect of T-614 on tumor necrosis factor-
694 alpha induced cytokine production and nuclear factor-kappaB activation in cultured human
695 synovial cells, J Rheumatol 28(12) (2001) 2591-6.

696 [4544] A. Herchenhan, F. Dietrich-Zagonel, P. Schjerling, M. Kjaer, P. Eliasson, Early Growth
697 Response Genes Increases Rapidly After Mechanical Overloading and Unloading in Tendon
698 Constructs, J Orthop Res 38(1) (2020) 173-181. <https://doi.org/10.1002/jor.24513>

699 [4645] Y. Wei, X. Sun, M. Hua, W. Tan, F. Wang, M. Zhang, Inhibitory Effect of a Novel
700 Antirheumatic Drug T-614 on the IL-6-Induced RANKL/OPG, IL-17, and MMP-3 Expression

701 in Synovial Fibroblasts from Rheumatoid Arthritis Patients, *Biomed Res Int* 2015 (2015)
702 214683. <https://doi.org/10.1155/2015/214683>

703 [4746] L. Shi, R. Kishore, M.R. McMullen, L.E. Nagy, Lipopolysaccharide stimulation of
704 ERK1/2 increases TNF-alpha production via Egr-1, *Am J Physiol Cell Physiol* 282(6) (2002)
705 C1205-11. <https://doi.org/10.1152/ajpcell.00511.2001>

706 [4847] C.E. Metzger, S. Anand Narayanan, P.H. Phan, S.A. Bloomfield, Hindlimb unloading
707 causes regional loading-dependent changes in osteocyte inflammatory cytokines that are
708 modulated by exogenous irisin treatment, *NPJ Microgravity* 6 (2020) 28.
709 <https://doi.org/10.1038/s41526-020-00118-4>

710 [4948] W. Sun, S. Chi, Y. Li, S. Ling, Y. Tan, Y. Xu, F. Jiang, J. Li, C. Liu, G. Zhong, D. Cao,
711 X. Jin, D. Zhao, X. Gao, Z. Liu, B. Xiao, Y. Li, The mechanosensitive Piezo1 channel is
712 required for bone formation, *Elife* 8 (2019). <https://doi.org/10.7554/elife.47454>

713 [5049] G. Thiel, A. Lesch, A. Keim, Transcriptional response to calcium-sensing receptor
714 stimulation, *Endocrinology* 153(10) (2012) 4716-28. <https://doi.org/10.1210/en.2012-1343>

715

716 **Figure legends**

717 **Figure 1. Effects of iguratimod on disuse osteoporosis in hindlimb-unloaded (HLU) mice.**
718 (A) Experimental protocol. (B) Body weight changes (in grams) in mice of each group. (C)
719 Representative microcomputed tomography images of the distal femur in the three groups after
720 the intervention. Scale bar: 500 μ m. (D) Quantification of trabecular bone parameters: bone
721 volume (BV)/tissue volume (TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and
722 trabecular separation (Tb.Sp). One-way ANOVA followed by Tukey's post-hoc analysis, **p
723 < 0.01, *p < 0.05 (vs. HLU mice). All data were expressed as mean \pm standard deviation for
724 eight mice in each group.

725

726 **Figure 2. Histological and histomorphometrical analyses of the distal femur for**
727 **osteoclasts, osteoblasts, and osteocytes in normal saline (NS) and hindlimb-unloaded**
728 **(HLU) mice with or without iguratimod (IGU) 5 days per week for 3 weeks.**

729 (A–C) Representative histological findings of trabecular and cortical bone in the distal femur
730 were examined using Tartrate-resistant acid phosphatase (TRAP) staining (A) and
731 immunohistochemical stains of osteocalcin (red arrows indicate osteocalcin-positive cells) (B)
732 and sclerostin (black arrows indicate sclerostin-positive osteocytes) (C) in each group (NS,
733 HLU + NS, and HLU + IGU). Scale bars: A 100 μ m, B,C 50 μ m. (D and E) Villanueva bone
734 staining of trabecular and cortical bone in the distal femur was conducted. The secondary
735 cancellous bone area was stained in green through computer image processing, whereas the
736 trabecular bone was stained in orange through computer image processing (D). Villanueva bone
737 staining was conducted under fluorescent light (E). Osteoclasts and trabecular bone were
738 stained in pink through computer image processing (G). Scale bars: D 500 μ m; E 100 μ m; F
739 10 μ m. Trabecular bone parameters, including bone formation rate per total volume (BFR/TV)
740 and erosion surface/bone surface (ES/BS) were quantified (F and H). One-way ANOVA
741 followed by Tukey's post-hoc analysis, **p < 0.01, *p < 0.05 (vs. HLU mice). All data were
742 expressed as the mean \pm standard deviation (A–C; n = 8, F and H; n = 3)

743

744 **Figure 3. Effects of iguratimod (IGU) on osteocytes and**
745 **osteoclastogenesis/osteoblastogenesis through osteocytes in vitro.**

746 (A) Effects of IGU on osteoclast-related gene expression in MLO-Y4 cells after tumor necrosis
747 factor (TNF)- α stimulation were analyzed using RT-qPCR analysis (data from three
748 independent experiments for each group). (B) Investigation of the effects of IGU on osteoclast
749 formation in cocultures of TNF α -stimulated MLO-Y4 and bone marrow cells as osteoclast
750 precursors (data from five to six independent experiments for each group). Scale bar: 500 μ m.

751 (C) RT-qPCR analysis of osteoblast-related gene expression in MC3T3-E1 cells treated with
752 BMP6 and sclerostin with or without IGU (data from three independent experiments for each
753 group). (D) Alkaline phosphatase (ALP) activity was measured using MC3T3-E1 cells cultured
754 with BMP6 and sclerostin with or without IGU (data from three independent experiments for
755 each group). (E) Effects of IGU on osteocyte-related gene expression in Saos-2 cells after
756 BMP2 stimulation were analyzed using RT-qPCR analysis (data from three independent
757 experiment data for each group). (F) Western blotting analysis of SOST using Saos-2 cells
758 treated with BMP2 with or without IGU. (G) Effects of IGU on the mitogen-activated protein
759 kinases signaling pathway in Saos-2 cells after BMP2 stimulation were analyzed using Western
760 blotting analysis. Statistical significance was determined using one-way ANOVA followed by
761 Tukey's post-hoc test (**p < 0.01, *p < 0.05). All data are presented as the mean \pm standard
762 deviation.

763

764 **Figure 4. Effects of iguratimod (IGU) on the NF- κ B pathway in Saos-2 cells.**

765 (A) Luciferase assay performed on Saos-2 cells cultured with TNF α /BMP2 with or without
766 IGU/dexamethasone (Dex). NF- κ B luciferase activity was expressed relative to that of the
767 control, set at 100% (data from five to seven independent experiments for each group). (B)
768 Western blotting analysis of p-p65 and p65 using nuclear and cytosolic proteins extracted from
769 Saos-2 cells cultured with BMP2 with or without IGU. β -actin and Lamin B1 were used as the
770 internal controls for the cytosolic and nuclear fractions, respectively. (C) Effects of IGU on the
771 nuclear translocation of NF- κ B p65 using immunofluorescence staining in Saos-2 cells
772 cultured with BMP2 with or without IGU. Scale bars: 30 μ m. The quantification of nuclear-
773 cytoplasmic intensity ratios was normalized to the control, set at 100 (data from eight
774 independent experiments for each group). Statistical significance was determined using one-
775 way ANOVA followed by Tukey's post-hoc test (**p < 0.01, *p < 0.05). All data are presented

776 as the mean \pm standard deviation.

777

778 **Figure 5. Potential of iguratimod to regulate early growth response protein 1 (EGR1)**
779 **expression identified through RNA sequencing analysis on Saos-2 cells.**

780 (A) This heat map visualizes the expression levels of differentially expressed genes identified
781 from RNA-seq datasets between BMP2-treated and BMP2 + IGU-treated groups. Green
782 represents replicates with low expression, whereas red represents those with high expression.
783 (B) Gene Ontology analysis of functional annotations for biological processes upregulated and
784 downregulated by IGU treatment. (C) The top 10 transcription factor genes whose expression
785 was significantly altered by IGU treatment. (D) Volcano plot displaying the differentially
786 expressed transcripts between the BMP2-treated and BMP2 + IGU-treated groups. (E)
787 Immunohistochemical staining of EGR1 in the distal femur cortical bone of each group (NS,
788 HLU + NS, and HLU + IGU) (data from eight independent experiments for each group). The
789 black arrows indicate EGR1-positive osteocytes. (F) RT-qPCR analysis of EGR1 gene
790 expression was performed using Saos-2 cells treated with or without BMP2 and IGU (data
791 from three independent experiments for each group). Statistical significance was determined
792 using one-way ANOVA followed by Tukey's post-hoc test (**p < 0.01, *p < 0.05). All data are
793 presented as the mean \pm standard deviation.

794

795 **Figure 6. Effects of iguratimod (IGU) on the production of sclerostin and RANKL from**
796 **osteocytes through the ERK/EGR1/TNF α pathway.**

797 (A) Immunohistochemical staining of heme oxygenase (HO)-1 in the distal femur of each
798 group (NS, HLU + NS, and HLU + IGU) (data from seven to eight independent experiments
799 for each group). (B) Western blotting analysis of ERK1/2 was performed on Saos-2 cells treated
800 with or without BMP2 and IGU. (C) Immunohistochemical staining of TNF α in the distal

801 femur cortical bone of each group (NS, HLU + NS, and HLU + IGU) (data from seven to eight
802 independent experiments for each group). The red arrows indicate TNF α -positive osteocytes.
803 (D) Saos-2 cells were transiently transfected with the EGR1 overexpression vector and treated
804 with or without IGU. RT-qPCR analysis of EGR1, TNF α , and osteocyte-related gene
805 expression (data from three independent experiment data for each group). (E) Saos-2 cells were
806 transiently transfected with EGR1-specific siRNA. RT-qPCR analysis of EGR1 and osteocyte-
807 related gene expression (data from three independent experiment data for each group). An
808 unpaired Student's t-test was employed for comparisons between two groups, whereas one-
809 way ANOVA followed by Tukey's post-hoc test was used for comparisons between more than
810 three groups. All data are expressed by the mean \pm standard deviation. Differences were
811 considered relevant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$).

812

813 **Figure 7. Hypothetic scheme summarizing the effects of iguratimod (IGU).**

814 Mechanical unloading may induce reactive oxygen species (ROS) and activate the downstream
815 ERK/EGR1/TNF α pathway in osteocytes. IGU may inhibit the phosphorylation of ERK1/2 and
816 downstream EGR1/TNF α pathway, thereby suppressing the expression of RANKL and
817 sclerostin, potentially ameliorating disuse osteoporosis.

818

819 **Supplemental Figure 1. Effects of iguratimod on cortical bone loss in hindlimb-unloaded**
820 **(HLU) mice.**

821 **(A) Tail-suspended hindlimb-unloaded mice confined in the cage, demonstrating the**
822 **suspension system. (B) Representative microcomputed tomography images of the distal femur**
823 **in the three groups after the intervention. Scale bars: 500 μ m. (C) Quantification of the cortical**
824 **bone parameter: cortical thickness (Ct.Th). One way ANOVA followed by Tukey's post hoc**
825 **analysis, ** $p < 0.01$ (vs. HLU mice). All data were expressed as mean \pm standard deviation for**

826 eight mice in each group.

827

828 **Supplemental Figure 2. Effects of iguratimod (IGU) on osteocyte-related gene, Dkk-1**
expression.

829

830 Western blotting analysis of Dkk-1 using Saos-2 cells treated with bone morphogenetic protein-
2 with or without IGU.

831

832

833 **Supplemental Figure 3. Effects of iguratimod (IGU) on osteocyte apoptosis.**

834 (A) Quantification of the relative number of empty lacunae in the cortical bone of the distal
femur obtained from each group (normal saline (NS), hindlimb unloaded (HLU) + NS, and
HLU + IGU). The black arrows indicate the empty lacunae. (B) Reverse transcription
quantitative polymerase chain reaction analysis of apoptosis related gene expression in Saos-2
cells treated with bone morphogenetic protein-2 (BMP2) with or without IGU (data from three
independent experiment data for each group). (C) Evaluation of cell proliferation in Saos-2
cells treated with different concentrations of IGU in the presence of BMP2 (data from five to
six independent experiments for each group). Statistical significance was determined using
one way ANOVA followed by Tukey's post hoc test (*p < 0.05). All data are presented as mean
+ standard deviation.

844

845

846

847 **Supplemental Figure 4. The heatmap of differentially expressed genes, which were**
detected by RNA sequencing analysis on Saos-2 cells from untreated, bone morphogenetic
protein-2 (BMP2)-treated, and BMP2 + iguratimod (IGU)-treated groups.

848 Hierarchical clustering analysis was performed to generate a gene expression profile map of
Saos-2 cells under untreated conditions, as well as conditions treated with BMP2 or BMP2 +
IGU. The gene expression levels are represented by different colors: red points indicate up-

851 regulated genes, green points indicate down-regulated genes and black points represent genes
852 with no change in expression.

853

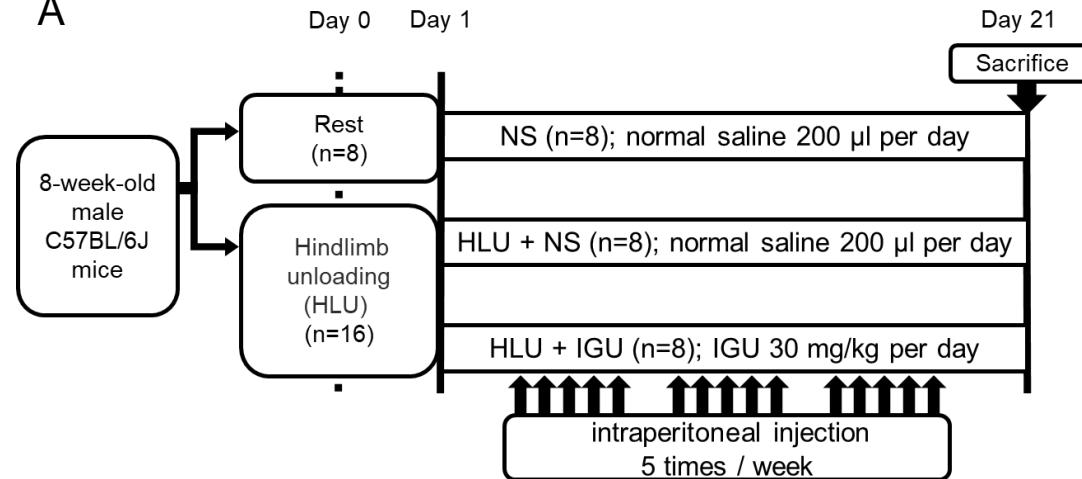
854 **Supplemental Figure 5. Effects of iguratimod (IGU) on Piezo1-related genes.**

855 (A) Immunohistochemical staining of Piezo1 in the distal femur cortical bone of each group
856 (normal saline (NS), hindlimb unloaded (HLU) + NS, and HLU + IGU) (data eight
857 independent experiments for each group). The black arrows indicate Piezo1-positive osteocytes.

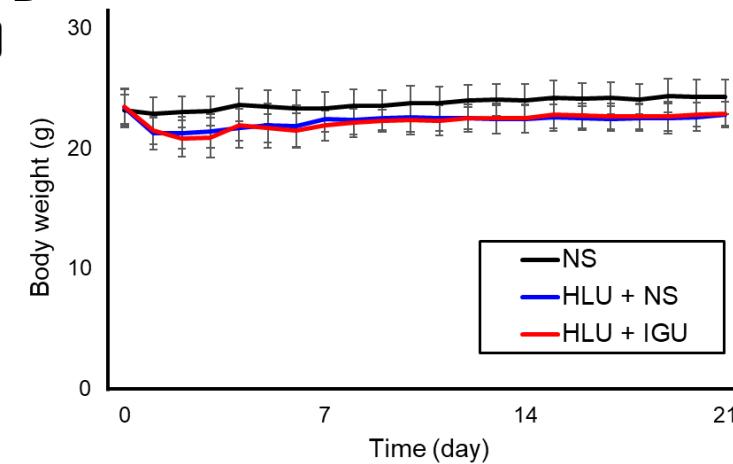
858 (B and C) Saos 2 cells were transiently transfected with the early growth response protein 1
859 (EGR1) overexpression vector (B) and the EGR1-specific siRNA (C). Reverse transcription
860 quantitative polymerase chain reaction analysis was conducted to examine the expression of
861 EGR1 and Piezo1 related genes (data from three independent experiment data for each group).
862 Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test
863 (**p < 0.01). All data are expressed as the mean ± standard deviation.

Figure 1

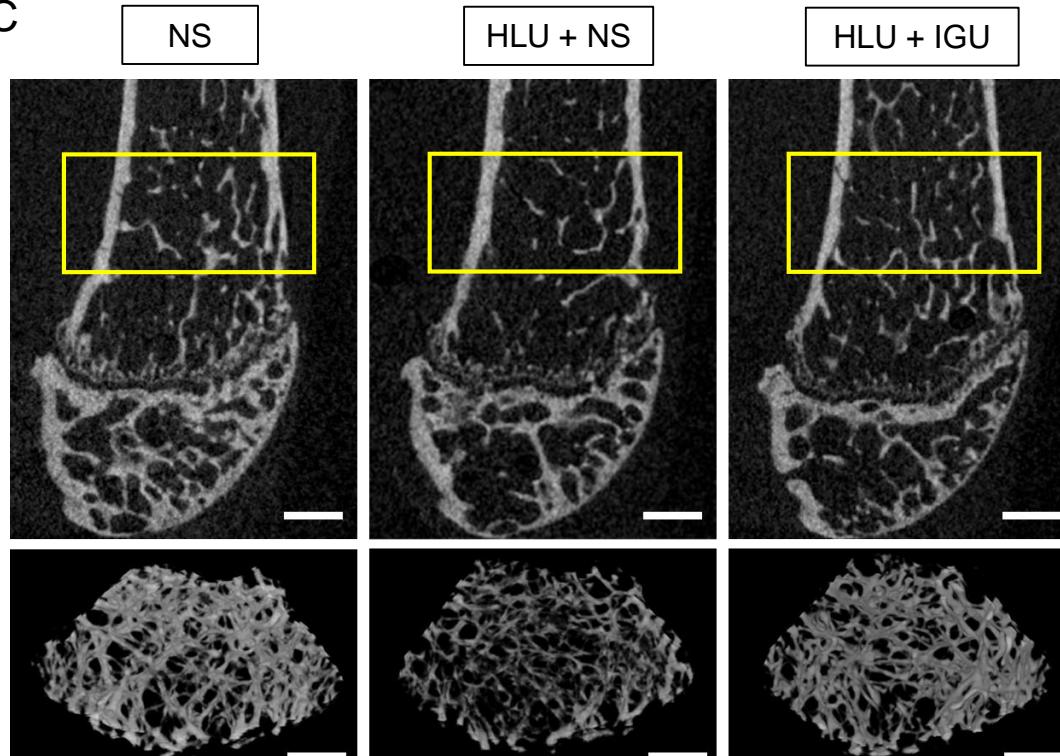
A



B



C



D

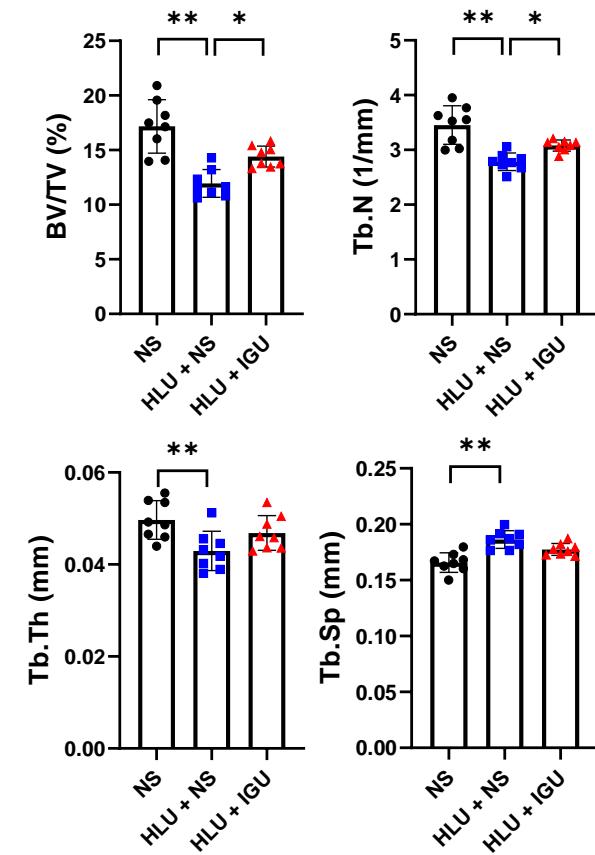


Figure 2

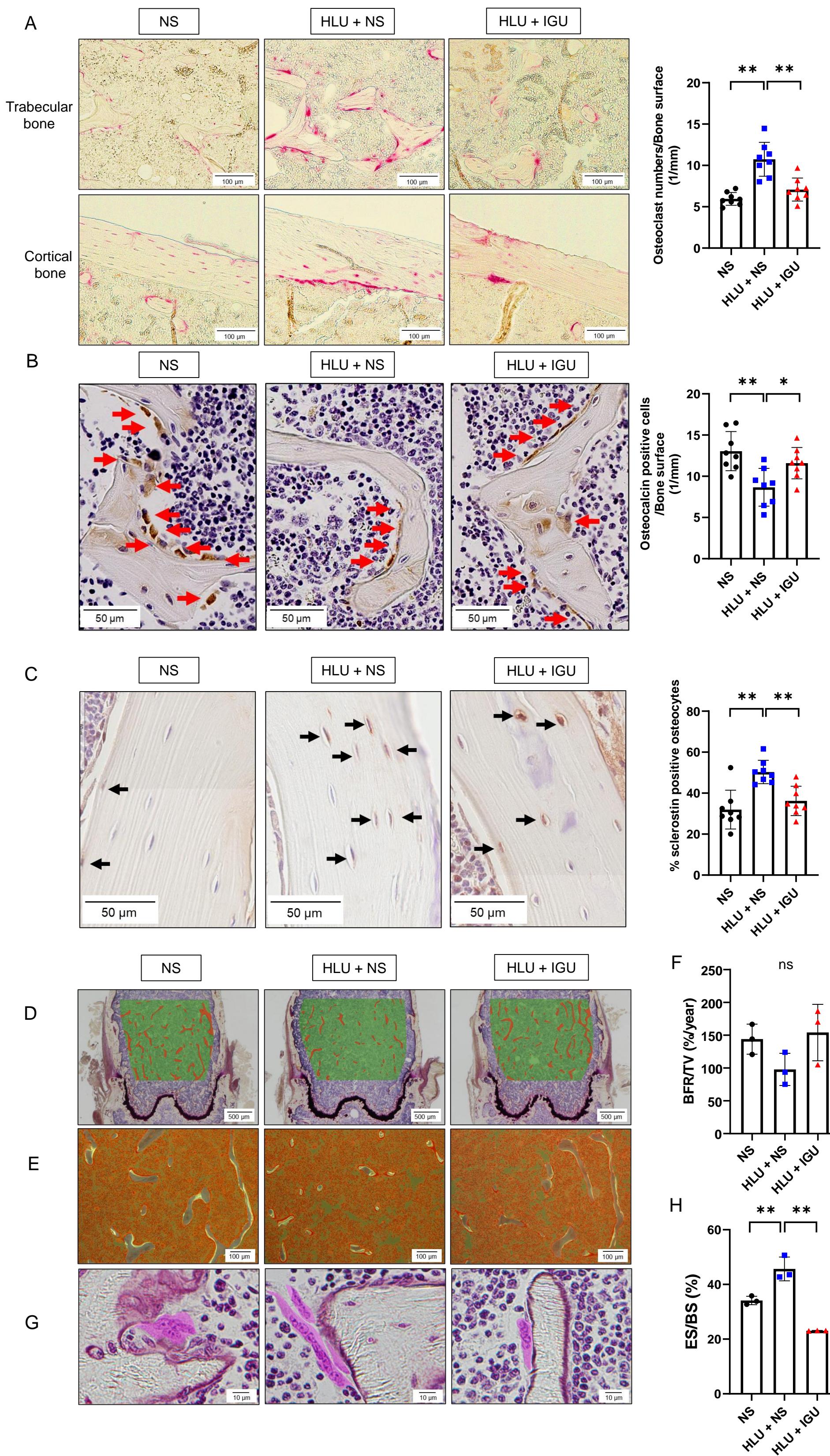
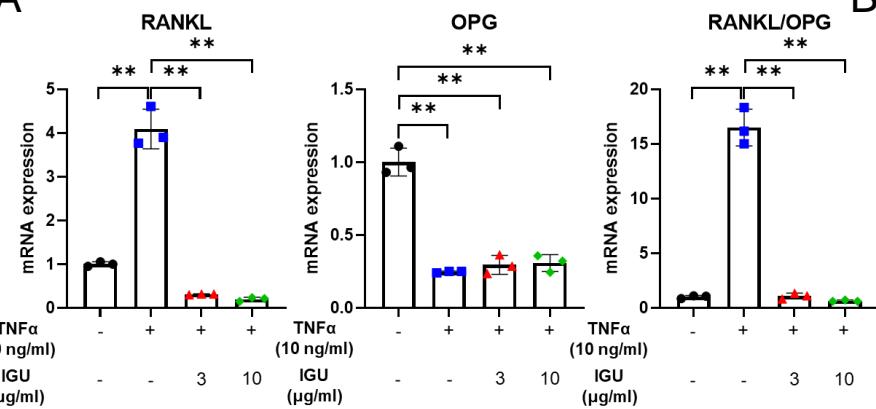


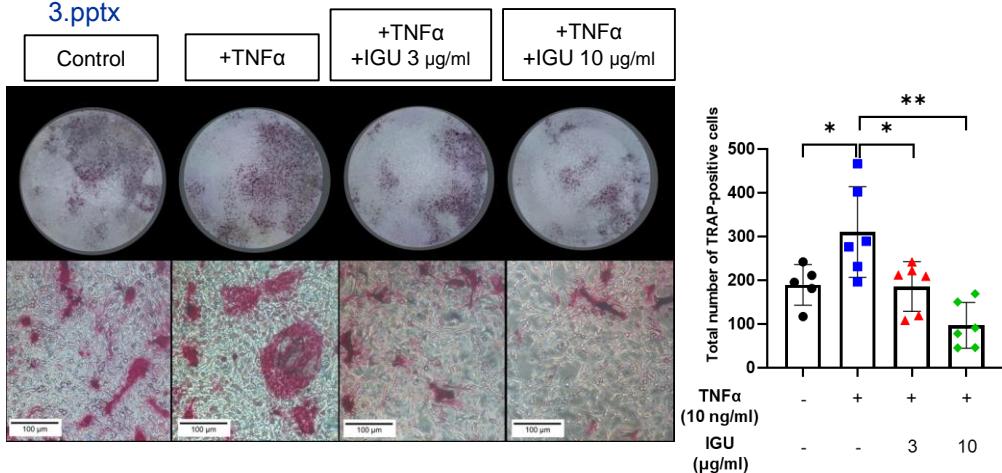
Figure 3

A

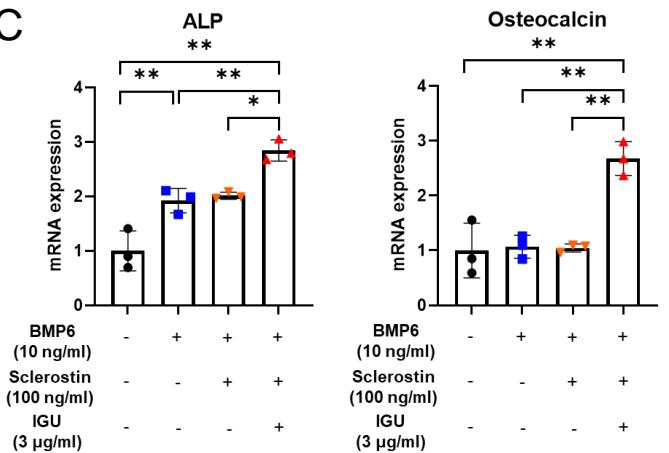


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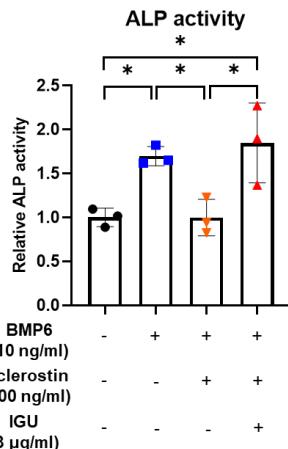
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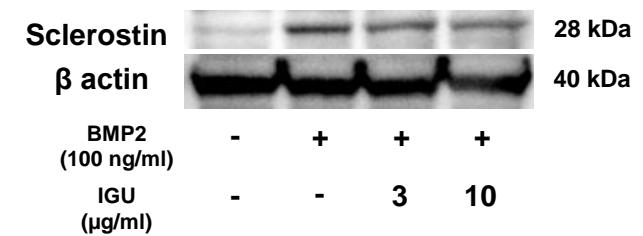
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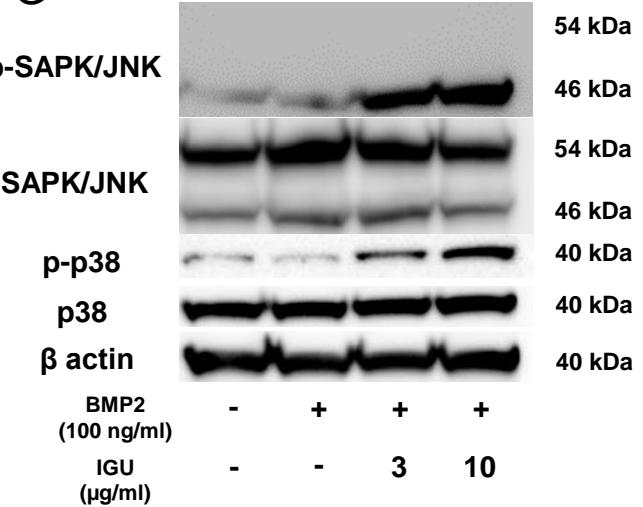
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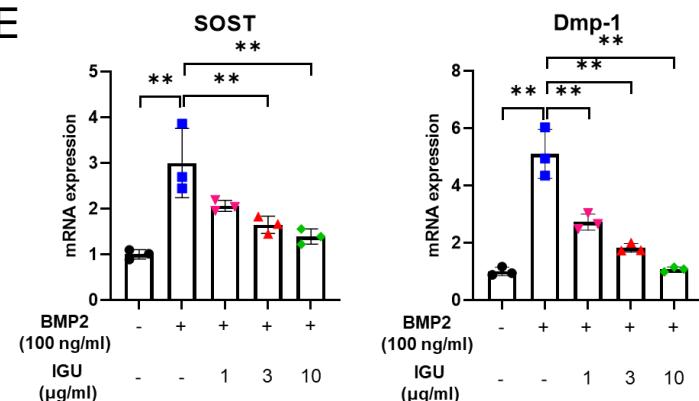
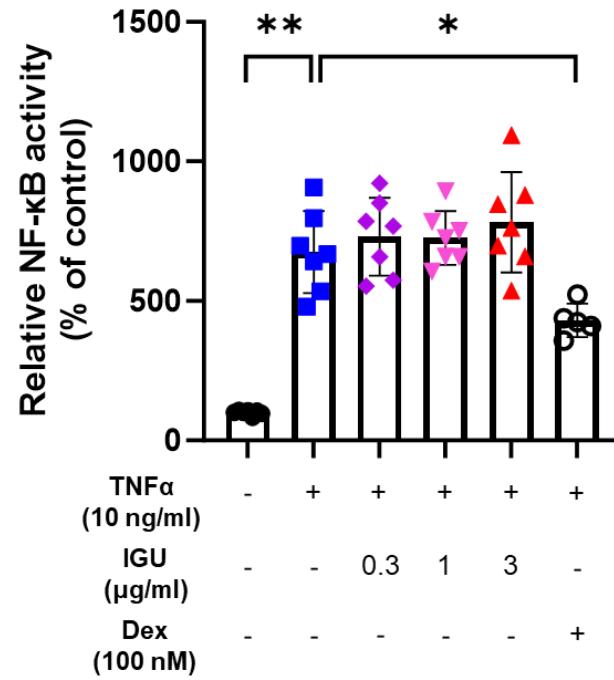
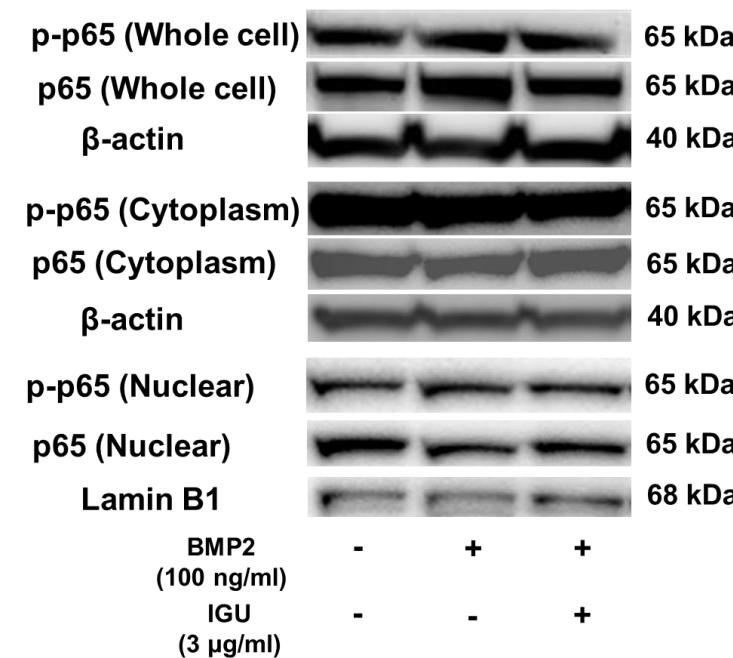


Figure 4

A



B



C

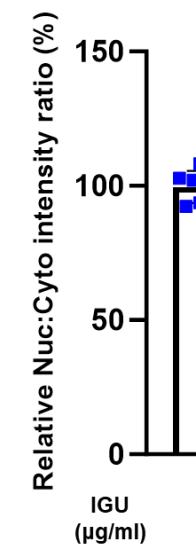
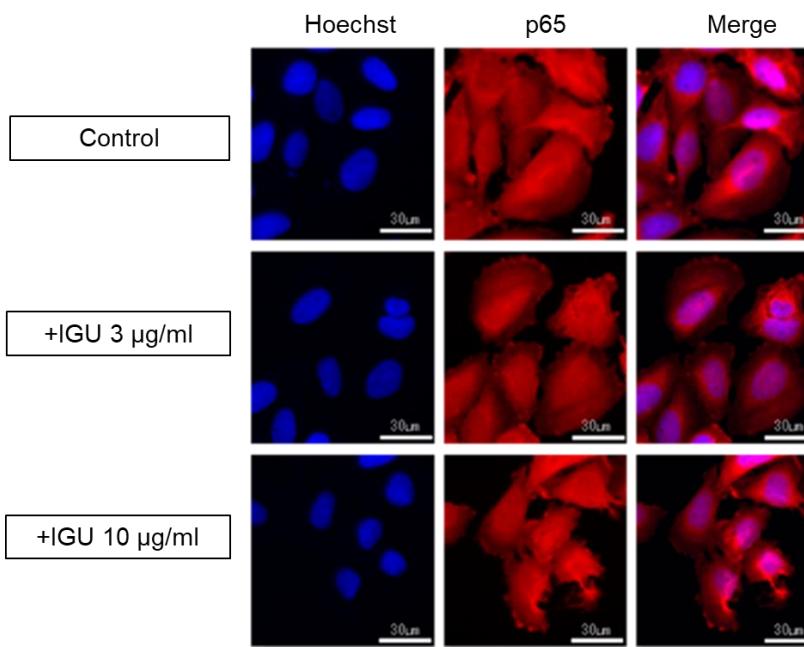
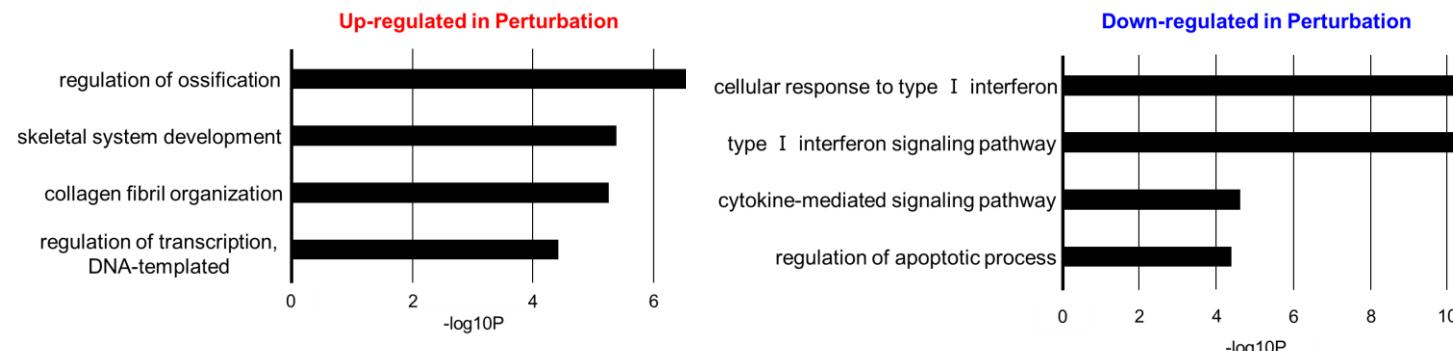


Figure 5

A



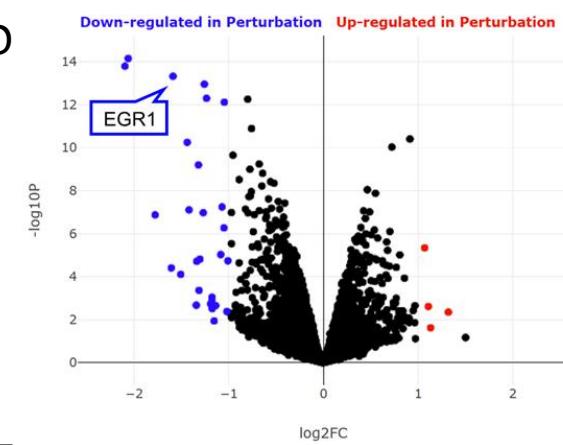
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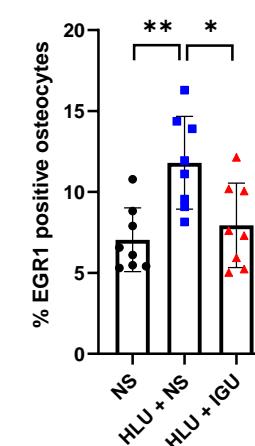
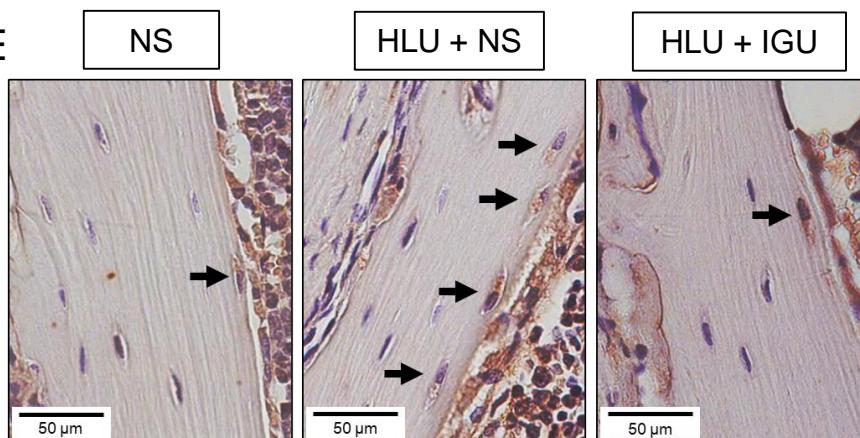
C

Gene	logFC	AveExpr	P-value	FDR	GO Biological Process
MEPE	-2.06	4.26	6.98E-15	9.67E-11	skeletal system development
DMP1	-2.09	4.52	1.61E-14	1.11E-10	ossification
EGR1	-1.59	5.60	4.75E-14	2.20E-10	regulation of dna-templated transcription
PLEKHA6	-1.25	6.63	1.11E-13	3.84E-10	N/A
TAC3	-1.23	6.40	5.03E-13	1.27E-09	signaling (neuropeptide)
SOST	-0.80	10.19	5.51E-13	1.27E-09	signaling (Wnt, BMP)
IBSP	-1.04	7.38	7.60E-13	1.50E-09	cell adhesion
KCNN3	-0.75	8.39	1.28E-11	2.22E-08	ion transport
IGFBP5	0.92	6.21	3.94E-11	6.07E-08	signaling (insulin-like growth factor)
FRZB	-1.44	3.99	5.59E-11	7.75E-08	signaling (Wnt)

D



E



F

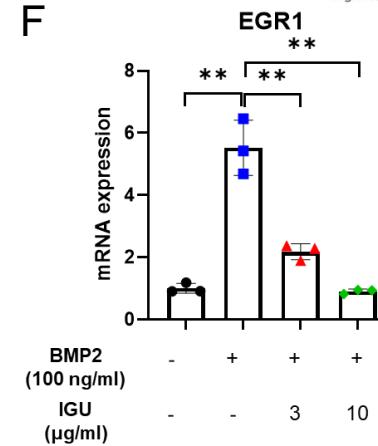


Figure 6

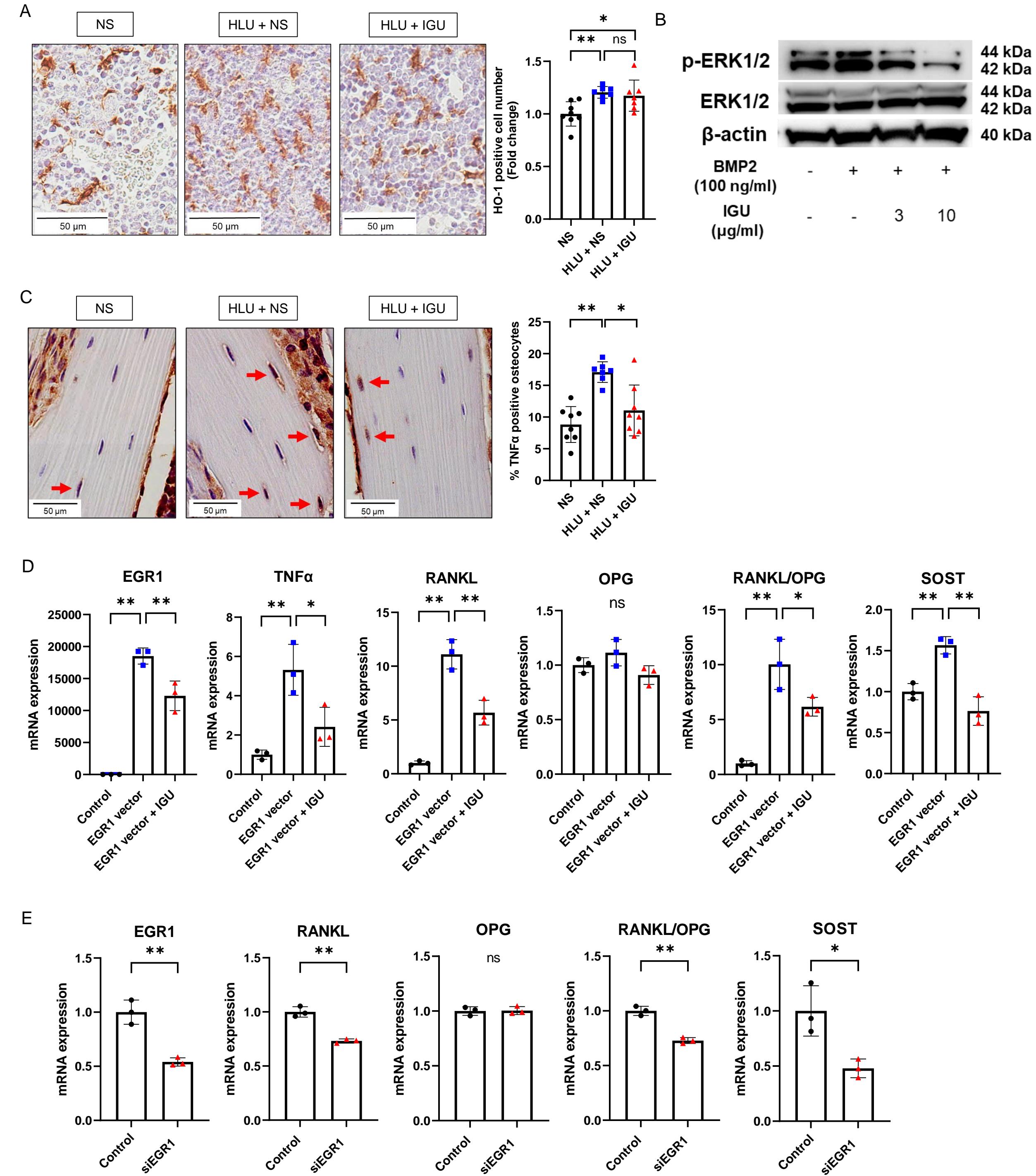


Figure 7

