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Crif1 Promotes Osteoporosis in Mice after Radiation

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40 **Abstract**

41 Radiation induces rapid bone loss and enhances bone resorption and RANKL expression.
42 RANKL provides the crucial signal to induce osteoclast differentiation and plays an
43 important role in bone resorption. However, the mechanisms of radiation-induced
44 osteoporosis are not fully understood. Here, we show that Crif1 expression increases in bone
45 marrow cells after radiation. Conditional Crif1 deletion in bone marrow cells causes
46 decreases in RANKL expression and the RANKL/OPG ratio, and relieves bone loss after
47 radiation in mice. We further demonstrated *in vitro* that Crif1 promotes RANKL secretion via
48 the cAMP/PKA pathway. Moreover, protein–protein docking screening identified five
49 compounds as Crif1 inhibitors; these compounds dramatically suppressed RANKL secretion
50 and CREB phosphorylation when cells were exposed to forskolin. This study enriches current
51 knowledge of the pathogenesis of osteoporosis and provides insights into potential
52 therapeutic strategies for osteoporosis treatment.

53

54 **Key words** Crif1, PKA, RANKL, Osteoporosis, Radiation

55

56 **Introduction**

57 Osteoporosis is a high-incidence disease closely associated with radiotherapy, hormonal
58 status, age and glucocorticoid treatment. It is characterized by an imbalance in skeletal
59 turnover with reduced bone formation and enhanced bone resorption, leading to an increased
60 risk of bone fracture(Khosla & Hofbauer, 2017; Phetfong et al., 2016). Osteoblasts are the
61 bone-forming cells derived from bone marrow mesenchymal stem/stromal cells (BM-MSCs)
62 and play an important role in the regulation of bone mass. Meanwhile, osteoclasts are the
63 principal cells capable of resorbing bone and play an essential role in bone
64 remodelling(Rachner, Khosla, & Hofbauer, 2011). Exposure to radiation, such as
65 radiotherapy for cancer, can cause rapid mineral loss and increase the number of osteoclasts
66 within metabolically active, cancellous bone tissue, leading to structural deficits. However,
67 the mechanisms of radiation-induced bone loss are not fully understood. Current treatment of
68 osteoporosis is based mainly on inhibiting bone resorption or stimulating bone generation to
69 increase bone mass, novel treatment strategies that have a dual mechanisms should be
70 developed.

71 Osteoclasts are large, multinucleated cells derived from haematopoietic progenitors of the
72 monocyte macrophage lineage(Boyle, Simonet, & Lacey, 2003). Their differentiation is
73 mainly regulated by macrophage colony-stimulating factor (M-CSF), RANK ligand
74 (RANKL), and osteoprotegerin (OPG)(Teitelbaum, 2000). M-CSF is required for osteoclast
75 survival and proliferation, but RANKL and OPG play central roles in the activation of
76 osteoclastogenesis(Boyce & Xing, 2007). By binding to its receptor RANK (on
77 haematopoietic progenitors), RANKL provides the crucial signal to induce osteoclast
78 differentiation from haematopoietic progenitor cells as well as to activate mature osteoclasts.
79 OPG is a soluble decoy receptor that can bind to RANKL and negatively regulate RANKL
80 binding to RANK(Wada, Nakashima, Hiroshi, & Penninger, 2006). BM-MSCs, osteocytes,
81 osteoblasts, adipocytes, and activated T and B lymphocytes are the main sources of RANKL
82 secretion. RANKL expression is promoted by radiation, inflammation, cytokines, hormones
83 and a number of other agents, including those that signal through cyclic adenosine
84 monophosphate (cAMP)/protein kinase A (PKA), glycoprotein 130 (gp130) and vitamin D
85 receptor (VDR)(Martin & Sims, 2015; Nakashima et al., 2011).

86 Crif1 is a multifunctional protein that can interact with many proteins to induce cell

87 cycle arrest, modulate oxidative stress, and regulate transcriptional activity through
88 interactions with the DNA-binding domains of transcription factors(Chen et al., 2017; Chung
89 et al., 2003; Kang, Hong, Kim, & Bae, 2010; Park et al., 2005; Ran et al., 2014). It is also the
90 constitutive protein of the large mitoribosomal subunit required for the synthesis and
91 insertion of mitochondrial-encoded OxPhos polypeptides into the mitochondrial
92 membrane(Greber et al., 2015). Crif1 deficiency in macrophages impairs mitochondrial
93 oxidative function and causes systemic insulin resistance and adipose tissue
94 inflammation(Jung et al., 2018). Our previous study showed that Crif1 promotes adipogenic
95 differentiation of BM-MSCs after radiation by modulating the cAMP/PKA signalling
96 pathway(Zhang et al., 2015).

97 In this study, we investigated the role of Crif1 in osteoporosis after radiation. To address
98 this question *in vivo*, we generated a *Crif1* bone marrow-specific conditional knockout mouse
99 model. Conditional *Crif1* deletion causes decreases in RANKL expression and the
100 RANKL/OPG ratio and reduces bone loss after radiation. In this study, we demonstrate that
101 Crif1 promotes osteoporosis by regulating RANKL expression via the cAMP/PKA pathway.
102 Through screening, we also identify five compounds that could effectively inhibit RANKL
103 expression.

104

105 **Results**

106 **Radiation induces osteoporosis in mice**

107 To confirm the extent of bone loss over the short term after irradiation, we irradiated mice
108 with a single dose of 5 Gy, and then, 7 days later, we harvested the left femurs. Micro-CT
109 analysis of the distal femurs of males and females at 12 weeks of age revealed significant
110 decreases in trabecular bone volume/total volume (BV/TV) (Figures 1A and 1B),
111 connectivity density (Conn.D) (Figure 1C), trabecular number (Tb.N*) (Figure 1D), and bone
112 mineral density (vBMD) (Figure 1E), but also showed significant increases in trabecular
113 spacing (Tb.Sp*) (Figure 1G) and structure model index (SMI) (Figure 1H). There was no
114 significant difference in trabecular thickness (Tb.Th*) (Figure 1F). Haematoxylin and eosin
115 (H&E) staining of femoral sections from irradiated mice showed significantly decreased
116 trabecular bone compared to controls (Figure 1I). Paraffin sections of femurs showed more
117 TRAP-positive cells in irradiated mice than in control mice (Figure 1J). These results
118 indicated that bone resorption was enhanced after radiation and validated the model of
119 radiation-induced osteoporosis. Moreover, RT-qPCR data revealed dramatic increases in
120 RANKL expression (Figure 1K) and the RANKL/OPG ratio in irradiated bone marrow cells
121 (Figure 1L). OPG expression was not affected by radiation treatment (Figure 1K). Notably,
122 expression of Crif1 also increased in irradiated bone marrow cells compared with control
123 cells 7 days after irradiation (Figure 1M).

124 **Conditional Crif1 deletion from bone marrow relieves bone loss after radiation**

125 To explore the possible relationship between Crif1 and RANKL, we constructed a bone
126 marrow cell-specific Crif1-deficient mouse model by crossing *Crif1^{fl/fl}* mice to
127 *B6.129P2-Lyz2tm1(cre)/Nju* mice to generate *Lyz2Cre;Crif1^{fl/fl}* mice (Figure 2A) and treated
128 the mice with a single dose of 5 Gy. RT-qPCR results demonstrated that *Lyz2Cre;Crif1^{fl/fl}* mice
129 had lower RANKL expression (Figure 2B) and RANKL/OPG ratio in bone marrow cells
130 after radiation compared to *Crif1^{fl/fl}* mice (Figure 2C). OPG expression in bone marrow cells
131 was not affected by *Crif1* deletion (Figure 2B). Therefore, we speculated that Crif1 may be
132 involved in the regulation of RANKL expression. To further assess the effects of *Crif1*
133 deletion on osteoporosis, trabecular bone parameters of femurs were evaluated by micro-CT

134 analysis. Conditional *Crif1* deletion from bone marrow had no effect on bone mass (Figures
135 2D-2K). Although trabecular bone volume/total volume, connectivity density and trabecular
136 number all decreased in *Crif1^{fl/fl}* mice and *Lyz2Cre;Crif1^{fl/fl}* mice after radiation compared to
137 nonirradiated mice, these three parameters were significantly higher in irradiated
138 *Lyz2Cre;Crif1^{fl/fl}* mice than in irradiated *Crif1^{fl/fl}* mice (Figures 2E, 2F and 2G). Notably,
139 trabecular spacing in *Crif1^{fl/fl}* mice and *Lyz2Cre;Crif1^{fl/fl}* mice both increased visibly after
140 radiation, but the increase in *Lyz2Cre;Crif1^{fl/fl}* mice was less than that of *Crif1^{fl/fl}* mice after
141 radiation treatment (Figure 2J). Furthermore, bone mineral density decreased (Figure 2H) and
142 structural model index increased (Figure 2K) only in irradiated *Crif1^{fl/fl}* mice compared to
143 nonirradiated controls, while no remarkable changes in these parameters were detected
144 between nonirradiated and irradiated *Lyz2Cre;Crif1^{fl/fl}* mice (Figure 2H and 2K). In addition,
145 trabecular thickness was not influenced by *Crif1* deletion or radiation treatment (Figure 2I).
146 H&E staining of paraffin sections of femurs confirmed the previously reported micro-CT
147 data depicting the reduced loss of trabecular tissue in *Lyz2Cre;Crif1^{fl/fl}* mice compared to
148 *Crif1^{fl/fl}* mice after radiation (Figure 2L). We also detected a decreased number of
149 TRAP-positive osteoclasts in irradiated *Lyz2Cre;Crif1^{fl/fl}* mice compared to irradiated *Crif1^{fl/fl}*
150 mice, indicating weakened bone resorption (Figure 2M). Taken together, these data suggested
151 that conditional *Crif1* deletion could alleviate bone resorption and reduce bone loss after
152 radiation.

153 **Overexpression of Crif1 in BM-MSCs increases RANKL secretion and the ratio of** 154 **RANKL to OPG**

155 To investigate the role of *Crif1* in osteoporosis *in vitro*, we transfected mouse BM-MSCs
156 with a *Crif1* lentiviral overexpression vector (Figure 3A). For osteoclast induction *in vitro*,
157 *Crif1*-overexpressing BM-MSCs and RAW264.7 cells were cocultured in a 12-well transwell
158 unit for 7 days. RT-qPCR results showed that the relative mRNA expression of RANKL and
159 RANKL/OPG ratio both increased in *Crif1*-overexpressing BM-MSCs compared to controls
160 after 7 days of coculture (Figure 3B and 3C). Concentrations of RANKL and OPG in
161 coculture medium were also detected by ELISA. *Crif1*-overexpressing BM-MSCs produced
162 high levels of RANKL compared to the control (Figure 3D), while there was no significant
163 difference in OPG concentration between the two groups (Figure 3E). The RANKL/OPG
164 ratio in *Crif1*-overexpressing BM-MSCs was higher than in the control (Figure 3F). We also
165 detected an increased number of tartrate-resistant alkaline phosphatase (TRAP)-positive cells
166 in RAW264.7 cells cocultured with *Crif1*-overexpressing BM-MSCs (Figures 3G and 3H).
167 These data suggested that *Crif1* could promote RANKL expression and may be involved in
168 osteoclast differentiation.

169 **Crif1 is involved in the regulation of RANKL expression after radiation**

170 To further confirm whether *Crif1* played an important role in osteoporosis after radiation, we
171 knocked out *Crif1* in RAW264.7 cells and BM-MSCs (Figures 4A and 4D), respectively.
172 Deletion of *Crif1* from RAW264.7 cells did not affect RANK expression or osteoclast
173 differentiation (Figures 4A, 4B and 4C). We previously demonstrated that *Crif1* expression
174 was upregulated after radiation (Zhang et al., 2015), and in this study, we found that RANKL
175 expression and the RANKL/OPG ratio were also elevated after radiation (Figures 4E, 4F, 4G
176 and 4I). Meanwhile, more TRAP-positive cells were found in RAW264.7 cells cocultured
177 with BM-MSCs after radiation (Figures 4J and 4K). However, knocking out *Crif1* in
178 BM-MSCs could significantly reduce RANKL expression and the RANKL/OPG ratio both
179 before and after radiation (Figures 4E, 4F, 4G and 4I). OPG expression was not affected by
180 *Crif1* deletion or radiation treatment (Figures 4E and 4H). Moreover, the number of

181 TRAP-positive cells also decreased in *Crif1* knockout BM-MSCs compared to the control
182 after 7 days of coculture (Figures 4J and 4K). These results further demonstrated that *Crif1*
183 can regulate RANKL expression, especially after radiation.

184 ***Crif1* mediates adipogenesis and RANKL secretion in adipocytes**

185 To determine whether *Crif1* affects RANKL expression in adipocytes, BM-MSCs were
186 grown in mouse mesenchymal stem cell adipogenic differentiation medium. Consistent with
187 our previous research (Zhang et al., 2015), more BM-MSCs became strongly predisposed to
188 adipogenesis (Figures 5A, 5B and 5C). Recently, it was reported that bone marrow
189 adipocytes can secrete RANKL (Fan et al., 2017). Here, we found an obvious increase in
190 RANKL expression and RANKL/OPG ratio in irradiated BM-MSCs after adipogenic
191 induction (Figures 5D, 5E, 5F and 5H). However, knocking out *Crif1* in BM-MSCs reduced
192 both adipogenesis and RANKL expression (Figures 5C, 5D and 5F). OPG expression was not
193 affected by *Crif1* deletion or radiation treatment (Figures 5D and 5G). These data suggested
194 that *Crif1* mediates adipogenesis and RANKL secretion in adipocytes.

195 ***Crif1* promotes RANKL secretion by modulating the cAMP/PKA signalling pathway**

196 To verify the mechanism underlying *Crif1*-mediated upregulation of RANKL expression,
197 PKA agonist (forskolin) and inhibitor (H89) were added to the coculture system. Although
198 RANKL expression and the RANKL/OPG ratio were both increased after treatment with 25
199 μM forskolin, these effects were significantly weakened in *Crif1* knockout BM-MSCs
200 (Figures 6A, 6B, 6C and 6E). In addition, RANKL expression and the RANKL/OPG ratio
201 were both decreased when *Crif1*-overexpressing BM-MSCs and controls were treated with
202 20 μM H89 (Figures 6F, 6G, 6H and 6J). OPG expression was not affected by forskolin or
203 H89 treatment (Figures 6A, 6D, 6F and 6H). The most TRAP-positive cells were found in the
204 coculture with forskolin-treated BM-MSCs (Figures 6K and 6L), the fewest TRAP-positive
205 cells were found in the coculture with H89-treated cells (Figures 6M and 6N). After the
206 addition of forskolin, CREB phosphorylation was significantly increased in the control
207 BM-MSCs, but was dramatically inhibited in *Crif1* knockout BM-MSCs (Figure 6O). We
208 also observed that CREB phosphorylation was suppressed in both *Crif1*-overexpressing
209 BM-MSCs and controls following exposure to H89 (Figure 6P). These results demonstrated
210 that *Crif1* promotes RANKL expression through cAMP/PKA signalling pathway.

211 ***Crif1* inhibitors effectively suppress RANKL secretion and CREB phosphorylation**

212 We used ClusPro and InterProSurf to investigate the interface in *Crif1*-PKA α complex, and
213 results showed that Thr¹⁹⁷, Gly²⁰⁰, Thr²⁰¹, Glu²⁰³, and Phe¹²⁹ of PKA α interact with Ile¹³²,
214 Met¹²⁸, Ile¹²¹, His¹²⁰, and Arg¹¹⁷ in the long alpha helical region of *Crif1*, forming the binding
215 interface (Figure 7A). A virtual screening using 462,608 compounds from the Life Chemicals
216 database around His¹²⁰ of *Crif1* was carried out using the program Autodock_vina. A set of
217 13 compounds was selected for experimental screening based on binding energy <-12.0
218 kcal/mol (Table S1). Initially a tetrazolium salt (WST-8) assay was carried out to study
219 potential toxic effects of these compounds on hBM-MSCs, vero cells and mouse BM-MSCs
220 (Figures S1-S3). The compounds F0382-0033, F3408-0076, F1430-0134, F3408-0031 and
221 F1430-0130 showed low toxicity to hBM-MSCs at concentrations of 25 μM (Figure 7G-7K).
222 The binding pattern in these ligand-protein complexes potentially contained multiple
223 interactions dominated by hydrophobic amino acids (Figure 7B-7F). To determine whether
224 these 5 *Crif1* inhibitors affected RANKL expression, hBM-MSCs were pretreated with these
225 compounds followed by treatment with forskolin. ELISA analysis of supernatant medium
226 revealed that RANKL expression was dramatically decreased by treatment with *Crif1*
227 inhibitors (Figure 7L). OPG expression could be significantly increased by F1430-0134

228 (Figure 7M). Moreover, RANKL/OPG ratios were also decreased by these 5 inhibitors
229 (Figure 7N). To further understand the mechanism, CREB phosphorylation was detected.
230 Western blotting analysis showed that CREB phosphorylation was inhibited by treatment
231 with the 5 inhibitors that showed suppressive effects on RANKL expression (Figure 7O).

232 Discussion

233 Radiation exposure (due to radiotherapy, accidental causes or terrorism) causes
234 irreparable damage to tissues and organs such as bone marrow. It suppresses bone formation
235 and elevates resorption, disturbing the balance between them and leading to a dramatic
236 decline in trabecular bone(D'Oronzo, Stucci, Tucci, & Silvestris, 2015; Jia, Gaddy, Suva, &
237 Corry, 2011). In this study, we treated mice with a single dose of 5 Gy to generate a model of
238 radiation-induced osteoporosis. RANKL expression and RANKL/OPG ratios actually
239 increased in the surviving bone marrow cells after radiation, which was consistent with a
240 previous study(Alwood et al., 2015). Meanwhile, expression of *Crif1* and bone resorption
241 also increased, indicating a relationship between RANKL and *Crif1* in osteoporosis.
242 BM-MSCs, osteoblasts, adipocytes and activated T lymphocytes in bone marrow are the
243 main source of RANKL secretion(Boyce & Xing, 2008; Honma et al., 2013); thus, to
244 elucidate the possible role of *Crif1* in RANKL expression, we generated bone marrow
245 cell-specific *Crif1*-deficient mice. Conditional *Crif1* deletion from bone marrow could reduce
246 RANKL expression and relieve bone loss after radiation, suggesting that *Crif1* is involved in
247 RANKL expression and osteoporosis.

248 BM-MSCs, which are a major and important component of the haematopoietic
249 microenvironment, secrete soluble RANKL and OPG(Horowitz, Xi, Wilson, & Kacena, 2001;
250 Morrison & Scadden, 2014). In our study, overexpression of *Crif1* in BM-MSCs resulted in
251 an increase in RANKL secretion, while deletion of *Crif1* from BM-MSCs significantly
252 reduced RANKL expression. BM-MSCs are pluripotent and are the progenitors of both bone
253 marrow osteocytes and adipocytes. The balance between osteogenic and adipogenic
254 differentiation of BM-MSCs also plays a pivotal role in maintaining bone homeostasis(Mori
255 et al., 2014; Yue, Zhou, Shimada, Zhao, & Morrison, 2016). Excessive numbers of
256 adipocytes are often found in the bone marrow of patients with osteoporosis. A recent study
257 revealed that bone marrow adipocytes can also secrete RANKL and accelerate
258 osteoclastogenesis(Fan et al., 2017). Moreover, after radiation exposure, the haematopoietic
259 red marrow gradually turns to yellow, a phenomenon known as bone marrow fattening.
260 Compared to other cells in bone marrow, BM-MSCs are more resistant to radiation; however,
261 radiation impairs the balance between osteogenic and adipogenic differentiation of
262 BM-MSCs, decreasing osteogenesis while increasing RANKL expression and
263 adipogenesis(Cao et al., 2011). We previously reported that *Crif1* can promote the
264 adipogenesis of BM-MSCs. Here, we found that deletion of *Crif1* from BM-MSCs can
265 reduce both adipogenesis and RANKL expression. Osteogenesis and adipogenesis in the
266 bone marrow are inversely correlated, so reduced adipogenesis results in an increase in the
267 osteoblast pool(Muruganandan, Roman, & Sinal, 2009; Nuttall & Gimble, 2000).

268 RANKL functions as an osteoclast-activating factor, and its binding to RANK induces
269 the activation of transcription factors such as c-fos, NFAT, and nuclear factor kappa B
270 (NF- κ B) in preosteoclasts and initiates several downstream signalling pathways, especially
271 the NF- κ B pathway(Nakashima, Hayashi, & Takayanagi, 2012). RANKL expression can be
272 upregulated by many agents, such as PTH and forskolin. Forskolin can stimulate RANKL
273 expression through the cAMP/PKA signalling pathway(Kondo, Guo, & Bringhurst, 2002;
274 Tseng et al., 2010). However, deletion of *Crif1* from BM-MSCs impairs the promotion of

275 RANKL expression by forskolin. Moreover, overexpression of *Crif1* in BM-MSCs does not
276 increase RANKL expression upon exposure to a PKA inhibitor. Our previous study revealed
277 that *Crif1* can interact with PKA and promote adipogenesis. Here, we further demonstrated
278 that *Crif1* can also promote RANKL expression through the cAMP/PKA signalling pathway.

279 Drugs for the treatment of osteoporosis could be divided into anabolic and antiresorptive
280 categories. Bisphosphonates (including alendronate and ibandronate), oestrogen, selective
281 oestrogen receptor modulators (SERMs), and denosumab are antiresorptive drugs, whereas
282 parathyroid hormone (PTH) and its analogues are anabolic agents (Andreopoulou & Bockman,
283 2015; Favus, 2010; Torstrick & Guldborg, 2014). However, the long-term adverse effects of
284 these anti-osteoporotic drugs should be considered, such as hypocalcaemia, arthralgia, nausea,
285 and especially the development of breast cancer and risks of cardiovascular events and
286 thromboembolism associated with treatment with oestrogen and SERMs (Black & Rosen,
287 2016; Khan, Cheung, & Khan, 2017). Therefore, it is necessary to look for new drugs with
288 high efficiency but few side effects. Because of the importance of RANKL in osteoclast
289 differentiation, RANKL-secreting cells, which play a central role in osteoclastogenesis, are
290 the targets of most antiresorptive agents (Dempster, Lambing, Kostenuik, & Grauer, 2012;
291 Tella & Gallagher, 2014). Future drug screening should target not only the regulation of the
292 balance between bone formation and bone resorption but also the balance between osteogenic
293 and adipogenic differentiation. Here, through screening, we identified five compounds that
294 effectively inhibit *Crif1* activity in the cAMP/PKA signalling pathway. These five
295 compounds may have dual actions on bone metabolism, both increasing bone formation and
296 decreasing bone resorption and adipogenesis.

297 In summary, we demonstrated that *Crif1* plays a crucial role in osteoporosis and that
298 conditional *Crif1* deletion reduces bone loss after radiation. Moreover, through screening, we
299 identified five *Crif1* inhibitors that could dramatically reduce RANKL secretion and CREB
300 phosphorylation. This study enriches current knowledge of the pathogenesis of
301 radiation-induced osteoporosis and provides insights into potential therapeutic strategies for
302 osteoporosis treatment.

303 **Materials and Methods**

304 **Mouse Strains**

305 Male and female C57BL/6 mice (aged 12-14 weeks) were purchased from Beijing HFK
306 Bio-Technology Co. Ltd. *B6.129P2-Lyz2tm1(cre)/Nju* mice were received as a gift from
307 Assistant Professor Tao Wang (Third Military Medical University). *Crif1^{fl/fl}* mice were
308 constructed by Nanjing BioMedical Research Institute of Nanjing University (NBRI).
309 *Lyz2Cre;Crif1^{fl/fl}* mice were generated by crossing *Crif1^{fl/fl}* mice to
310 *B6.129P2-Lyz2tm1(cre)/Nju* transgenic mice. For radiation treatment, mice were irradiated
311 with 5 Gy Co-60 at a rate of 0.69 Gy/minute. Blood was collected at 7 days after irradiation,
312 and serum from each mouse was analysed individually in triplicate.

313 **Cell culture and treatment**

314 For *in vitro* study, mouse bone marrow mesenchymal stem/stromal cells (BM-MSCs)
315 purchased from Cyagen Biosciences were cultured in mouse mesenchymal stem cell medium
316 (MUCMX-90011, Cyagen Biosciences) at 37°C and 5% CO₂. For radiation treatment, mouse
317 BM-MSCs were irradiated with 9 Gy Co-60 at a rate of 0.69 Gy/minute.

318 RAW264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone)
319 supplemented with 10% FBS.

320 For osteoclast induction, RAW264.7 cells and mouse BM-MSCs were cocultured in a
321 12-well transwell unit (0.4 µm) for 7 days with or without forskolin (25 µM) or H89 (20 µM)

322 treatment. After 7 days of coculture, cells were collected for RT-qPCR and Western blotting;
323 meanwhile, supernatant medium was collected for ELISA.

324 Human bone marrow mesenchymal stem/stromal cells (hBM-MSCs) (catalogue No. 7500,
325 ScienCell) were cultured in mesenchymal stem cell medium (catalogue No. 7501, ScienCell)
326 at 37 °C and 5% CO₂.

327 ***Crif1* knockout and overexpression in vitro**

328 For *Crif1* overexpression, mouse BM-MSCs were transfected with a *Crif1* lentiviral
329 overexpression vector (pLV[Exp]-EGFP:T2A:Puro-EF1A>mGadd45gip1
330 [NM_183358.4]) constructed by Cyagen Biosciences (vector ID: VB180112-1182ypt) and
331 selected with 5 µg/ml puromycin dihydrochloride (A1113803, Invitrogen). An empty vector
332 (pLV[Exp]-EGFP:T2A:Puro-Null, vector ID: VB160420-1011mqh, Cyagen Biosciences) was
333 included as a control.

334 For *Crif1* knockout, mouse BM-MSCs were first transfected with lentiCas9-Blast vector
335 (Genomeditech) and selected with 5 µg/ml blasticidin S HCl (A1113903, Invitrogen). Then,
336 cells were transfected with CRISPR/Cas9 M_Gadd45gip1 gRNA vector (target sequence:
337 GCGGGGGCGCACGGTAGCTG, Genomeditech) and selected with 5 µg/ml puromycin
338 dihydrochloride. An empty vector (LentiGuide-Puro-Scramble-gRNA, Genomeditech) was
339 included as a control.

340 ***In vitro* adipogenic differentiation**

341 To induce adipogenesis, mouse BM-MSCs were treated in mouse mesenchymal stem cell
342 adipogenic differentiation medium (MUCMX-90031, Cyagen Biosciences). After
343 differentiation, we preserved the supernatant medium for ELISA and fixed the cells
344 with 2 ml of 4% formaldehyde solution for 30 minutes. Then, the cells were stained with 1
345 ml oil red O working solution (catalogue No. S0131, Cyagen Biosciences) for 30 minutes and
346 visualized with a light microscope (Leica DMIRB, Heidelberg, Germany). The dye from oil
347 red O staining was extracted using isopropanol, and the optical density at 510 nm was
348 measured using a Varioskan FLASH microplate reader.

349 **Micro-CT analysis**

350 Femurs were dissected, fixed overnight in 4% paraformaldehyde, and stored in 1%
351 paraformaldehyde at 4°C. Trabecular bone parameters were measured in the distal metaphysis
352 of the femur. We started analysing slices at the bottom of the distal growth plate, where the
353 epiphyseal cap structure completely disappeared and continued for 95 slices (10.5 µm/slice,
354 using SCANCO VivaCT40) towards the proximal end of the femur.

355 **Isolation of bone marrow cells**

356 Femurs were collected and cleaned in sterile PBS, and both ends of each femur were trimmed
357 off. Bones were placed in a 0.6-mL microcentrifuge tube that was cut open at the bottom and
358 nested inside a 1.5-mL microcentrifuge tube. Fresh bone marrow was spun out by brief
359 centrifugation (from 0 to 10,000 rpm, 9 s). Red blood cells were lysed using RBC lysis buffer
360 (catalogue NO. RT122-02, TIANGEN). After centrifugation (3,000 rpm, 5 minutes), cells at
361 the bottom layer were collected for Western blotting and RT-qPCR assays(Fan et al., 2017).

362 **Western blotting and antibodies**

363 Protein expression in the samples was analysed by Western blotting. Total protein lysates
364 were extracted with cell lysis buffer for Western blotting and immunoprecipitation (catalogue
365 No. P0013, Beyotime) and denatured by boiling. Protein samples were resolved on 12%
366 SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (PVDF
367 Western Blotting Membranes; Roche). Membranes were blocked in PBS containing 5% (w/v)
368 nonfat dry milk and 0.1% TWEEN 20 and then incubated with the appropriate primary

369 antibodies overnight at 4 °C. Membranes were washed with TBST three times, and then
370 incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1
371 h at 24 °C. Immunoreactive bands were detected by the BeyoECL Plus reagent (P0018,
372 Beyotime) using a Photo-Image System (Molecular Dynamics, Sunnyvale, CA, USA). The
373 primary antibodies used for blotting were as follows: Crif1 (M-222) (sc-134882; Santa Cruz),
374 RANK (H-7) (sc-374360; Santa Cruz), A-FABP (AP2, sc-18661; Santa Cruz), PPAR γ
375 (sc-7273; Santa Cruz), β -actin (sc-47778; Santa Cruz), phospho-CREB rabbit mAb (#9198;
376 Cell Signaling Technology), and CREB rabbit mAb (#9197; Cell Signaling Technology).

377 **RT-qPCR**

378 Real-time quantitative polymerase chain reaction (RT-qPCR) was used to analyse the mRNA
379 levels of selected genes in collected samples. Total RNA was extracted using TRIzol Reagent
380 (catalogue No. 10296010, Invitrogen) according to the manufacturer's instructions.
381 First-strand cDNA was synthesized from 1 μ g of RNA using the PrimeScript RT Reagent Kit
382 with gDNA Eraser (catalogue No. RR047A, TaKaRa). qPCR was performed in triplicate in
383 20- μ l reactions containing SYBR Premix Ex Taq II (catalogue No. RR820A, TaKaRa). The
384 reaction protocol was as follows: heating for 30 s at 95°C, followed by 40 cycles of
385 amplification (5 s at 95°C, 30 s at 60°C).

386 The sequences of the RT-PCR primers were as follows:

387 M-actin-F: AGCCATGTACGTAGCCATCC,

388 M-actin-R: CTCAGCTGTGGTGGTGAA,

389 M-Rankl-F: GCTCCGAGCTGGTGAAGAAA,

390 M-Rankl-R:CCCCAAAGTACGTCGCATCT,

391 M-OPG-F: GTTCCTGCACAGCTTCACAA,

392 M-OPG-R: AAACAGCCCAGTGACCATTC.

393 **ELISA**

394 The concentrations of RANKL and OPG were measured using the Mouse RANKL (Receptor
395 Activator of Nuclear Factor Kappa B Ligand) ELISA Kit (E-EL-M0644c, elabscience),
396 Human sRANKL (Soluble Receptor Activator of Nuclear factor-kB Ligand) ELISA Kit
397 (E-EL-H5558c, elabscience), Mouse OPG (Osteoprotegerin) ELISA Kit (E-EL-M0081c,
398 elabscience) and Human OPG (Osteoprotegerin) ELISA Kit (E-EL-H1341c, elabscience)
399 according to the manufacturer's instructions. Assays were performed in triplicate.

400 **Tartrate-resistant acid phosphatase (TRAP) staining**

401 After the 7-day coculture period, cells were washed once with PBS, fixed in 10% formalin
402 for 10 minutes, and incubated with a substrate solution, naphthol AS-BI phosphate (catalogue
403 No. 387, Sigma), in the presence of 50 mM sodium tartrate at 37 °C for 1 h. The resulting
404 mononuclear and multinuclear TRAP-positive cells were visualized by light microscopy and
405 quantified.

406 **Histochemistry and histomorphometric analysis**

407 Femurs were dissected, fixed overnight in 4% paraformaldehyde, decalcified in 10% EDTA
408 (pH 7.0) for 20 days and embedded in paraffin. Longitudinally oriented sections of bone 4
409 μ m thick, including the metaphysis and diaphysis, were processed for histochemistry and
410 haematoxylin and eosin (H&E) staining. Dewaxed sections were also stained for
411 tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts. Sections were
412 incubated in TRAP stain for 45 minutes at 37°C.

413 **Crif1 inhibitor screening**

414 ClusPro and InterProSurf were used to investigate the interface in Crif1-PKA α complex. A
415 virtual screening using 462,608 compounds from the Life Chemicals database around His¹²⁰

416 of Crif1 was carried out using the program Autodock_vina. For inhibitor screening,
417 hBM-MSCs were cultured in 6-well plates and pretreated with 5 different compounds (25
418 μM). After 3 hours of pretreatment, forskolin (25 μM) was added to the medium. After 1
419 hour of forskolin treatment, total protein lysates were extracted for CREB phosphorylation
420 detection, and 3 days later, supernatant medium was collected for ELISA.

421 **Tetrazolium salt (WST-8) assay**

422 A tetrazolium salt (WST-8) assay was carried out to study the toxicity of compounds to
423 hBM-MSCs. hBM-MSCs seeded at a density of 3000 cells per well in 96-well plate were
424 treated with 5 different compounds at 8 final concentrations (3.125 μM , 6.25 μM , 12.5 μM ,
425 25 μM , 50 μM , 100 μM , 200 μM , 400 μM). Three days later, 10 μl cell counting kit-8 solution
426 was added to each well. After 4 hours of incubation, the absorbance at 450 nm was measured
427 using a Varioskan FLASH microplate reader (Thermo).

428 **Statistical analysis**

429 The mRNA expression levels of RANKL and OPG in the tested samples were determined as
430 the cycle threshold (CT) level, and normalized copy numbers (relative quantification) were
431 calculated using the $\Delta\Delta\text{CT}$ equation as follows: $\Delta\Delta\text{CT} = \Delta\text{CT}$ of the bone marrow sample
432 $-\Delta\text{CT}$ of β -actin, and then the normalized copy number (relative quantification) $= 2^{-\Delta\Delta\text{CT}}$. Data
433 are presented as the mean \pm SD. Statistical significance was assessed using a two-tailed paired
434 Student's t-test. The results were considered significant when * $p < 0.05$ or ** $p < 0.01$.

435 **Study approval**

436 All animal studies performed were approved by the Institutional Animal Care and Use
437 Committee at the Third Military Medical University.

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442 **Author Contributions:** Z.L., L.X., Q.R. and L.C.: conceived the project; L.X. and Q.R.:
443 designed and performed most experiments and data analysis with input; Y.X., F.L., M.Z.,
444 L.C., Y.X., L.Z., J.F.Z. and S.C.L.: assisted with experiments; L.X.: wrote and edited the
445 manuscript.

446 **Declaration of Interests:** The authors have declared that no conflict of interest exists.

447 **References**

- 448 Alwood, J. S., Shahnazari, M., Chicana, B., Schreurs, A. S., Kumar, A., Bartolini, A., . . .
449 Globus, R. K. (2015). Ionizing Radiation Stimulates Expression of
450 Pro-Osteoclastogenic Genes in Marrow and Skeletal Tissue. *J Interferon Cytokine*
451 *Res*, 35(6), 480-487. doi:10.1089/jir.2014.0152
- 452 Andreopoulou, P., & Bockman, R. S. (2015). Management of postmenopausal osteoporosis.
453 *Annu Rev Med*, 66, 329-342. doi:10.1146/annurev-med-070313-022841
- 454 Black, D. M., & Rosen, C. J. (2016). Postmenopausal Osteoporosis. *N Engl J Med*, 374(21),
455 2096-2097. doi:10.1056/NEJMc1602599
- 456 Boyce, B. F., & Xing, L. (2007). Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res*
457 *Ther*, 9 Suppl 1, S1. doi:10.1186/ar2165
- 458 Boyce, B. F., & Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and
459 remodeling. *Arch Biochem Biophys*, 473(2), 139-146.

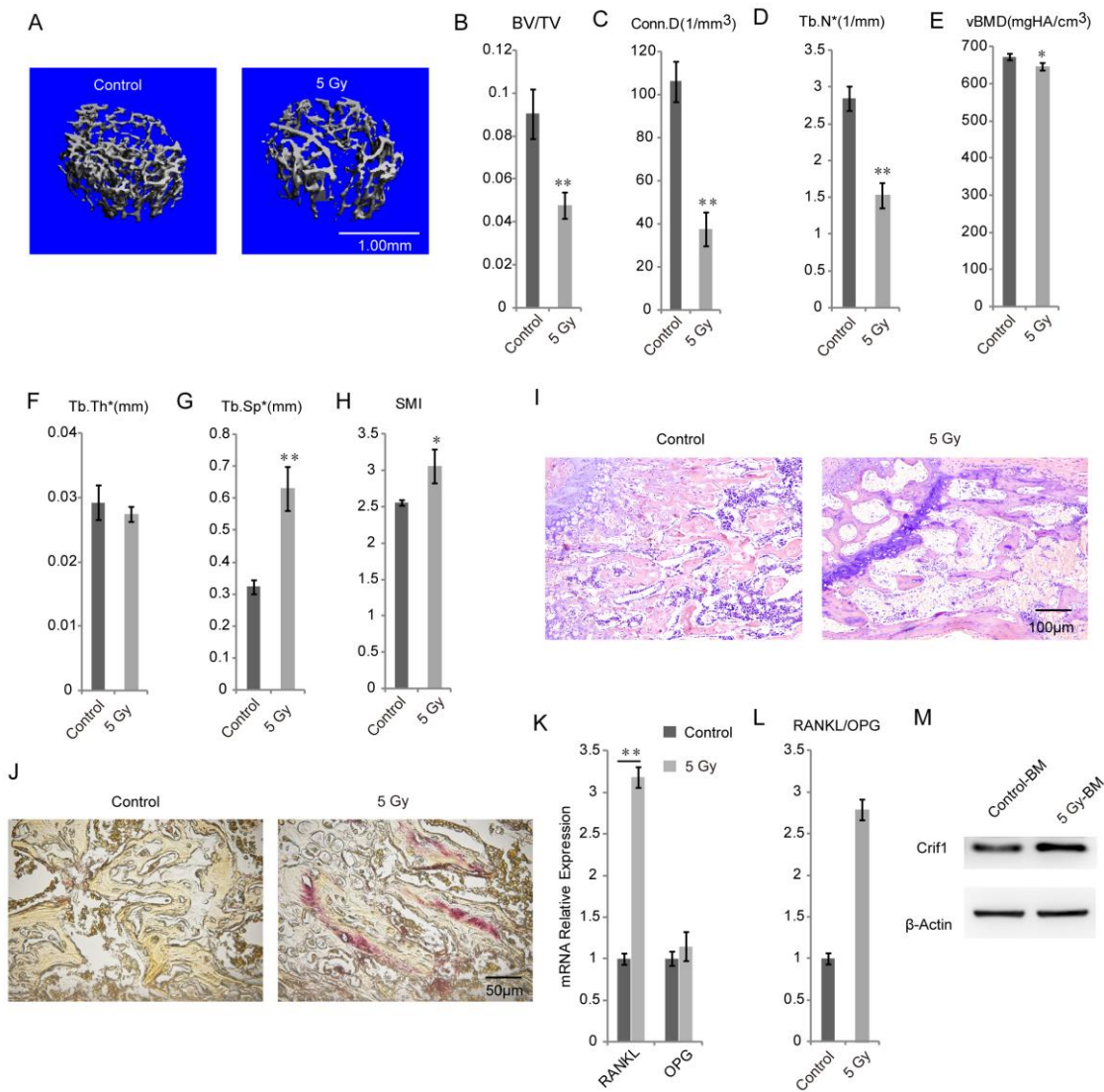
- 460 doi:10.1016/j.abb.2008.03.018
- 461 Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation.
462 *Nature*, 423(6937), 337-342. doi:10.1038/nature01658
- 463 Cao, X., Wu, X., Frassica, D., Yu, B., Pang, L., Xian, L., . . . Frassica, F. J. (2011). Irradiation
464 induces bone injury by damaging bone marrow microenvironment for stem cells.
465 *Proc Natl Acad Sci U S A*, 108(4), 1609-1614. doi:10.1073/pnas.1015350108
- 466 Chen, L., Ran, Q., Xiang, Y., Xiang, L., Li, F., Wu, J., . . . Li, Z. (2017). Co-Activation of PKC- δ
467 by CRIF1 Modulates Oxidative Stress in Bone Marrow Multipotent Mesenchymal
468 Stromal Cells after Irradiation by Phosphorylating NRF2 Ser40. *Theranostics*, 7(10),
469 2634-2648. doi:10.7150/thno.17853
- 470 Chung, H. K., Yi, Y. W., Jung, N. C., Kim, D., Suh, J. M., Kim, H., . . . Shong, M. (2003).
471 CR6-interacting factor 1 interacts with Gadd45 family proteins and modulates the
472 cell cycle. *J Biol Chem*, 278(30), 28079-28088. doi:M212835200 [pii]
473 10.1074/jbc.M212835200
- 474 D'Oronzo, S., Stucci, S., Tucci, M., & Silvestris, F. (2015). Cancer treatment-induced bone
475 loss (CTIBL): pathogenesis and clinical implications. *Cancer Treat Rev*, 41(9),
476 798-808. doi:10.1016/j.ctrv.2015.09.003
- 477 Dempster, D. W., Laming, C. L., Kostenuik, P. J., & Grauer, A. (2012). Role of RANK ligand
478 and denosumab, a targeted RANK ligand inhibitor, in bone health and osteoporosis:
479 a review of preclinical and clinical data. *Clin Ther*, 34(3), 521-536.
480 doi:10.1016/j.clinthera.2012.02.002
- 481 Fan, Y., Hanai, J. I., Le, P. T., Bi, R., Maridas, D., DeMambro, V., . . . Lanske, B. (2017).
482 Parathyroid Hormone Directs Bone Marrow Mesenchymal Cell Fate. *Cell Metab*,
483 25(3), 661-672. doi:10.1016/j.cmet.2017.01.001
- 484 Favus, M. J. (2010). Bisphosphonates for osteoporosis. *N Engl J Med*, 363(21), 2027-2035.
485 doi:10.1056/NEJMct1004903
- 486 Greber, B. J., Bieri, P., Leibundgut, M., Leitner, A., Aebersold, R., Boehringer, D., & Ban, N.
487 (2015). Ribosome. The complete structure of the 55S mammalian mitochondrial
488 ribosome. *Science*, 348(6232), 303-308. doi:10.1126/science.aaa3872
- 489 Honma, M., Ikebuchi, Y., Kariya, Y., Hayashi, M., Hayashi, N., Aoki, S., & Suzuki, H. (2013).
490 RANKL subcellular trafficking and regulatory mechanisms in osteocytes. *J Bone*
491 *Miner Res*, 28(9), 1936-1949. doi:10.1002/jbmr.1941
- 492 Horowitz, M. C., Xi, Y., Wilson, K., & Kacena, M. A. (2001). Control of osteoclastogenesis
493 and bone resorption by members of the TNF family of receptors and ligands.
494 *Cytokine Growth Factor Rev*, 12(1), 9-18.
- 495 Jia, D., Gaddy, D., Suva, L. J., & Corry, P. M. (2011). Rapid loss of bone mass and strength
496 in mice after abdominal irradiation. *Radiat Res*, 176(5), 624-635.
- 497 Jung, S. B., Choi, M. J., Ryu, D., Yi, H. S., Lee, S. E., Chang, J. Y., . . . Shong, M. (2018).
498 Reduced oxidative capacity in macrophages results in systemic insulin resistance.
499 *Nat Commun*, 9(1), 1551. doi:10.1038/s41467-018-03998-z
- 500 Kang, H. J., Hong, Y. B., Kim, H. J., & Bae, I. (2010). CR6-interacting factor 1 (CRIF1)
501 regulates NF-E2-related factor 2 (NRF2) protein stability by proteasome-mediated
502 degradation. *J Biol Chem*, 285(28), 21258-21268. doi:M109.084590 [pii]
503 10.1074/jbc.M109.084590
- 504 Khan, M., Cheung, A. M., & Khan, A. A. (2017). Drug-Related Adverse Events of
505 Osteoporosis Therapy. *Endocrinol Metab Clin North Am*, 46(1), 181-192.
506 doi:10.1016/j.ecl.2016.09.009

- 507 Khosla, S., & Hofbauer, L. C. (2017). Osteoporosis treatment: recent developments and
508 ongoing challenges. *Lancet Diabetes Endocrinol*, *5*(11), 898-907.
509 doi:10.1016/S2213-8587(17)30188-2
- 510 Kondo, H., Guo, J., & Bringhurst, F. R. (2002). Cyclic adenosine monophosphate/protein
511 kinase A mediates parathyroid hormone/parathyroid hormone-related protein
512 receptor regulation of osteoclastogenesis and expression of RANKL and
513 osteoprotegerin mRNAs by marrow stromal cells. *J Bone Miner Res*, *17*(9),
514 1667-1679. doi:10.1359/jbmr.2002.17.9.1667
- 515 Martin, T. J., & Sims, N. A. (2015). RANKL/OPG; Critical role in bone physiology. *Rev Endocr*
516 *Metab Disord*, *16*(2), 131-139. doi:10.1007/s11154-014-9308-6
- 517 Mori, K., Suzuki, K., Hozumi, A., Goto, H., Tomita, M., Koseki, H., . . . Osaki, M. (2014).
518 Potentiation of osteoclastogenesis by adipogenic conversion of bone
519 marrow-derived mesenchymal stem cells. *Biomed Res*, *35*(2), 153-159.
- 520 Morrison, S. J., & Scadden, D. T. (2014). The bone marrow niche for haematopoietic stem
521 cells. *Nature*, *505*(7483), 327-334. doi:10.1038/nature12984
- 522 Muruganandan, S., Roman, A. A., & Sinal, C. J. (2009). Adipocyte differentiation of bone
523 marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic
524 program. *Cell Mol Life Sci*, *66*(2), 236-253. doi:10.1007/s00018-008-8429-z
- 525 Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-Hora, M., Feng, J. Q., . . .
526 Takayanagi, H. (2011). Evidence for osteocyte regulation of bone homeostasis
527 through RANKL expression. *Nat Med*, *17*(10), 1231-1234. doi:10.1038/nm.2452
- 528 Nakashima, T., Hayashi, M., & Takayanagi, H. (2012). New insights into osteoclastogenic
529 signaling mechanisms. *Trends Endocrinol Metab*, *23*(11), 582-590.
530 doi:10.1016/j.tem.2012.05.005
- 531 Nuttall, M. E., & Gimble, J. M. (2000). Is there a therapeutic opportunity to either prevent
532 or treat osteopenic disorders by inhibiting marrow adipogenesis? *Bone*, *27*(2),
533 177-184.
- 534 Park, K. C., Song, K. H., Chung, H. K., Kim, H., Kim, D. W., Song, J. H., . . . Shong, M. (2005).
535 CR6-interacting factor 1 interacts with orphan nuclear receptor Nur77 and inhibits
536 its transactivation. *Mol Endocrinol*, *19*(1), 12-24. doi:10.1210/me.2004-0107
- 537 Phetfong, J., Sanvoranart, T., Nartprayut, K., Nimsanor, N., Seenprachawong, K.,
538 Prachayasittikul, V., & Supokawej, A. (2016). Osteoporosis: the current status of
539 mesenchymal stem cell-based therapy. *Cell Mol Biol Lett*, *21*, 12.
540 doi:10.1186/s11658-016-0013-1
- 541 Rachner, T. D., Khosla, S., & Hofbauer, L. C. (2011). Osteoporosis: now and the future.
542 *Lancet*, *377*(9773), 1276-1287. doi:10.1016/S0140-6736(10)62349-5
- 543 Ran, Q., Hao, P., Xiao, Y., Xiang, L., Ye, X., Deng, X., . . . Li, Z. (2014). CRIF1 interacting with
544 CDK2 regulates bone marrow microenvironment-induced G0/G1 arrest of leukemia
545 cells. *PLoS One*, *9*(2), e85328. doi:10.1371/journal.pone.0085328
- 546 Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science*, *289*(5484), 1504-1508.
- 547 Tella, S. H., & Gallagher, J. C. (2014). Prevention and treatment of postmenopausal
548 osteoporosis. *J Steroid Biochem Mol Biol*, *142*, 155-170.
549 doi:10.1016/j.jsbmb.2013.09.008
- 550 Torstrick, F. B., & Guldberg, R. E. (2014). Local strategies to prevent and treat osteoporosis.
551 *Curr Osteoporos Rep*, *12*(1), 33-40. doi:10.1007/s11914-014-0191-6
- 552 Tseng, W., Graham, L. S., Geng, Y., Reddy, A., Lu, J., Effros, R. B., . . . Tintut, Y. (2010).
553 PKA-induced receptor activator of NF-kappaB ligand (RANKL) expression in vascular

- 554 cells mediates osteoclastogenesis but not matrix calcification. *J Biol Chem*, 285(39),
555 29925-29931. doi:10.1074/jbc.M110.117366
- 556 Wada, T., Nakashima, T., Hiroshi, N., & Penninger, J. M. (2006). RANKL-RANK signaling in
557 osteoclastogenesis and bone disease. *Trends Mol Med*, 12(1), 17-25.
558 doi:10.1016/j.molmed.2005.11.007
- 559 Yue, R., Zhou, B. O., Shimada, I. S., Zhao, Z., & Morrison, S. J. (2016). Leptin Receptor
560 Promotes Adipogenesis and Reduces Osteogenesis by Regulating Mesenchymal
561 Stromal Cells in Adult Bone Marrow. *Cell Stem Cell*, 18(6), 782-796.
562 doi:10.1016/j.stem.2016.02.015
- 563 Zhang, X., Xiang, L., Ran, Q., Liu, Y., Xiang, Y., Xiao, Y., . . . Li, Z. (2015). Crif1 Promotes
564 Adipogenic Differentiation of Bone Marrow Mesenchymal Stem Cells After
565 Irradiation by Modulating the PKA/CREB Signaling Pathway. *Stem Cells*, 33(6),
566 1915-1926. doi:10.1002/stem.2019

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601 **Figures:**



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603 **Figure 1. Radiation induces osteoporosis in mice**

604 (A) Micro-CT images of the distal metaphysis of the femur.

605 (B-H) Micro-CT analysis of the trabecular bone volume/total volume (B), connectivity
606 density (C), trabecular number (D), bone mineral density (E), trabecular thickness (F),
607 trabecular spacing (G), and structure model index (H).

608 (I) H&E staining of femoral sections from irradiated mice and controls.

609 (J) TRAP staining of femoral sections from irradiated mice and controls.

610 (K) RT-qPCR analysis of RANKL and OPG mRNA expression in flushed whole bone
611 marrow.

612 (L) RANKL/OPG ratio of RT-qPCR results.

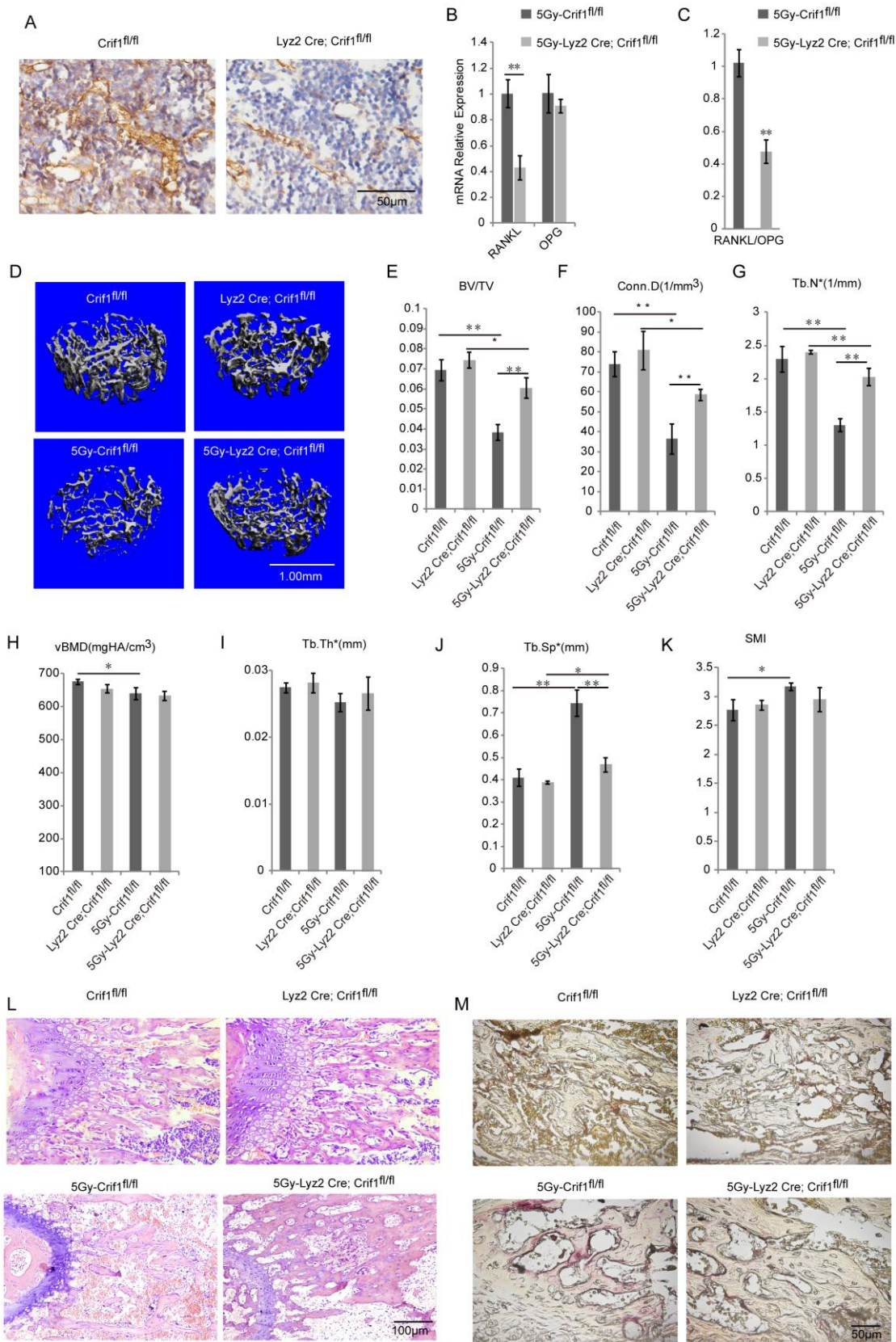
613 (M) Western blotting analysis of Crif1 expression in flushed whole bone marrow.

614 **P* < 0.05, ***P* < 0.01. All experiments were performed in triplicate, and the bars represent
615 the mean ± SD.

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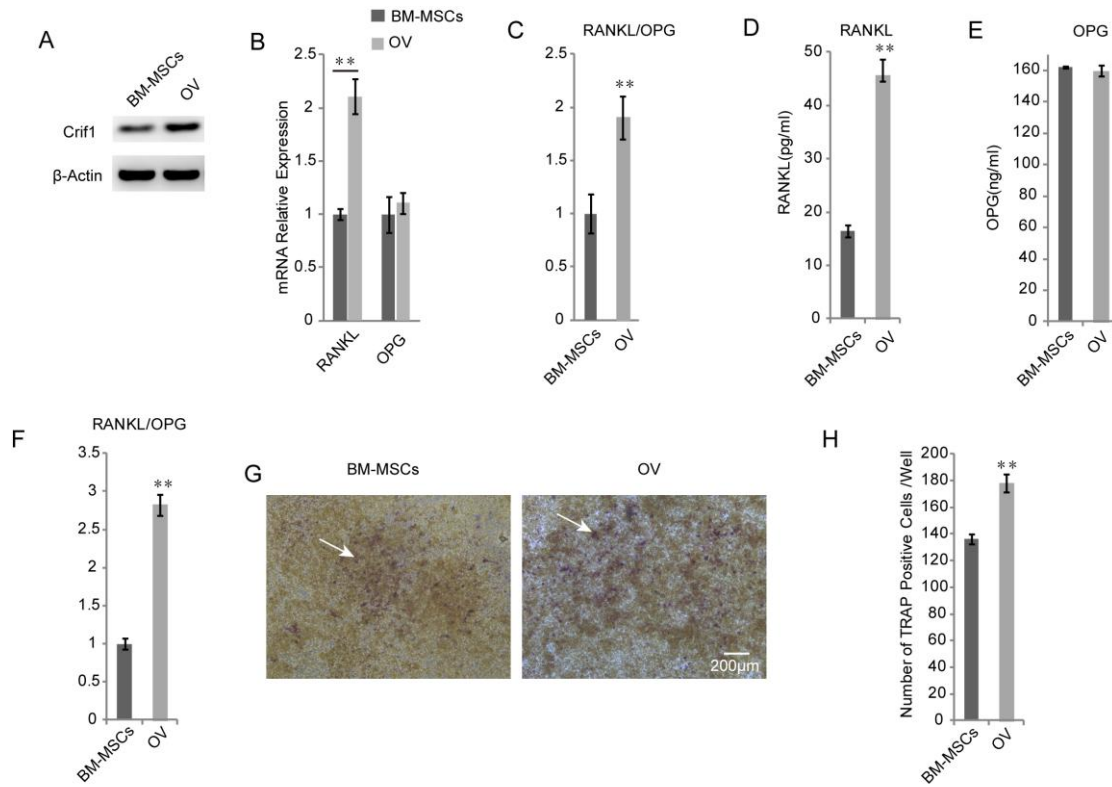
620 **Figure 2. Conditional *Crif1* deletion from bone marrow reduces bone loss after**
 621 **radiation**

622 (A) Histochemical staining analysis of *Crif1* expression in femoral bone marrow.

623 (B) RT-qPCR analysis of RANKL and OPG mRNA expression in flushed whole bone
 624 marrow of *Crif1^{fl/fl}* mice and *Lyz2Cre;Crif1^{fl/fl}* mice after 5 Gy radiation.

625 (C) RANKL/OPG ratio of RT-qPCR results.
626 (D) Micro-CT images of the distal metaphysis of the femur from *Lyz2Cre;Crif1^{fl/fl}* mice and
627 *Crif1^{fl/fl}* mice.
628 (E-K) Micro-CT analysis of the trabecular bone volume/total volume (E), connectivity
629 density (F), trabecular number (G), bone mineral density (H), trabecular thickness (I),
630 trabecular spacing (J), and structure model index (K).
631 (L) H&E staining of femoral sections from *Crif1^{fl/fl}* mice and *Lyz2Cre;Crif1^{fl/fl}* mice.
632 (M) TRAP staining of femoral sections from *Crif1^{fl/fl}* mice and *Lyz2Cre;Crif1^{fl/fl}* mice.
633 * $P < 0.05$, ** $P < 0.01$. All experiments were performed in triplicate, and the bars represent
634 the mean \pm SD.

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661 **Figure 3. Overexpression of Crif1 in BM-MSCs results in increases in RANKL secretion**
662 **and the ratio of RANKL to OPG**

663 (A) Western blotting analysis of Crif1 expression in mouse BM-MSCs. Mouse BM-MSCs
664 were transfected with a Crif1 lentiviral overexpression vector.

665 (B) RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and
666 Crif1-overexpressing BM-MSCs (OV). BM-MSCs and OV were cocultured with RAW264.7.

667 (C) RANKL/OPG ratio of RT-qPCR results.

668 (D) ELISA analysis of RANKL protein levels in coculture supernatant medium.

669 (E) ELISA analysis of OPG protein levels in coculture supernatant medium.

670 (F) RANKL/OPG ratio in coculture supernatant medium.

671 (G) TRAP staining of RAW264.7 after 7 days in coculture.

672 (H) Average number of TRAP-positive cells/well (arrow) from RAW264.7 cells in coculture.

673 * $P < 0.05$, ** $P < 0.01$. All experiments were performed in triplicate, and the bars represent
674 the mean \pm SD.

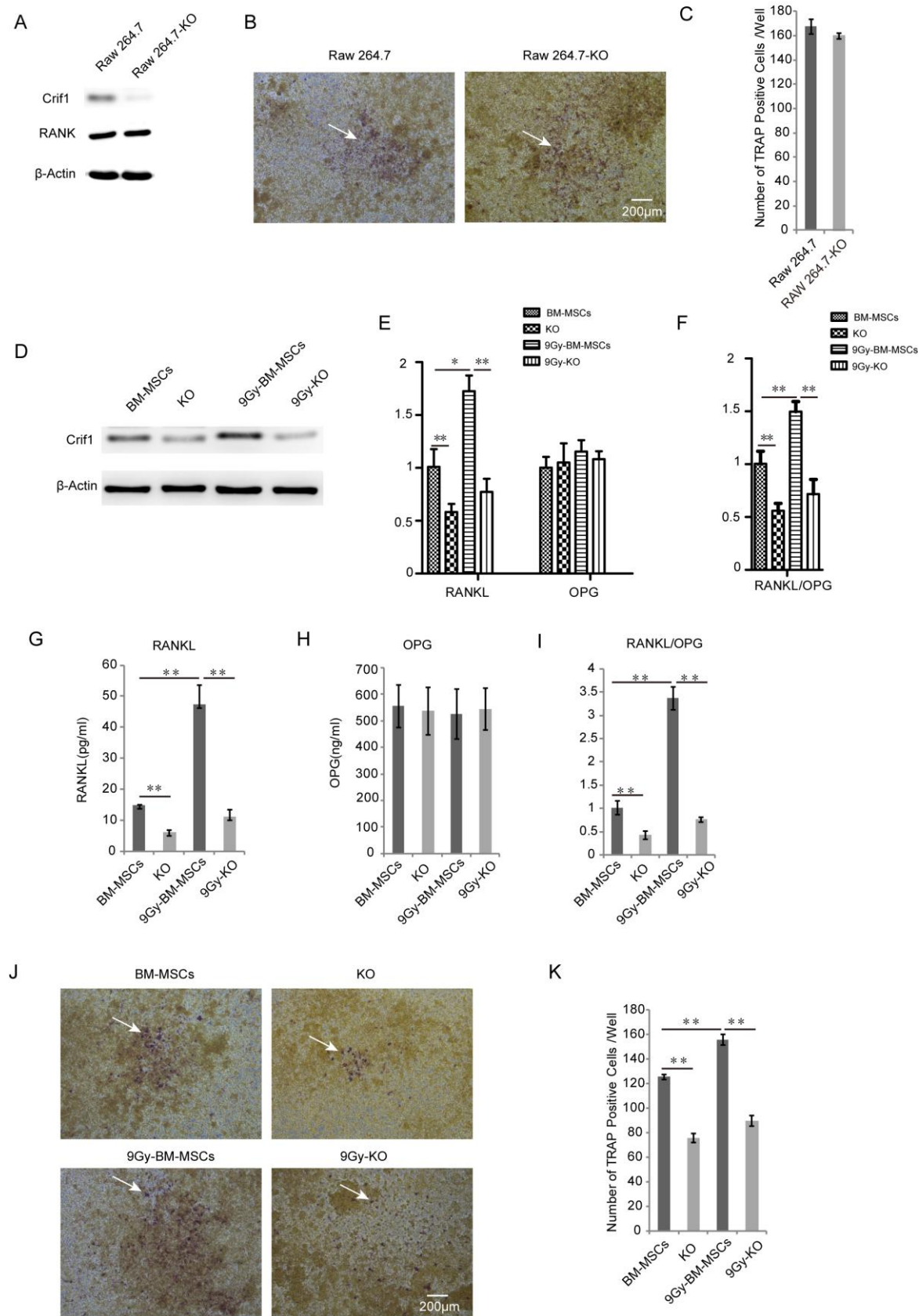
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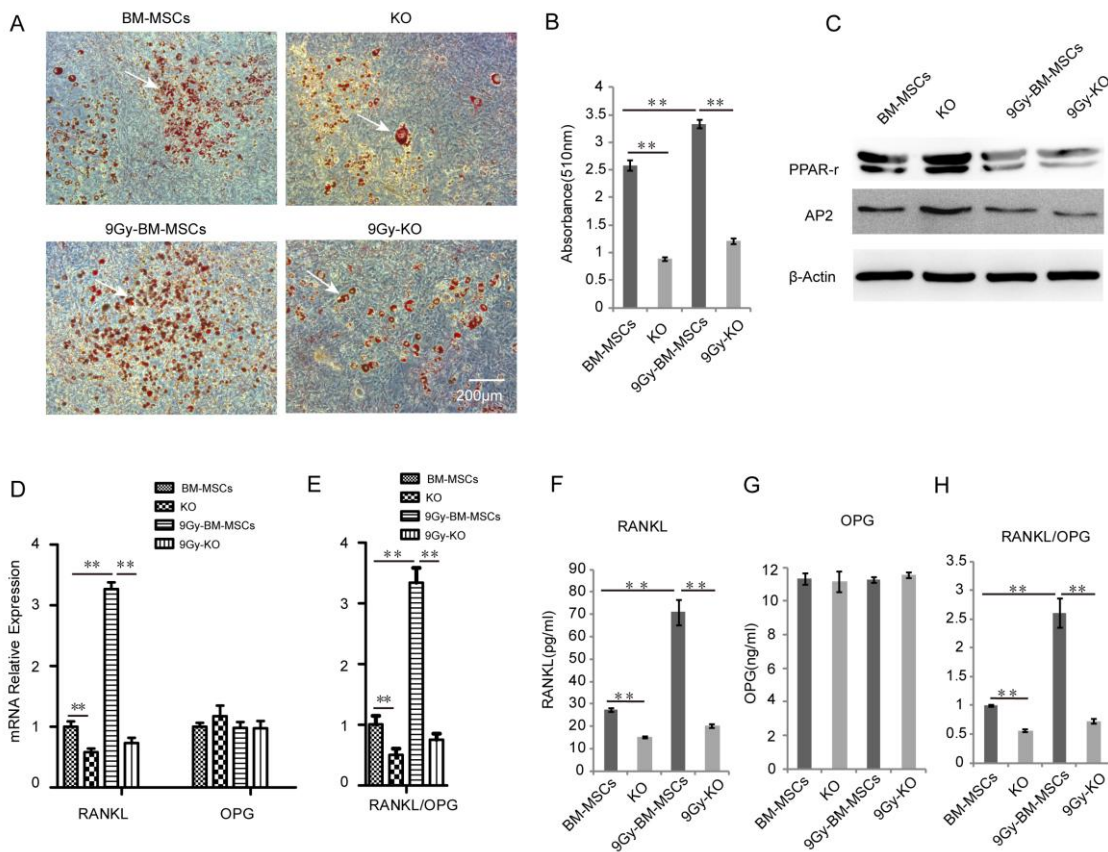


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Figure 4. Crif1 is involved in the regulation of RANKL expression after radiation
 (A) Western blotting analysis of Crif1 and RANK expression in RAW264.7 cells. Crif1 was
 knocked out from RAW264.7 cells (RAW264.7-KO).
 (B) TRAP staining of RAW264.7-KO and controls after 7 days in coculture with mouse
 BM-MSCs.

- 687 (C) Average number of TRAP-positive cells/well (arrow) from RAW264.7-KO and controls
688 after 7 days in coculture with mouse BM-MSCs.
- 689 (D) Western blotting analysis of Crif1 expression in BM-MSCs. Crif1 was knocked out from
690 mouse BM-MSCs (KO), and KO and controls were irradiated with 9 Gy Co-60.
- 691 (E) RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and Crif1
692 knockout BM-MSCs (KO). BM-MSCs and KO were cocultured with RAW264.7
- 693 (F) RANKL/OPG ratio of RT-qPCR results.
- 694 (G) ELISA analysis of RANKL protein levels in coculture supernatant medium.
- 695 (H) ELISA analysis of OPG protein levels in coculture supernatant medium.
- 696 (I) RANKL/OPG ratio in coculture supernatant medium.
- 697 (J) TRAP staining of RAW264.7 after 7 days in coculture.
- 698 (K) Average number of TRAP-positive cells/well (arrow) from RAW264.7 in coculture.
- 699 * $P < 0.05$, ** $P < 0.01$. All experiments were performed in triplicate, and the bars represent
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722 **Figure 5. Crif1 mediates adipogenesis and RANKL secretion in adipocytes**

723 (A) Oil red O staining analysis of mouse BM-MSCs after 21 days of adipogenic
 724 differentiation. Crif1 was knocked out from mouse BM-MSCs (KO), and KO and controls
 725 were irradiated with 9 Gy Co-60. KO and controls were treated with mouse mesenchymal
 726 stem cell adipogenic differentiation medium (Ad) to induce adipogenesis.

727 (B) The dye from oil red O staining was extracted using isopropanol, and the optical density
 728 at 510 nm was measured using a Benchmark Plus.

729 (C) Western blotting analysis of adipogenesis-related markers and transcription factors
 730 PPAR γ and AP2 in mouse BM-MSCs after 21 days of adipogenic differentiation.

731 (D) RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and Crif1
 732 knockout BM-MSCs (KO).

733 (E) RANKL/OPG ratio of RT-qPCR results.

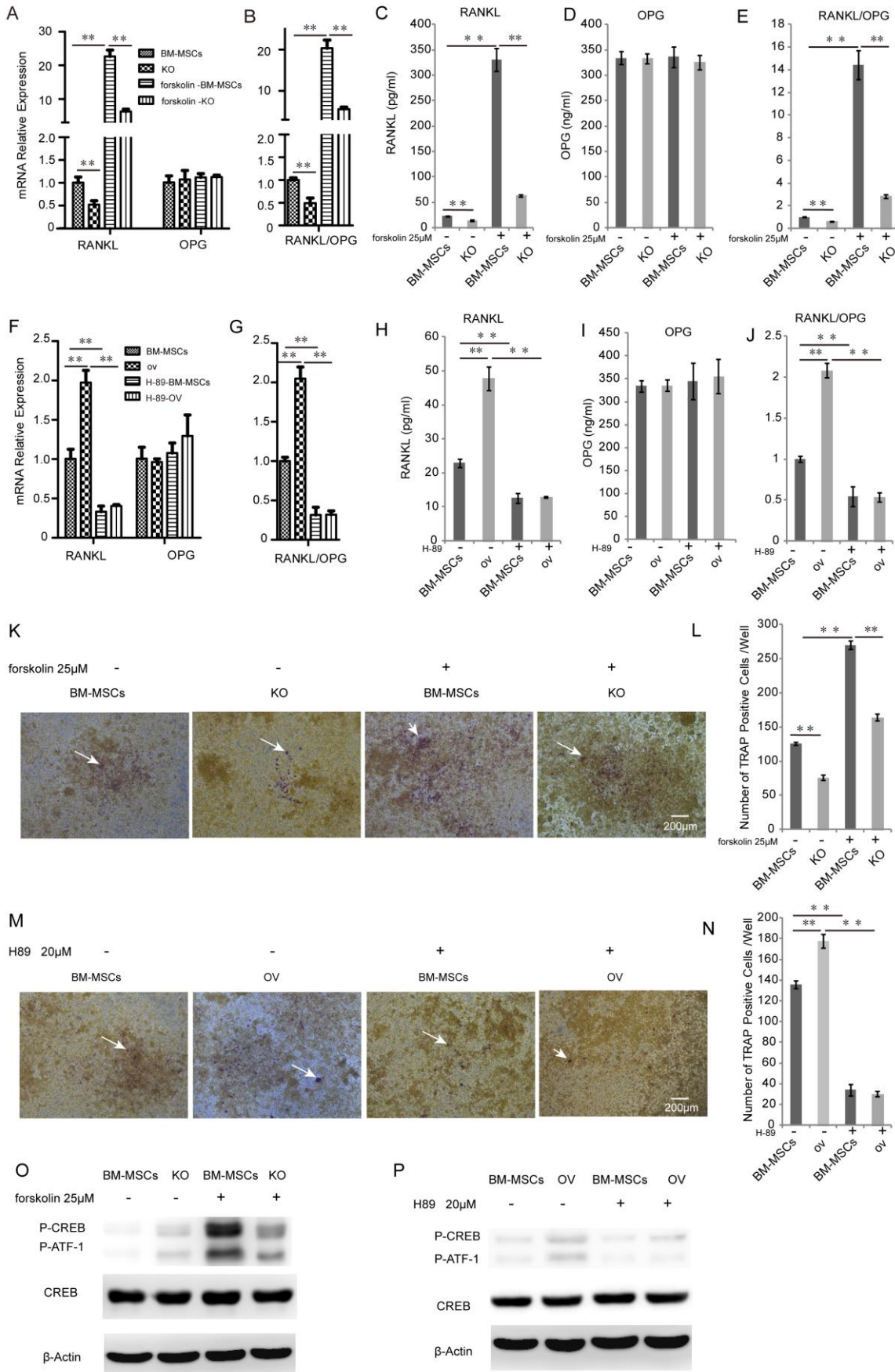
734 (F) ELISA analysis of RANKL protein levels in supernatant adipogenic differentiation
 735 medium.

736 (G) ELISA analysis of OPG protein levels in supernatant adipogenic differentiation medium.

737 (H) RANKL/OPG ratio in supernatant adipogenic differentiation medium.

738 * $P < 0.05$, ** $P < 0.01$. All experiments were performed in triplicate, and the bars represent
 739 the mean \pm SD.

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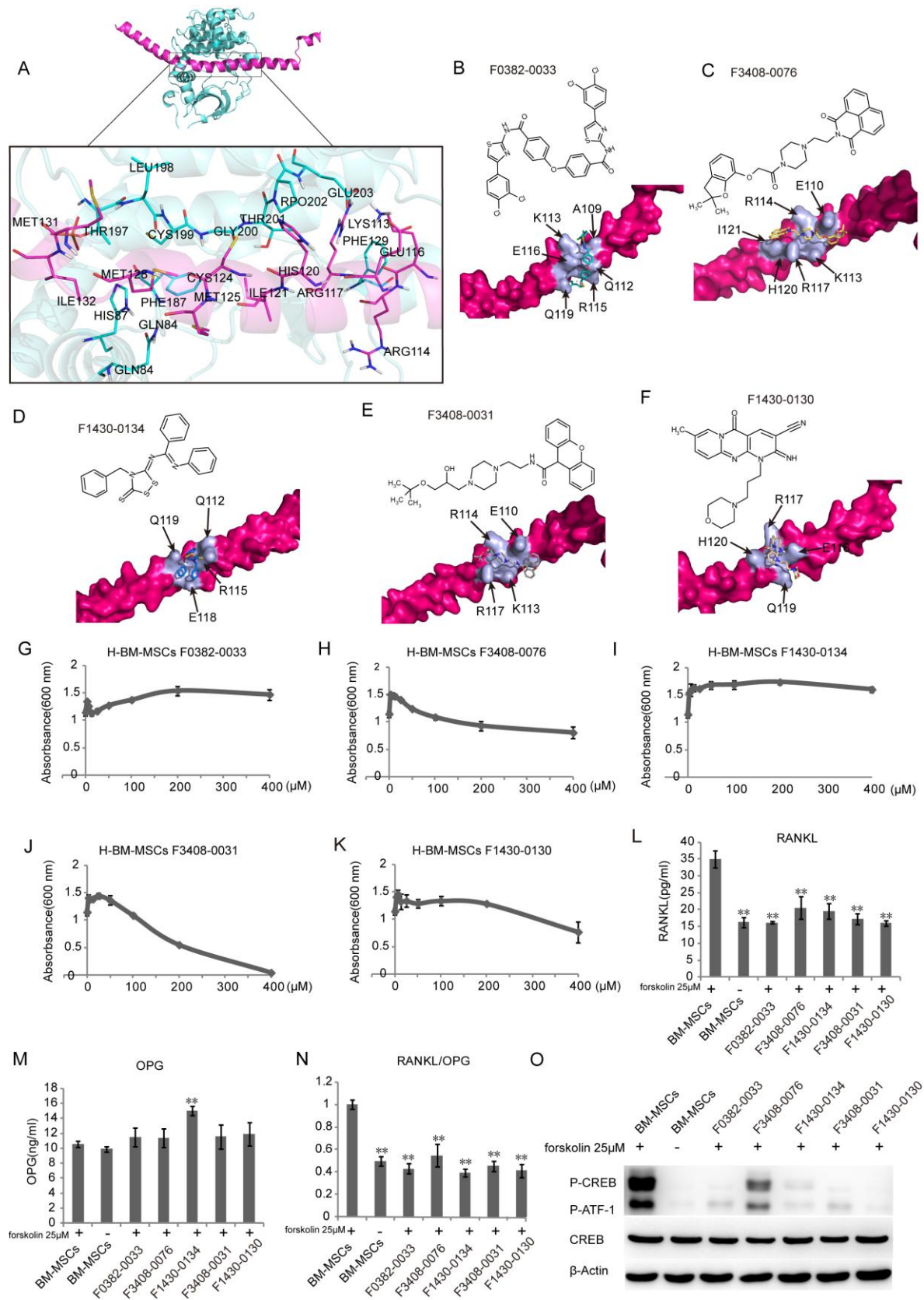
Figure 6. Crif1 promotes RANKL secretion by modulating the cAMP/PKA signalling pathway

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(A) RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and Crif1 knockout BM-MSCs (KO). BM-MSCs and KO were cocultured with RAW264.7 with or

745

- 746 without 25 μ M forskolin respectively.
- 747 (B) RANKL/OPG ratio of RT-qPCR results.
- 748 (C) ELISA analysis of RANKL protein levels in coculture supernatant medium with or
749 without 25 μ M forskolin.
- 750 (D) ELISA analysis of OPG protein levels in coculture supernatant medium with or without
751 25 μ M forskolin.
- 752 (E) RANKL/OPG ratio in coculture supernatant medium with or without 25 μ M forskolin.
- 753 (F) RT-qPCR analysis of RANKL and OPG mRNA expression in BM-MSCs and
754 Crif1-overexpressing BM-MSCs (OV). BM-MSCs and OV were cocultured with RAW264.7
755 with or without 20 μ M H89 respectively.
- 756 (G) RANKL/OPG ratio of RT-qPCR results.
- 757 (H) ELISA analysis of RANKL protein levels in coculture supernatant medium with or
758 without 20 μ M H89.
- 759 (I) ELISA analysis of OPG protein levels in coculture supernatant medium with or without 20
760 μ M H89.
- 761 (J) RANKL/OPG ratio in coculture supernatant medium with or without 20 μ M H89.
- 762 (K) TRAP staining of RAW264.7 in coculture treated with or without 25 μ M forskolin.
- 763 (L) Average number of TRAP-positive cells/well (arrow) from RAW264.7 in coculture
764 treated with or without 25 μ M forskolin.
- 765 (M) TRAP staining of RAW264.7 in coculture treated with or without 20 μ M H89.
- 766 (N) Average number of TRAP-positive cells/well (arrow) from RAW264.7 in coculture
767 treated with or without 20 μ M H89.
- 768 (O) Western blotting analysis of CREB phosphorylation levels in BM-MSCs in coculture
769 treated with or without 25 μ M forskolin.
- 770 (P) Western blotting analysis of CREB phosphorylation levels in BM-MSCs in coculture
771 treated with or without 20 μ M H89.
- 772 * $P < 0.05$, ** $P < 0.01$..All experiments were performed in triplicate, and the bars represent
773 the mean \pm SD.



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777 **Figure 7. Crif1 inhibitors effectively suppress RANKL secretion and CREB**
 778 **phosphorylation**

779 (A) Crif1-PKA α interaction model showing Crif1 (colored in rose red) and PKA α (colored
 780 in cyan). Interface amino acids are shown as sticks and colored in rose red (for Crif1) and

781 cyan (for PKA α) and indicated as a zoomed-in view in the inset figure.
782 (B-F) Chemical structure of each inhibitor molecule and their docked pose on Crif1 (colored
783 in rose red, surface view). Docked molecule (stick) and the amino acids involved in the
784 hydrophobic interactions (light purple) are shown. F0382-0033 (B), F3408-0076 (C),
785 F1430-0134 (D), F3408-0031 (E) and F1430-0130 (F).
786 (G-K) A tetrazolium salt (WST-8) assay was carried out to study the toxicity effect of
787 compounds on the hBM-MSCs. F0382-0033 (G), F3408-0076 (H), F1430-0134 (I),
788 F3408-0031 (J) and F1430-0130 (K).
789 (L) ELISA analysis of RANKL protein levels in supernatant medium. hBM-MSCs were
790 pretreated with 5 different compounds followed by treatment with forskolin, and supernatant
791 medium was collected for ELISA after 3 days.
792 (M) ELISA analysis of OPG protein levels in supernatant medium.
793 (N) RANKL/OPG ratio in supernatant medium.
794 (O) Western blotting analysis of CREB phosphorylation levels. hBM-MSCs were pretreated
795 with 5 different compounds followed by treatment with forskolin and total protein lysates
796 were extracted for CREB phosphorylation detection after 1 hour.
797 * $P < 0.05$, ** $P < 0.01$. All experiments were performed in triplicate, and the bars represent
798 the mean \pm SD.

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828 **Supplementary data**

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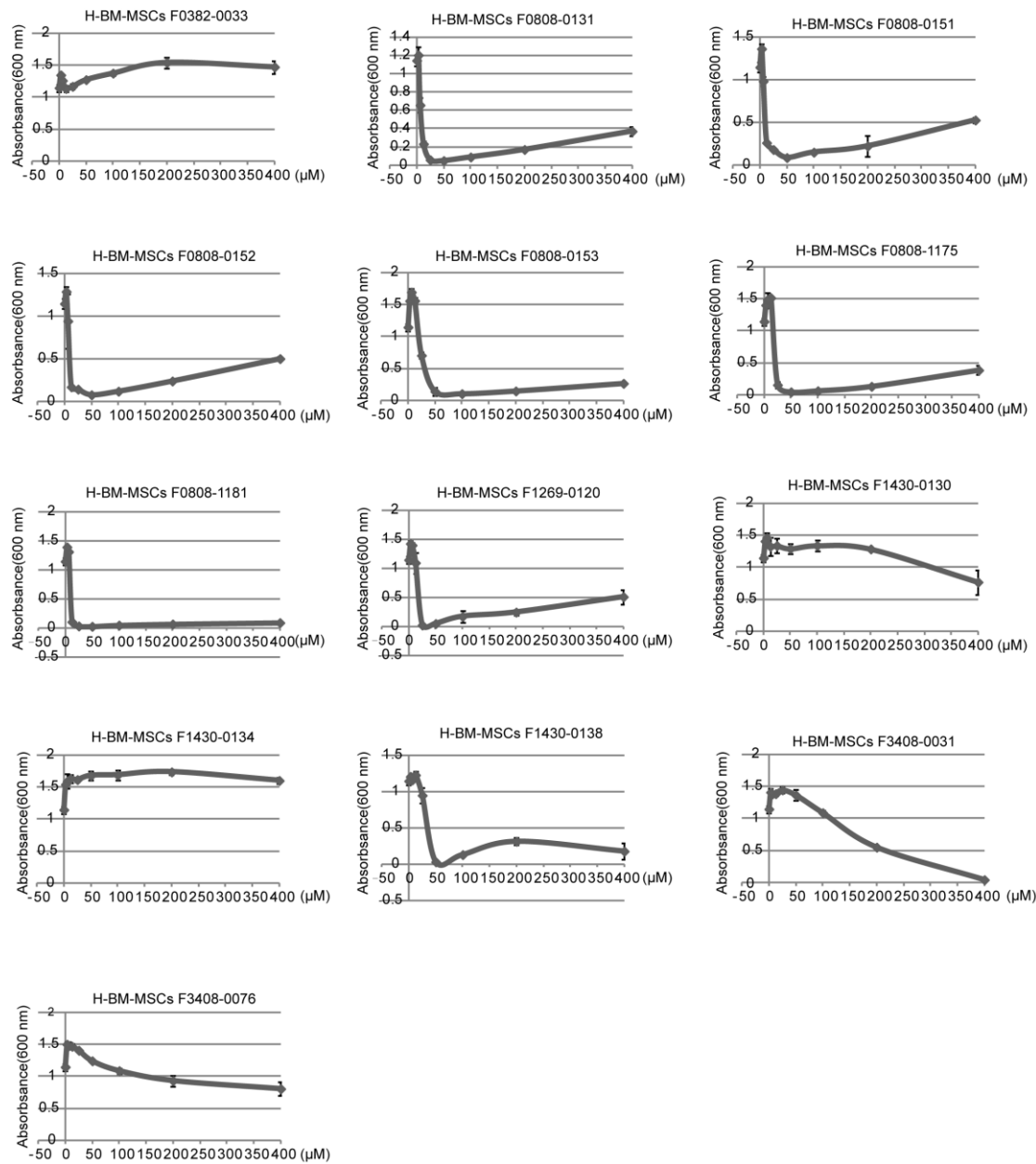
Compound Name	Affinity (kcal/mol)
F3408-0076	-14.6
F0382-0033	-13.1
F1430-0130	-12.9
F0808-0153	-12.5
F0808-0131	-12.5
F0808-0151	-12.4
F0808-1181	-12.4
F0808-1175	-12.4
F1269-0120	-12.3
F0808-0152	-12.2
F3408-0031	-12.2
F1430-0134	-12
F1430-0138	-12

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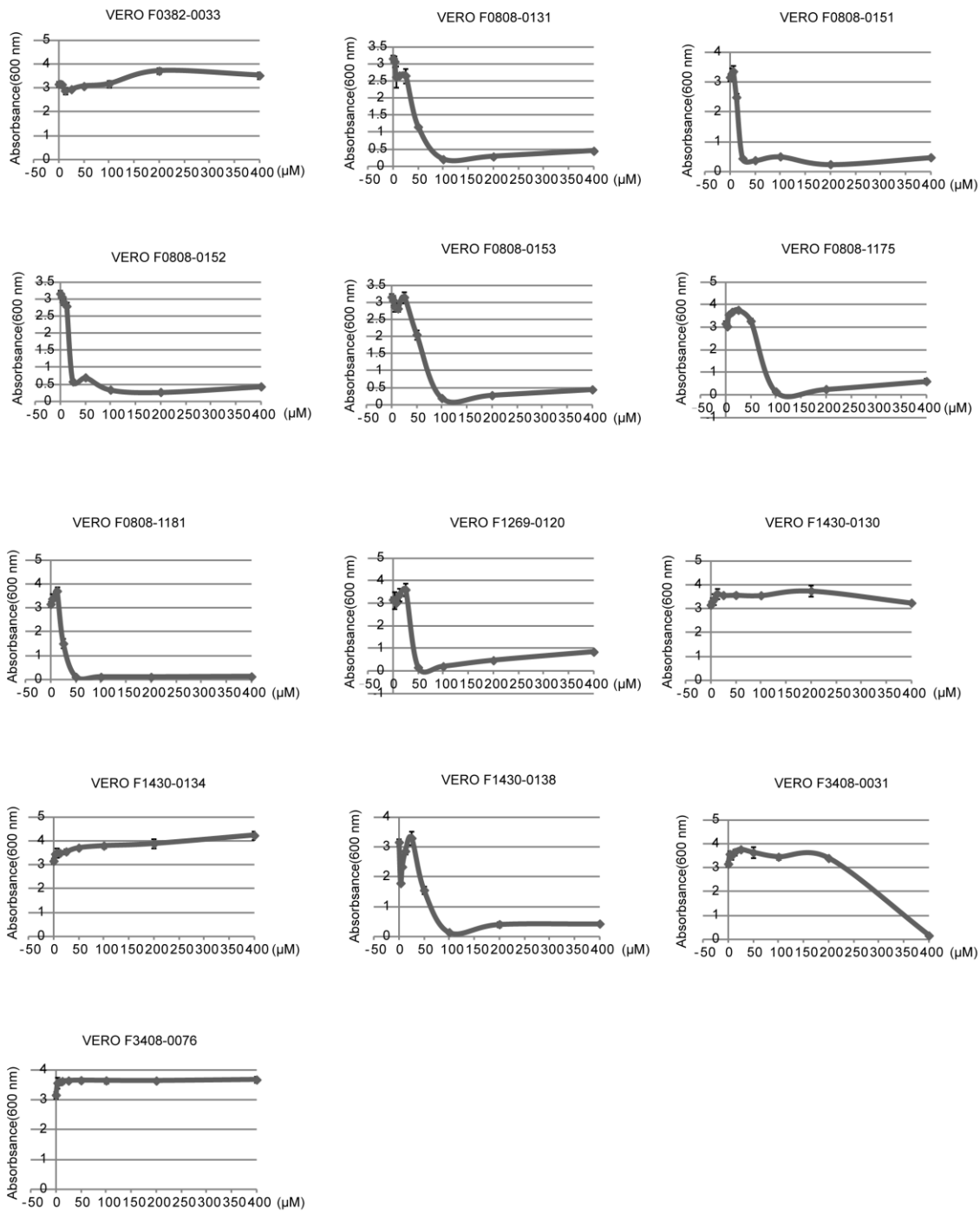
833 Table S1. 13 compounds were chosen based on binding energy <-12.0 kcal/mol.

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Figure S1. A tetrazolium salt (WST-8) assay was carried out to study the toxic effects of compounds on H-BM-MSCs.



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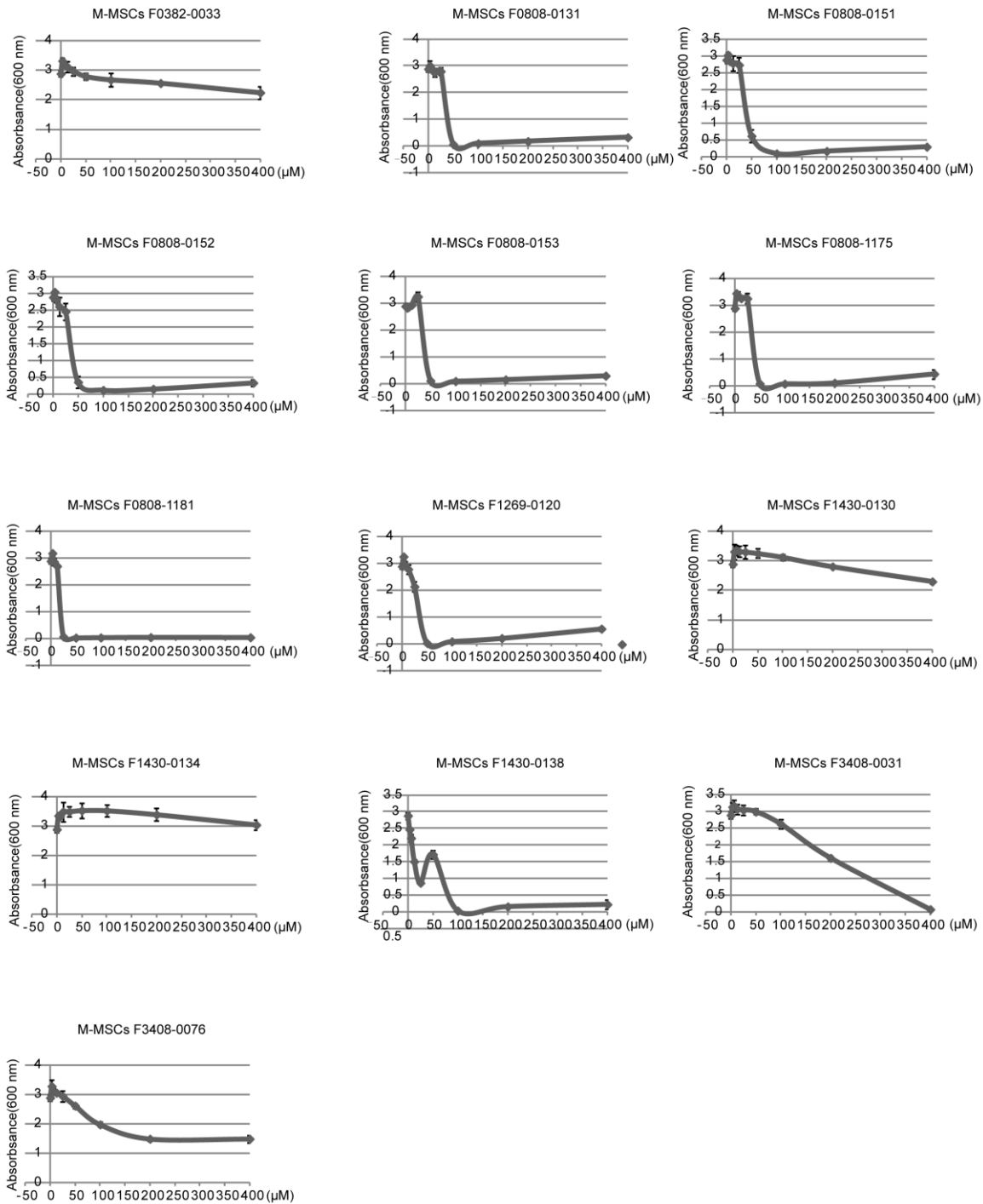
850 Figure S2. A tetrazolium salt (WST-8) assay was carried out to study the toxic effects of
851 compounds on vero cells.

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859 Figure S3. A tetrazolium salt (WST-8) assay was carried out to study the toxic effects of
860 compounds on mouse BM-MSCs.

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