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1 **Original Article**

2 IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways *in*

3 *vitro*

4

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21 **Keywords**

22 Interleukin-6, osteoblast differentiation, MEK2, Akt2, signaling pathway

23

24 **Abstract**

25 It has been suggested that interleukin-6 (IL-6) plays a key role in the pathogenesis of rheumatoid arthritis
26 (RA), including osteoporosis not only in inflamed joints but also in the whole body. However, previous *in*
27 *vitro* studies regarding the effects of IL-6 on osteoblast differentiation are inconsistent. The aim of this
28 study was to examine the effects and signal transduction of IL-6 on osteoblast differentiation in
29 MC3T3-E1 cells and primary murine calvarial osteoblasts. IL-6 and its soluble receptor significantly
30 reduced alkaline phosphatase (ALP) activity, the expression of osteoblastic genes (Runx2, osterix, and
31 osteocalcin), and mineralization in a dose-dependent manner, which indicates negative effects of IL-6 on
32 osteoblast differentiation. Signal transduction studies demonstrated that IL-6 activated not only two major
33 signaling pathways, SHP2/MEK/ERK and JAK/STAT3, but also the SHP2/PI3K/Akt2 signaling
34 pathway. The negative effect of IL-6 on osteoblast differentiation was restored by inhibition of MEK as
35 well as PI3K, while it was enhanced by inhibition of STAT3. Knockdown of MEK2 and Akt2 transfected
36 with siRNA enhanced ALP activity and gene expression of Runx2. These results indicate that IL-6
37 negatively regulates osteoblast differentiation through SHP2/MEK2/ERK and SHP2/PI3K/Akt2
38 pathways, while affecting it positively through JAK/STAT3. Inhibition of MEK2 and Akt2 signaling in
39 osteoblasts might be of potential use in the treatment of osteoporosis in RA.

40

41

42 **Introduction**

43 Inflammation-mediated bone loss is a major feature of various bone diseases, including rheumatoid
44 arthritis (RA). Interleukin-6 (IL-6) contributes to the development of arthritis and is present at high
45 concentrations in the serum and synovial fluid of patients with RA [1-4]. Soluble IL-6 receptor (sIL-6R)
46 is also elevated in the serum and synovial fluid of RA patients [5, 6], and IL-6 exerts its action by binding
47 either to its membrane-bound receptor (mIL-6R) or to sIL-6R. Moreover, IL-6 is closely associated with
48 the expression of receptor activator of NF- κ B ligand (RANKL) in osteoblasts [7]. That is to say, IL-6 acts
49 indirectly on osteoclastogenesis by stimulating the release of RANKL by cells within bone tissues such as
50 osteoblasts [8]. It can unquestionably be said that IL-6 plays a major role in the pathogenesis of RA
51 [9-12], including osteoporosis not only in inflamed joints but also in the whole body.

52 There have been several studies on the effect of IL-6 on bone turnover in animal models. In IL-6
53 knock-out mice, microstructure abnormalities in cortical bones and delayed fracture healing were
54 observed [13, 14], in spite of the evident normal phenotype [15]. Also, bone loss after estrogen depletion
55 was mitigated in IL-6-deficient mice, while a high level of IL-6 and bone loss are seen in wild-type mice
56 [13]. Moreover, IL-6-overexpressed-transgenic mice develop osteopenia and defective ossification, in
57 which the activity of mature osteoblasts is significantly decreased [16]. All these findings, together with
58 studies on human RA patients [17, 18], indicate that IL-6 plays a major role in bone turnover and is an
59 important regulator of bone homeostasis.

60 Recently, several biological agents have been introduced for the treatment of RA and have
61 demonstrated not only potent anti-inflammatory effects but also inhibitory effects on joint destruction.
62 Among these biological agents, tocilizumab, an anti- IL-6 receptor antibody, has been reported to increase
63 serum bone formation markers in RA patients [19], suggesting that IL-6 has a negative effect on
64 osteoblast differentiation. However, previous reports regarding the effects of IL-6 on osteoblast
65 differentiation *in vitro* have been inconsistent [20]. IL-6 has been shown to decrease the expression of
66 differentiation markers in osteoblasts [21, 22] and to inhibit bone formation [23], while it has been shown
67 to induce osteoblast differentiation [24, 25].

68 Binding of IL-6 with sIL-6R or mIL-6R leads to subsequent homodimerization of the
69 signal-transducing molecule gp130, followed by activation of two major intracellular signaling pathways,
70 Janus protein tyrosine kinase (JAK)/ signal transducer and activator of transcription factors (STAT) 3, or
71 Src-homology domain 2 containing protein-tyrosine phosphatase (SHP2)/ mitogen-activated protein
72 kinase-extracellular signal-regulated kinase kinase (MEK)/ mitogen-activated protein kinase (MAPK),
73 also called extracellular signal-regulated kinase (ERK) [26]. There have been many reports in which the
74 effects of IL-6 on JAK/STAT3 and SHP2/ERK signal transduction pathways have been studied in
75 osteoblasts, though it is still controversial whether differentiation is enhanced by IL-6 [9, 20]. SHP2 can
76 also form a tertiary complex with the scaffolding proteins Gab1/2 and the p85 subunit of
77 phosphatidylinositol-3-kinase (PI3K) [27], which leads to activation of the Akt pathway. Several papers

78 have so far reported that the PI3K/Akt pathway triggered by IL-6 plays important roles in various cells
79 [28-32], but no reports have been published regarding the effect of IL-6 on this pathway in osteoblasts.

80 The purpose of this study was to clarify the effect of IL-6 on osteoblast differentiation *in vitro*,
81 with consideration of intracellular signaling pathways in murine MC3T3-E1 osteoblastic cells and
82 primary murine calvarial osteoblasts.

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93 **Materials and methods**

94 **Ethics statement**

95 Prior to the study, all experimental protocols were approved by the Ethics Review Committee for Animal
96 Experimentation of Osaka University School of Medicine.

97

98 **Cell culture**

99 MC3T3-E1 osteoblastic cells were purchased from Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells
100 were cultured in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS;
101 Equitech-Bio, Kerrville, TX, USA) and 1% penicillin and streptomycin at 37°C in a humidified
102 atmosphere of 5% CO₂. All media were purchased from Life Technologies Japan (Tokyo, Japan). Murine
103 primary osteoblasts were isolated from the calvariae of 3-days-old C57BL/6 mice (Charles River
104 Laboratories Japan, Inc, Osaka, Japan) by sequential collagenase digestion as described previously [33].

105 MC3T3-E1 cells and murine calvarial osteoblasts were seeded at 1×10^5 cells per well in 12-well
106 plates. After the cells reached confluence, the medium was replaced to induce osteoblast differentiation.
107 The differentiation medium contained 10% FBS, 10 mM β -glycerophosphate, and 50 μ g/ml ascorbic acid
108 in the absence or presence of recombinant mouse (rm) IL-6 (R&D Systems, Inc., Minneapolis, MN,

109 USA) (10, 50 ng/mL), and rm sIL-6R (R&D Systems) (100 ng/mL). The medium and reagents were
110 renewed every 3 days.

111 To study signal transduction, the following inhibitors or vehicle (DMSO) (Sigma-Aldrich,
112 St.Louis, MO, USA) were added to culture medium at several concentrations; MEK inhibitor (U0126; 1,
113 2.5, 5 μ M; Cell Signaling Technology, Danvers, MA, USA), STAT3 inhibitor (V Stattic; 2.5, 5 μ M;
114 Calbiochem, La Jolla, CA, USA), PI3K inhibitor (LY294002; 1, 2.5, 5 μ M; Cell Signaling Technology),
115 and SHP2 inhibitor (PHPS1; 5, 20, 40 μ M; Sigma-Aldrich). These inhibitors were added 1 h before
116 treatment with IL-6/sIL-6R. All inhibitors were maintained until the end of the culture period at the
117 indicated concentrations.

118

119 **Alkaline phosphatase (ALP) staining and activity**

120 MC3T3-E1 cells and murine calvarial osteoblasts were treated with or without IL-6/sIL-6R and signal
121 pathway inhibitors after the cells reached confluence and were incubated for 6 days.

122 For ALP staining, after fixation with 10% formalin, cells were washed twice with
123 phosphate-buffered saline (PBS) (pH 7.4) and incubated with ALP substrate solution, 0.1 mg/ml naphthol
124 AS-MX (Sigma-Aldrich), and 0.6 mg/ml fast violet B salt (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 8.5)
125 for 20 min.

126 To measure ALP activity, cells were washed twice with PBS and lysed in Mammalian Protein
127 Extraction Reagent (Pierce, Rockford, IL, USA) according to manufacturer's protocol. ALP activity was
128 assayed using *p*-nitrophenylphosphate as a substrate by an Alkaline Phosphatase Test Wako (Wako Pure
129 Chemicals Industries, Ltd., Osaka, Japan), and the protein content was measured using the Bicinchoninic
130 Acid Protein Assay Kit (Pierce).

131

132 **Proliferation assay**

133 MC3T3-E1 cells were cultured in 96-well plates at a concentration of 2.0×10^4 cells/cm² in α -MEM
134 containing 10% FBS. Cells were incubated for 1 day, after which the medium was treated with
135 IL-6/sIL-6R for 3 days. Cell proliferation was assessed using the Premix WST-1 Cell Proliferation Assay
136 System (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. We performed this
137 assay every 24 h.

138

139 **Alizarin red staining**

140 After fixation with 10% formalin, MC3T3-E1 cells and murine calvarial osteoblasts were washed with
141 distilled water, and stained with alizarin red S solution (Sigma-Aldrich) (pH 6.0) for 10 min, followed by
142 incubation in 100 mM cetylpyridinium chloride for 1 h at room temperature to dissolve and release

143 calcium-bound alizarin red. The absorbance of the released alizarin red was then measured at 570 nm
144 [34]. To measure the value of absorbance for alizarin red, the absorbance data were normalized by total
145 DNA content. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf,
146 Germany).

147

148 **Knockdown of MEK1, MEK2, Akt1 and Akt2 using RNA interference**

149 MC3T3-E1 cells were transfected with small interfering RNAs (siRNA) using Lipofectamine RNAiMAX
150 (Life Technologies Japan) according to the reverse transfection method in the manufacturer's protocol.

151 The siRNAs for MEK2, Akt1 and Akt2 and that for MEK1 were purchased from Cell Signaling
152 Technology and Qiagen, respectively, with negative controls for each molecule. MC3T3-E1 cells
153 transfected with siRNA were seeded in 24-well plates at a concentration of 1.0×10^4 cells/cm² for 48 h.
154 The medium was then replaced with differentiation medium with vehicle or with 20 ng/ml IL-6 and 100
155 ng/ml sIL-6R and the cells were incubated for 3 days prior to use for further experiments.

156

157 **Western blotting**

158 Cells cultured in 6-well plates for 2 days were washed twice with PBS and then homogenized with 100 μ
159 of Kaplan buffer (150 mM NaCl, 50 mM Tris-HCL pH 7.4, 1% NP40, 10% glycerol, and 1 tablet per 50
160 ml buffer of protease inhibitor cocktail and phosphatase inhibitor cocktail). The lysates were centrifuged
161 at 13,000 rpm for 20 min at 4°C, and the supernatants were used for electrophoresis after a protein assay
162 using bovine serum albumin as standard. Western blotting was performed by use of the following
163 antibodies purchased from Cell Signaling Technology, except for phosphate anti-Akt2 antibody from
164 Enogene Biotech (New York, NY, USA): phosphate anti-STAT3 (Tyr705) (1:2000) and anti-STAT3
165 (1:1000); phosphate anti-Akt (Ser473) (1:2000), phosphate anti-Akt2 (Ser474) (1:1000), anti-Akt1,
166 anti-Akt2, and anti-Akt (1:1000); phosphate anti-ERK (Thr202/Tyr204) (1:2000), anti-MEK1,
167 anti-MEK2 and anti-ERK (1:1000); and phosphate anti-SHP2 (Tyr542) (1:1000). To control for protein
168 loading, blots were additionally stained with anti- β actin antibody (1:1000).

169

170 **Reverse transcription polymerase chain reaction (RT-PCR)**

171 Total RNA was extracted from cells with an RNeasy Mini Kit (Qiagen), and first-strand cDNA was
172 synthesized using SuperScript II RNase H-reverse transcriptase (Life Technologies Japan). Then PCR
173 was performed using Ex Taq (Takara Bio) and the following primers:

174 *Osteocalcin* (forward primer 5'-CTCACTCTGCTGGCCCTG-3'; reverse primer

175 5'-CCGTAGATGCGTTTGTAGGC-3');

176 *Osterix* (forward primer 5'-AGGCACAAAGAAGCCATAC-3'; reverse primer

177 5'-AATGAGTGAGGGAAGGGT-3');

178 *Runx2* (forward primer 5'-GCTTGATGACTCTAAACCTA-3'; reverse primer

179 5'-AAAAAGGGCCCAGTTCTGAA-3');

180 *GAPDH* (forward primer 5'-TGAACGGGAAGCTCACTGG-3'; reverse primer

181 5'-TCCACCACCCTGTTGCTGTA-3').

182

183 **Quantitative real-time PCR analysis**

184 We obtained cDNA by reverse transcription as mentioned above, and proceeded with real-time PCR

185 using a Light Cycler system (Roche Applied Science, Basel, Switzerland). The SYBR Green assay using

186 a Quantitect SYBR Green PCR Kit (Qiagen), in which each cDNA sample was evaluated in triplicate 20

187 μ l reactions, was used for all target transcripts. Expression values were normalized to GAPDH.

188

189 **Statistical analysis**

190 The results are expressed as the mean \pm standard error (SE). Between-group differences were assessed
191 using the ANOVA test. A probability value of <0.05 was considered to indicate statistical significance.

192 **Results**

193 **IL-6/sIL-6R does not affect proliferation, but significantly reduces ALP activity and expression of** 194 **osteoblastic genes in MC3T3-E1 cells**

195 We first measured the proliferation of MC3T3-E1 cells with IL-6. Cell proliferation did not show
196 significant difference in any culture condition (Fig. 1a).

197 To investigate the influence of IL-6 treatment on osteoblast differentiation, we examined ALP
198 activity in MC3T3-E1 cells. As shown in Figs. 1b and 1c, IL-6/sIL-6R significantly reduced ALP activity
199 in a dose-dependent manner. The single addition of sIL-6R did not show a significant difference as
200 compared to the negative control with vehicle. As shown in Figs. 1d and 1e, gene expression of Runx2,
201 osterix and osteocalcin was significantly down-regulated by IL-6/sIL-6R in a dose-dependent manner.
202 Again, the single addition of sIL-6R did not show significant difference as compared to the negative
203 control with vehicle.

204

205 **IL-6/sIL-6R significantly inhibits mineralization of extracellular matrix (ECM) in MC3T3-E1 cells**

206 As shown in Fig. 2a, IL-6/sIL-6R significantly inhibited the mineralized area in a dose-dependent
207 manner. The single addition of sIL-6R did not show a significant difference as compared to the negative
208 control with vehicle (Fig. 2a). Quantitative analysis of mineralization by measuring the absorbance of
209 alizarin red revealed significant decrease by IL-6/sIL-6R in a dose-dependent manner (Fig. 2b).

210

211 **IL-6/sIL-6R activates ERK, STAT3 and Akt2 signal transduction pathways in MC3T3-E1 cells**

212 When MC3T3-E1 cells were incubated in the presence of IL-6/sIL-6R, phosphorylation of ERK, STAT3
213 and Akt was clearly observed at 15 min, and their activation became weaker at 30 min. When only
214 sIL-6R was added, there was no apparent activation of ERK, STAT3, or Akt as compared to the negative
215 control (Fig. 3a). As for Akt, the phosphorylation by IL-6/sIL-6R was recognized more strikingly as early
216 as 5 min in a dose-dependent manner, both for whole and for Akt2 only, one of its three isoforms (Fig.
217 3b).

218

219 **IL-6-induced activation of ERK is enhanced by blocking the STAT3 signaling pathway, and**

220 **IL-6-induced ERK and Akt signaling pathways negatively regulate each other reciprocally.**

221 The SHP2 inhibitor PHPS1 [35] inhibited IL-6-induced phosphorylation of ERK and Akt to the
222 constitutive level, but did not inhibit STAT3 (Fig. 4a and Supplementary Fig. S1a), suggesting that the

223 downstream pathways of SHP2 are ERK and Akt, not STAT3. The STAT3 inhibitor V Stattic inhibited
224 the phosphorylation of STAT3 but enhanced ERK significantly (Fig. 4a and Supplementary Fig. S1a),
225 suggesting that STAT3 could negatively regulate ERK, which is consistent with previous reports [36].
226 The MEK/ERK inhibitor U0126 completely inhibited both constitutive and IL-6-induced phosphorylation
227 of ERK but enhanced those of Akt. Moreover, the PI3K/Akt inhibitor LY294002 completely inhibited
228 both constitutive and IL-6-induced phosphorylation of Akt but enhanced those of ERK (Fig. 4b and
229 Supplementary Fig. S1b). From these findings, we concluded that IL-6-induced ERK and Akt signaling
230 pathways, both of which are downstream of SHP2, can negatively regulate each other reciprocally.

231

232 **The negative effects of IL-6 on osteoblast differentiation are restored by inhibition of MEK, PI3K**
233 **and SHP2, while they are enhanced by inhibition of STAT3.**

234 To identify the intracellular signaling pathways associated with the down-regulation of osteoblast
235 differentiation, the effects of various signal transduction inhibitors, consisting of a MEK inhibitor
236 (U0126), PI3K inhibitor (LY294002), SHP2 inhibitor (PHPS1), and STAT3 inhibitor (V Stattic), were
237 assessed for ALP activity, the expression of osteoblastic genes (Runx2, osterix and osteocalcin), and the
238 mineralization of ECM.

239 The negative effect of IL-6/sIL-6R on ALP activity was restored by treatment with either U0126,
240 LY294002, or PHPS1 in a dose-dependent manner. On the other hand, the negative effect of IL-6/sIL-6R
241 on ALP activity was enhanced by treatment with V Static (Fig. 5a). These results indicate that the
242 SHP2-associated signal transduction molecules MEK/ERK and PI3K/Akt have a negative effect on
243 osteoblast differentiation, whereas the JAK-associated molecule STAT3 has a positive effect.

244 The negative effect of IL-6/sIL-6R on the expression of osteoblastic genes (Runx2, osterix and
245 osteocalcin) was also restored by treatment with either U0126, LY294002, or PHPS1 in a dose-dependent
246 manner, while it was enhanced by treatment with V Static (Fig. 5b). Moreover, a high dose of PHPS1, 20
247 μM , caused significantly up-regulated expression of osteocalcin.

248 For mineralization of ECM, the negative effect of IL-6/sIL-6R was restored by treatment with
249 either U0126, LY294002, or PHPS1. As with ALP activity and osteoblastic gene expression, the negative
250 effect of IL-6/sIL-6R on mineralization was enhanced by treatment with V Static (Figs. 6a and 6b). ALP
251 activity, osteoblastic gene expression, and mineralization of ECM in cells treated only with each inhibitor
252 demonstrated the same behavior (Figs. 5 and 6).

253 Furthermore, the negative effects of ALP activity, osteoblastic gene expression and mineralization
254 of ECM by stimulation with IL-6/sIL-6R were compared between in the presence and in the absence of
255 each inhibitor. The negative effects on osteoblast differentiation by IL-6/sIL-6R showed a tendency to
256 decrease in the presence of each inhibitor, as compared to the absence of inhibitors (Figs. 5 and 6). The

257 negative effects were decreased by 15-44%, 20-61%, 7-140%, and 21-80% in the presence of U0126,
258 LY294002, PHPS1 and V Static, respectively, as compared to the absence of inhibitors. These results
259 indicate that the effects of IL-6/sIL-6R on osteoblast differentiation might be mediated either by
260 MEK/ERK, PI3K/Akt, or JAK/STAT3 pathways.

261

262 **Knockdown of MEK2 and Akt2 via siRNA transfection restores ALP activity and Runx2 gene**
263 **expression**

264 To further confirm the effects of MEK and Akt inhibition on osteoblast differentiation in MC3T3-E1 cells,
265 we studied cell differentiation after knockdown of MEK and Akt. For each protein, RNAs of two
266 isoforms were separately blocked: MEK1 and MEK2 for MEK, and Akt1 and Akt2 for Akt.

267 The protein expression level of each molecule was found to be diminished selectively at 48 h after
268 transfection of the respective siRNAs (Fig. 7a). The ALP activity in MC3T3-E1 cells treated with
269 IL-6/sIL-6R was restored by knockdown of MEK2 and Akt2 as compared to that in cells transfected with
270 negative control siRNA. On the other hand, knockdown of MEK1 and Akt1 enhanced the negative effects
271 of IL-6/sIL-6R on ALP activity (Fig. 7b). (ALP activity after transfection with each siRNA without
272 IL-6/sIL-6R demonstrated the same behavior; Fig. 7b.) Quantitative real-time PCR analysis revealed that
273 the gene expressions of Runx2, osterix, and osteocalcin were restored by knockdown of MEK2. On the

274 other hand, knockdown of Akt2 also restored Runx2, but decreased osteocalcin expression (Fig. 7c),
275 while knockdown of Akt2 without IL-6/sIL-6R caused no significant difference in Runx2 expression (Fig.
276 7b). As was recognized for ALP activity, knockdown of MEK1 and Akt1 enhanced the down-regulation
277 of osteocalcin expression (Figs. 7b and 7c). Also, the negative effects of IL-6/sIL-6R on osteoblast
278 differentiation showed some tendency to decrease with each knockdown compared to those without
279 knockdown. The negative effects were decreased by 2-24%, 4-27%, 7-43%, and 21-26% with knockdown
280 of MEK1, MEK2, Akt1, and Akt2, respectively, as compared to those without knockdown. These results
281 indicate that IL-6 may suppress osteoblast differentiation through MEK2 and Akt2.

282

283 **IL-6/sIL-6R inhibits the differentiation of primary murine calvarial osteoblasts by activating**
284 **phosphorylation of ERK, Akt2, and STAT3**

285 Experiments were repeated with murine calvarial osteoblasts isolated from the calvariae of 3-day-old
286 C57BL/6 mice. As was recognized in MC3T3-E1 cells, IL-6 inhibited ALP activity (Fig. 8a), the
287 expression of osteoblastic genes (Fig. 8b), and mineralization (Figs. 8c and 8d) in a dose-dependent
288 manner. Furthermore, IL-6 induced phosphorylation of ERK, Akt2, and STAT3 (Fig. 8e), which was
289 exactly the same as with MC3T3-E1 cells.

290

291

292

293 **Discussion**

294 We examined the effects of IL-6 and its soluble receptor on the proliferation and differentiation of murine
295 MC3T3-E1 osteoblastic cells and primary murine calvarial osteoblasts. Our results showed that they
296 significantly reduced ALP activity, bone mineralization, and expression of the osteoblastic genes Runx2
297 osterix and osteocalcin, in a dose-dependent manner. From these experiments, we clearly demonstrated
298 that IL-6 inhibited osteoblast differentiation of MC3T3-E1 cells and primary murine calvarial osteoblasts.

299 It has been demonstrated that the JAK/STAT3 signaling pathway has important roles both *in vivo*
300 and *in vitro* in the differentiation of osteoblasts [37, 38]. Our results are consistent with previous reports
301 and imply that the activation of STAT3 induced by IL-6 may induce osteoblast differentiation.

302 IL-6 activates another major intracellular signaling pathway, SHP2/ERK, and can also lead to the
303 activation of an additional signaling cascade involving SHP2/PI3K/Akt. IL-6-induced activation of PI3K
304 and downstream protein kinase Akt/PKB has been reported to play important roles in the proliferation of
305 prostate cancer cells [30, 31], hepatoma cells [32], and multiple myeloma cells [29]. They were also
306 reported to associate with neuroendocrine differentiation of prostate cancer cells induced by IL-6 [32]. In
307 this study, we focused on the PI3K/Akt pathway triggered by IL-6, because no reports have demonstrated

308 the role of IL-6 in the activation of PI3K/Akt signaling pathway in osteoblasts. We have demonstrated for
309 the first time that IL-6-induced activation of Akt2, one of the downstream pathways of SHP2, may be a
310 key player in the negative regulation of osteoblast differentiation induced by IL-6. Among the three
311 isoforms of Akt, Akt1 and Akt2 are highly expressed in osteoblasts [39]. Mice lacking Akt1, the major
312 isoform in bone tissue, exhibit osteopenia [40, 41], and the impact of Akt1 deficiency in osteoblast
313 differentiation and bone development have also been published [39, 42-44], all of which are consistent
314 with our results showing that knockdown of Akt1 signaling by siRNA inhibited osteoblast differentiation.
315 In contrast, Mukherjee et al. reported enhanced osteogenic differentiation in the absence of Akt1 in cell
316 lines [44]. Moreover, they reported that Akt2 was required for BMP2-initiated osteoblast differentiation
317 of cultured murine mesenchymal stem cells but that Akt1 was dispensable in this assay [45], which is
318 inconsistent with our results showing that knockdown of Akt2 signaling by siRNA promoted osteoblast
319 differentiation. These discrepancies might be due to the difference between cell types, i.e.
320 intramembranous (calvariae) cells and endochondral (long bones) cells.

321 In this study, gene expression of osteocalcin, a late osteoblastic differentiation marker, was
322 up-regulated by treatment with a PI3K/Akt inhibitor, but was down-regulated by knockdown of both Akt1
323 and Akt2. Moreover, complete blockade with a high dose (more than 10 μ M) of the PI3K/Akt inhibitor
324 conversely down-regulated the expression of osteocalcin (data not shown). This discrepancy may be due
325 to the difference between the temporary or partial blockade by the inhibitor and constitutive knockdown

326 by siRNA. Since bone formation has been reported to increase without impairment of mineralization and
327 resorption even in osteocalcin-deficient mice [46], the expression of osteocalcin may not directly affect
328 bone formation.

329 We have previously reported that osteoblast differentiation was significantly promoted by MEK
330 inhibitor in BMP-2-treated C2C12 cells and MC3T3-E1 cells [47]. Our findings in the present study are
331 consistent with our previous report and others [47-49] at the point that IL-6-induced activation of ERK
332 significantly down-regulated osteoblast differentiation. In addition, our results suggest that there might be
333 different roles in osteoblast differentiation between MEK1 and MEK2. Constitutively active expression of
334 MEK1 has been reported to accelerate bone development both *in vitro* [50] and *in vivo* [51], which is
335 consistent with the results showing that knockdown of MEK1 inhibited osteoblast differentiation in the
336 present study. As for MEK2, there are no reports concerning its roles in osteoblast differentiation, and we
337 are the first to demonstrate that MEK2 may also be a key player in the negative regulation of osteoblast
338 differentiation induced by IL-6. The effects of a MEK inhibitor that inhibits both MEK1 and MEK2 on
339 bone formation are still controversial [52]. These controversies might be due to different roles played
340 between MEK1 and MEK2 in osteoblast differentiation, and the effects of MEK inhibitors could depend
341 on which pathway is predominantly inhibited in each study.

342 With respect to intracellular signaling pathways, our results showed that IL-6 triggers three
343 signaling pathways, one of which has a conflicting function with the others. SHP2/ERK and SHP2/Akt2

344 negatively affects osteoblast differentiation, whereas JAK/STAT3 positively affects it (Fig. 9). In other
345 cells, it is often that simultaneous activation of the SHP2/ERK and JAK/STAT3 cascades generate
346 opposing, or at least different signals. In osteoclasts, for example, SHP2/ERK activation inhibits
347 osteoclastogenesis [53], whereas STAT3 is a pro-osteoclastic molecule after phosphorylation on
348 serine727 [54]. In myeloid leukemic M1 cells, STAT3 induces differentiation *in vitro* [55], whereas the
349 SHP2/ERK pathway promotes their proliferation [56]. These examples suggest that the integration of
350 opposing activities transduced by more than one pathway could provide a biologically balanced state in
351 the end, remaining availability to respond to another physiological situation. Indeed, Hirano and
352 colleagues have proposed a “signaling orchestration” model in a single cell, where the balance or
353 interplay of simultaneously generated contradictory signals eventually determines the biological outcome
354 [57]. Thus, the inconsistent results regarding the effects of IL-6 on osteoblast differentiation in previous
355 reports could be explained by which intracellular signaling pathway was predominantly activated in each
356 study. The balance of three signaling pathways could be influenced by such conditions as the variety of
357 cultured cells, the stage of cell differentiation, and the employed culture conditions.

358 To the best of our knowledge, this is the first report of signal crosstalk in which IL-6-induced ERK
359 and Akt signaling pathways negatively regulated each other in cultured osteoblastic cells. In this study,
360 however, cancellation of the negative effects of IL-6/sIL-6R on osteoblast differentiation by inhibitors
361 was incomplete as compared to the absence of inhibitor (Figs. 5 and 6). This might be because ERK, Akt
362 and STAT3 are all critical pathways in osteoblast differentiation even in the absence of IL-6/sIL-6R, and

363 even though one pathway is blocked, another pathway is enhanced by reciprocal regulation in the
364 crosstalk between IL-6-activated signaling pathways (Fig. 9). Our results demonstrated that a STAT3
365 inhibitor significantly enhanced IL-6-induced activation of ERK and SHP2, but not of Akt (Fig. 4a).
366 SHP2 could predominantly lead to the activation of the ERK signaling pathway as compared to Akt, and
367 the enhanced signaling of ERK may restrain the enhancement of the Akt signaling pathway in a negative
368 feedback manner.

369 The results obtained from the present study show that SHP2, MEK and PI3K inhibitors would be
370 of potential use for the treatment of osteoporotic changes in RA patients. In particular, SHP2 inhibitors
371 not only could inhibit the negative effect of IL-6-induced MEK/ERK and PI3K/Akt2 signaling, but also
372 enhance the positive effect of IL-6-induced STAT3 signaling on osteoblast differentiation [37]. However,
373 since a pro-inflammatory effect of STAT3 on synovitis has been reported [36, 58], selective inhibition of
374 MEK2 and Akt2 signaling in osteoblasts may be more promising in order to avoid the enhancement of
375 synovitis and consequent joint destruction.

376 In conclusion, our study provides new insights in the pathophysiology as well as potential
377 treatment options for bone loss in RA, focusing on osteoblast differentiation *in vitro*. Our results
378 demonstrated that IL-6 could inhibit osteoblast differentiation through MEK2/ERK and PI3K/Akt2
379 signaling pathways, both of which are SHP2-dependent downstream signaling pathways.

380

381 **Conflict of interest**

382 All authors have no conflicts of interest.

383

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546 Figure legends

547 **Fig. 1**

548 IL-6/sIL-6R significantly reduced ALP activity and expression of osteoblastic genes in MC3T3E1 cells,

549 but did not affect proliferation.

550 (a) Proliferation of MC3T3-E1 cells with IL-6/sIL-6R was examined. Cells were pre-incubated for 1 day

551 and then the medium was treated with or without IL-6/sIL-6R for 3 days. Cell proliferation assay was

552 performed daily throughout the 4 days of incubation. Cell proliferation did not show significant

553 differences in any culture condition. (b) ALP staining was performed in MC3T3-E1 cells treated with or

554 without IL-6/sIL-6R for 6 days. Apparently significant reduction of ALP staining was recognized in cells

555 treated with either 10 ng/ml or 50 ng/ml IL-6. (c) ALP activity of the lysates of MC3T3-E1 cells treated

556 with or without IL-6/sIL-6R for 6 days was measured using p-nitrophenylphosphate as a substrate.

557 IL-6/sIL-6R significantly reduced ALP activity in a dose-dependent manner. (d) Total RNA was

558 extracted from MC3T3-E1 cells treated with or without IL-6/sIL-6R for 6 days and subjected to RT-PCR

559 for osteoblastic genes Runx2, osterix, and osteocalcin. Apparently significant reduction of osteoblastic

560 gene expression was recognized in cells treated with either 10 ng/ml or 50 ng/ml IL-6. (e) Real-time PCR
561 for Runx2, osterix, and osteocalcin was performed for quantitative analysis. Data were normalized to
562 GAPDH expression and are shown as the ratio of expression compared to control cells treated with
563 vehicle. The expression of osteoblastic genes was significantly down-regulated by IL-6/sIL-6R in a
564 dose-dependent manner. Representative data from at least 3 independent experiments are shown. Data are
565 shown as means \pm SE. *n.s.* not significant; **P* < 0.05; ***P* < 0.001; ****P* < 0.001

566 **Fig. 2**

567 IL-6/sIL-6R significantly inhibited mineralization of ECM in MC3T3E1 cells.

568 MC3T3-E1 cells were treated with or without IL-6/sIL-6R and were incubated for 21 days. (a) After
569 fixation, the cells were stained with alizarin red solution. Apparently significant reduction of alizarin red
570 staining was recognized in the cells treated with either 10 ng/ml, 25 ng/ml, or 50 ng/ml IL-6. (b) Matrix
571 mineralization was quantified by the measurement of absorbance of alizarin red and normalized by total
572 DNA content. Matrix mineralization was significantly reduced by IL-6/sIL-6R in a dose-dependent
573 manner. Representative data from at least 3 independent experiments are shown. Data are shown as
574 means \pm SE. *n.s.* not significant; **P* < 0.05; ***P* < 0.001; ****P* < 0.001.

575 **Fig. 3**

576 IL-6/sIL-6R activated ERK, STAT3, and Akt2 signal transduction pathways in MC3T3-E1 cells.

577 (a) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml or 50 ng/ml IL-6 and 100 ng/ml sIL-6R in
578 a time-course experiment (0, 15, and 30 min). Western blot analysis was performed using cell lysates for
579 the detection of ERK, STAT3, and Akt, either phosphorylated or not. IL-6/sIL-6R significantly induced
580 the phosphorylation of ERK, STAT3, and Akt in a dose-dependent manner. (b) MC3T3-E1 cells were
581 incubated with increasing concentrations of IL-6 and 100 ng/ml sIL-6R for 5 min. Western blotting was
582 performed using cell lysates for the detection of ERK, STAT3, as well as Akt, either non-phosphorylated,
583 phosphorylated, or the phosphorylated isoform Akt2. The phosphorylation of both whole Akt and Akt2
584 by IL-6/sIL-6R was recognized more strikingly in a dose-dependent manner. Representative data from at
585 least 3 independent experiments are shown.

586 **Fig. 4**

587 IL-6-induced activation of ERK was enhanced by blocking the STAT3 signaling pathway, and
588 IL-6-induced ERK and Akt signaling pathways negatively regulated each other reciprocally.

589 (a) MC3T3-E1 cells were stimulated with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (15 min) after
590 pre-treatment either with PHPS1 (5, 20, 40 μ M; 1 h), with U0126 (5 μ M; 1 h), or with V Static (5 μ M; 1
591 h), and the cell lysates were subjected to Western blotting. PHPS1 inhibited IL-6-induced
592 phosphorylation of ERK and Akt to the constitutive level, but not of STAT3. IL-6-induced activation of
593 ERK was enhanced by V Static. (b) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml IL-6 and
594 100 ng/ml sIL-6R (15 min) after pre-treatment either with U0126 (5 μ M; 1 h) or with LY294002 (10 μ M;

595 1 h), and the cell lysates were subjected to Western blotting. Both constitutive and IL-6-induced
596 phosphorylation of Akt and ERK were enhanced by treatment with U0126 and LY294002, respectively.
597 Representative data from at least 3 independent experiments are shown.

598 **Fig. 5**

599 The negative effects of IL-6 on ALP activity and the expression of osteoblastic genes were restored by
600 inhibition of MEK, PI3K, and SHP2, while they were enhanced by inhibition of STAT3.

601 MC3T3-E1 cells were pre-treated either with U0126 (1, 2.5, 5 μ M; 1 h), LY294002 (1, 2.5, 5 μ M; 1 h),
602 PHPS1 (5, 20 μ M; 1 h), or V Stattic (5 μ M; 1 h), then stimulated either with 10 ng/ml IL-6 and 100 ng/ml
603 sIL-6R or with vehicle and incubated for 6 days. (a) ALP activity of the cell lysates was measured using
604 p-nitrophenylphosphate as a substrate. The negative effect of IL-6 on ALP activity was restored by
605 treatment with either U0126, LY294002, or PHPS1 in a dose-dependent manner, while it was enhanced
606 by treatment with V Stattic. (b) Total RNA was extracted and real-time PCR for Runx2, osterix, and
607 osteocalcin was performed. Data were normalized to GAPDH expression and are shown as the ratio of
608 gene expression compared to control cells treated with vehicle. The negative effect of IL-6 on expression
609 of osteoblastic genes was restored by treatment either with U0126, LY294002, or PHPS1 in a
610 dose-dependent manner, while it was enhanced by treatment with V Stattic. Representative data from at
611 least 3 independent experiments are shown. Data are shown as means \pm SE. *n.s.* not significant; [#]*P* < 0.05;

612 $^{##}P < 0.001$; $^{###}P < 0.001$, compared to the group treated with vehicle. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P <$
613 0.001 , compared to group treated with IL-6/sIL-6R.

614 **Fig. 6**

615 The negative effect of IL-6 on mineralization of ECM was restored by inhibition of MEK, PI3K, and
616 SHP2, while it was enhanced by inhibition of STAT3.

617 MC3T3-E1 cell were pre-treated either with U0126 (1 μ M; 1 h), LY294002 (1 μ M; 1 h), PHPS1 (20 μ M;
618 1 h), or V Stattic (2.5 μ M; 1 h), then stimulated with either 10 ng/ml IL-6 and 100 ng/ml sIL-6R or with

619 vehicle and incubated for 21 days. (a) After fixation, the cells were stained with alizarin red solution. The

620 reduction of alizarin red staining by IL-6/sIL-6R was restored in cells treated with either U0126,

621 LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. (b) Quantification of matrix

622 mineralization was by measurement of absorbance for alizarin red normalized by total DNA content. The

623 reduction of matrix mineralization by IL-6/sIL-6R was restored in cells treated with either U0126,

624 LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. Representative data from at

625 least 3 independent experiments are shown. Data are shown as means \pm SE. *n.s.* not significant; $^{#}P < 0.05$;

626 $^{##}P < 0.001$; $^{###}P < 0.001$, compared to the group treated with vehicle. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P <$
627 0.001 , compared to group treated with IL-6/sIL-6R.

628 **Fig. 7**

629 Knockdown of MEK2 and Akt2 in cells transfected with siRNA restored ALP activity and Runx2 gene
630 expression.

631 (a) MC3T3-E1 cells transfected with respective siRNAs were cultured for 48 h. Western blotting was
632 performed using cell lysates stimulated with vehicle or with 20 ng/ml IL-6 and 100 ng/ml sIL-6R (15
633 min). Expression levels of each protein, MEK1, MEK2, Akt1, and Akt2, were selectively diminished at
634 48 h after transfection with respective siRNAs. (b) MC3T3-E1 cells transfected with respective siRNAs
635 were incubated for 48 h after which the medium was changed to differentiation medium with vehicle or
636 with 20 ng/ml IL-6 and 100 ng/ml sIL-6R. The cells were then incubated for 3 days to evaluate osteoblast
637 differentiation. ALP activity in MC3T3-E1 cells treated with IL-6/sIL-6R was restored by knockdown of
638 MEK2 and Akt2 as compared to that in cells transfected with negative control siRNA. (c) Expression of
639 osteoblastic genes in MC3T3-E1 cells transfected with respective siRNAs was assessed by real-time PCR.
640 The expression of each gene was normalized against GAPDH expression. The gene expressions of Runx2,
641 osterix, and osteocalcin were restored by knockdown of MEK2. Knockdown of Akt2 also restored Runx2,
642 but decreased osteocalcin. Representative data from at least 3 independent experiments are shown. Data
643 are shown as means \pm SE. *n.s.* not significant; [#]*P* < 0.05; ^{##}*P* < 0.001; ^{###}*P* < 0.001, compared to negative
644 control group treated with vehicle. **P* < 0.05; ***P* < 0.001; ****P* < 0.001, compared to negative control
645 group treated with IL-6/sIL-6R.

646 **Fig. 8**

647 IL-6/sIL-6R inhibited the differentiation of primary murine calvarial osteoblasts with the activated
648 phosphorylation of ERK, Akt2, and STAT3.

649 (a) ALP activity of lysates of murine calvarial osteoblasts treated with or without IL-6/sIL-6R for 6 days
650 was measured using p-nitrophenylphosphate as a substrate. IL-6/sIL-6R significantly reduced ALP
651 activity in a dose-dependent manner. (b) Total RNA was extracted from murine calvarial osteoblasts
652 treated with or without IL-6/sIL-6R for 6 days, and real-time PCR for Runx2, osterix, and osteocalcin was
653 performed. Data were normalized to GAPDH expression and are shown as the ratio of gene expression as
654 compared to control cells treated with vehicle. The expression of osteoblastic genes was significantly
655 down-regulated by IL-6/sIL-6R in a dose-dependent manner. (c) Murine calvarial osteoblasts were treated
656 with or without IL-6/sIL-6R and were cultured for 21 days. After fixation, the cells were stained with
657 alizarin red solution. Apparently significant reduction of alizarin red staining was recognized in cells
658 treated with either 10 ng/ml or 50 ng/ml IL-6. (d) Matrix mineralization was quantified by measurement
659 of absorbance for alizarin red normalized by total DNA content. IL-6/sIL-6R significantly inhibited
660 mineralization of ECM in a dose-dependent manner. (e) Primary murine calvarial osteoblasts were treated
661 with vehicle or 10 ng/ml or 50 ng/ml IL-6 and 100 ng/ml sIL-6R in a time-course experiment (5, 15, and
662 30 min). Western blotting was performed using cell lysates. IL-6 significantly induced the
663 phosphorylation of ERK, Akt2, and STAT3 in a dose-dependent manner. Representative data from at

664 least 3 independent experiments are shown. Data are shown as means \pm SE. *n.s.* not significant; **P* <
665 0.05; ***P* < 0.001; ****P* < 0.001.

666

667 **Fig. 9**

668 Schematic presentation of signaling pathways involved in osteoblast differentiation induced by IL-6.

669 IL-6-induced novel SHP2/MEK2/ERK and SHP2/PI3K/Akt2 signal crosstalk in osteoblastic cells; ERK

670 and Akt signaling pathways, both of which are downstream of SHP2, negatively regulate each other

671 reciprocally. On the other hand, the STAT3 signaling pathway negatively regulates the ERK signaling

672 pathway. MEK2/ERK and PI3K/Akt2 have negative effects on osteoblast differentiation, whereas STAT3

673 has a positive effect. Overall, IL-6 inhibits osteoblast differentiation through MEK2 and Akt2 signaling

674 pathways.

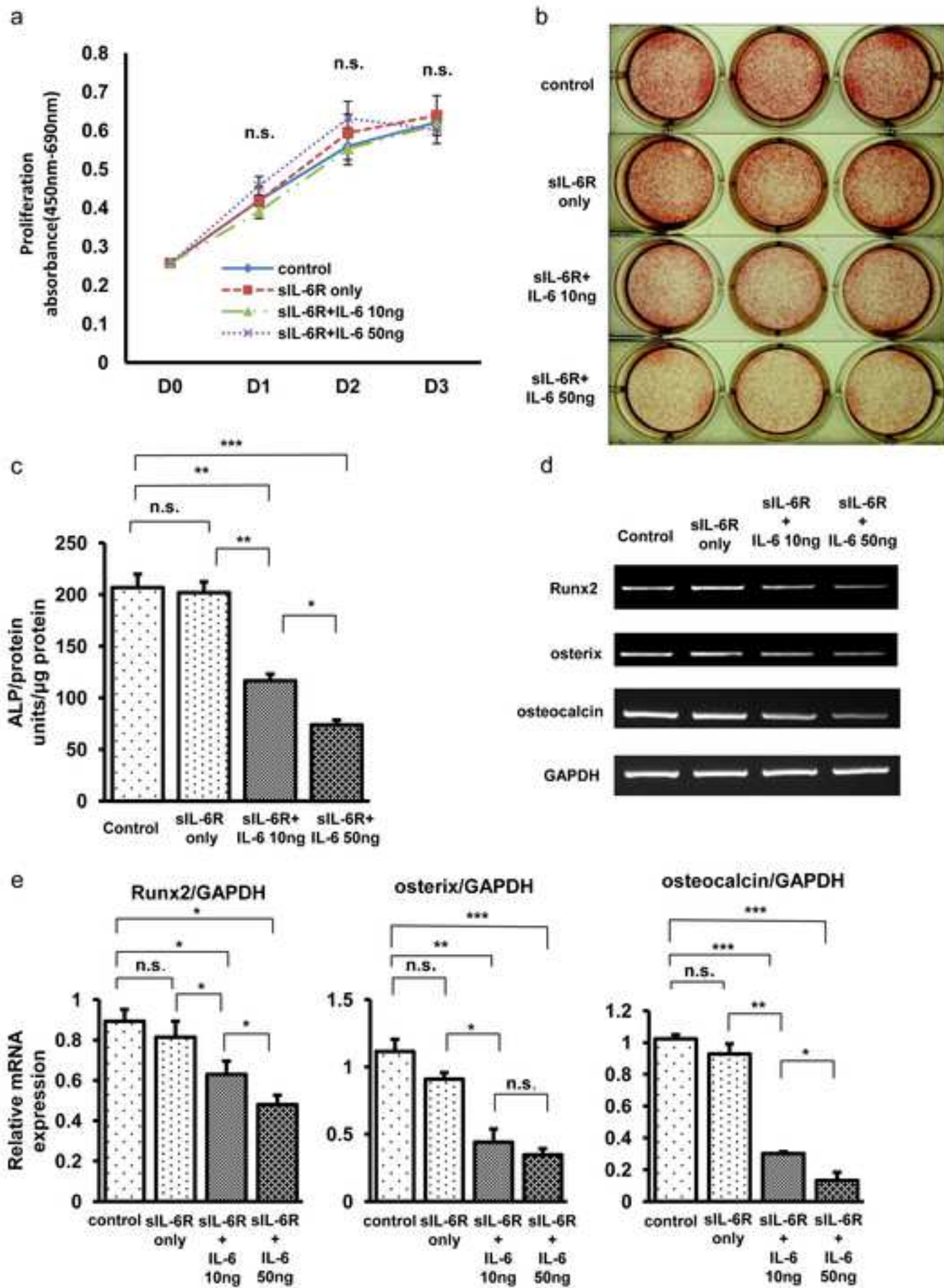
675

676 **Supplementary Fig. S1**

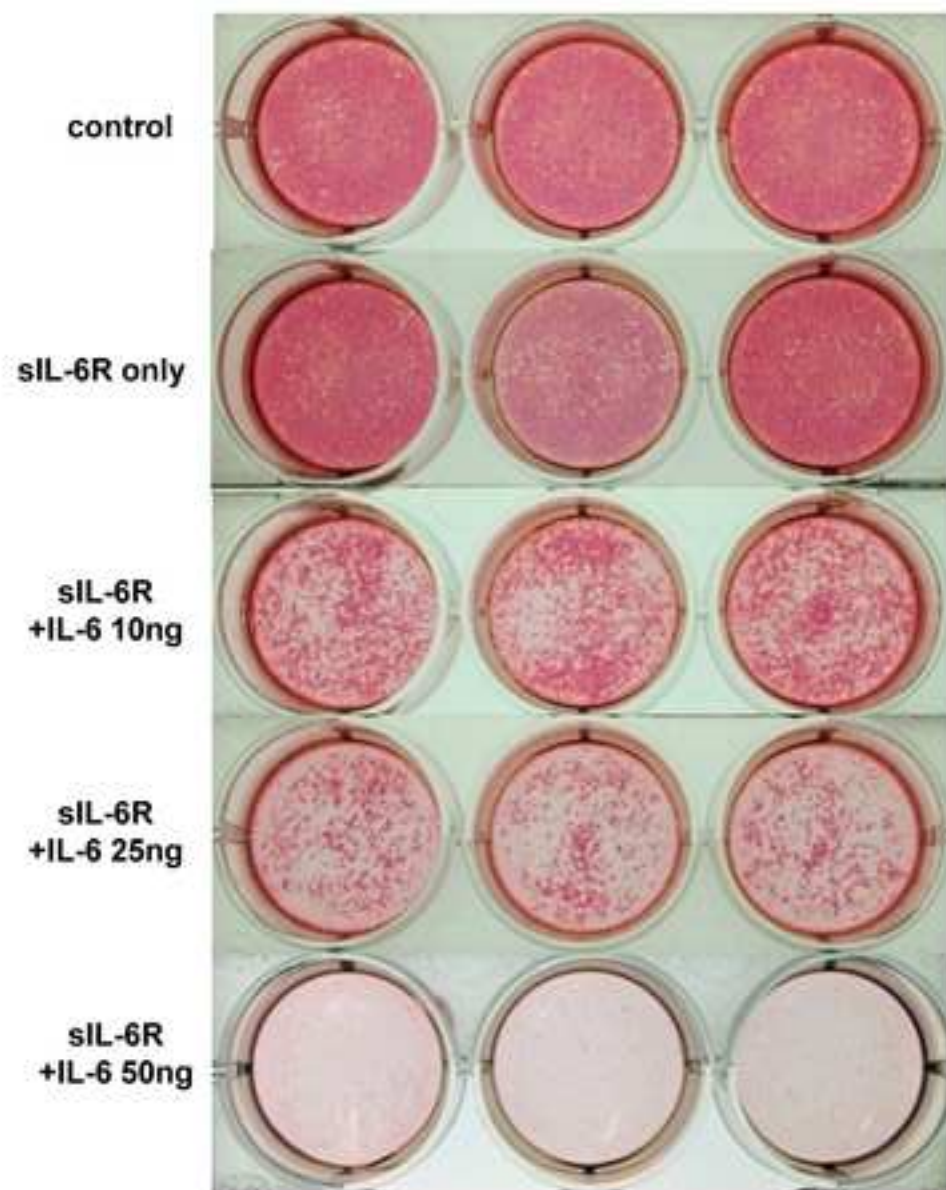
677 IL-6-induced activation of ERK was enhanced by blocking the STAT3 signaling pathway, and

678 IL-6-induced ERK and Akt signaling pathways negatively regulated each other reciprocally.

679 (a) MC3T3-E1 cells were stimulated with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (30 min) after
680 pre-treatment either with PHPS1 (5, 20, 40 μ M; 1 h), U0126 (5 μ M; 1 h), or V Static (5 μ M; 1 h), and the
681 cell lysates were subjected to Western blotting. PHPS1 inhibited IL-6-induced phosphorylation of ERK
682 and Akt to the constitutive level, but not phosphorylation of STAT3. IL-6-induced activation of ERK was
683 enhanced by V Static. (b) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml IL-6 and 100
684 ng/ml sIL-6R (30 min) after pre-treatment with either U0126 (5 μ M; 1 h) or LY294002 (10 μ M; 1 h), and
685 and the cell lysates were subjected to Western blotting. Both constitutive and IL-6-induced
686 phosphorylation of Akt and ERK were enhanced by treatment with U0126 and LY294002, respectively.
687 Representative data from at least 3 independent experiments are shown.



a



b

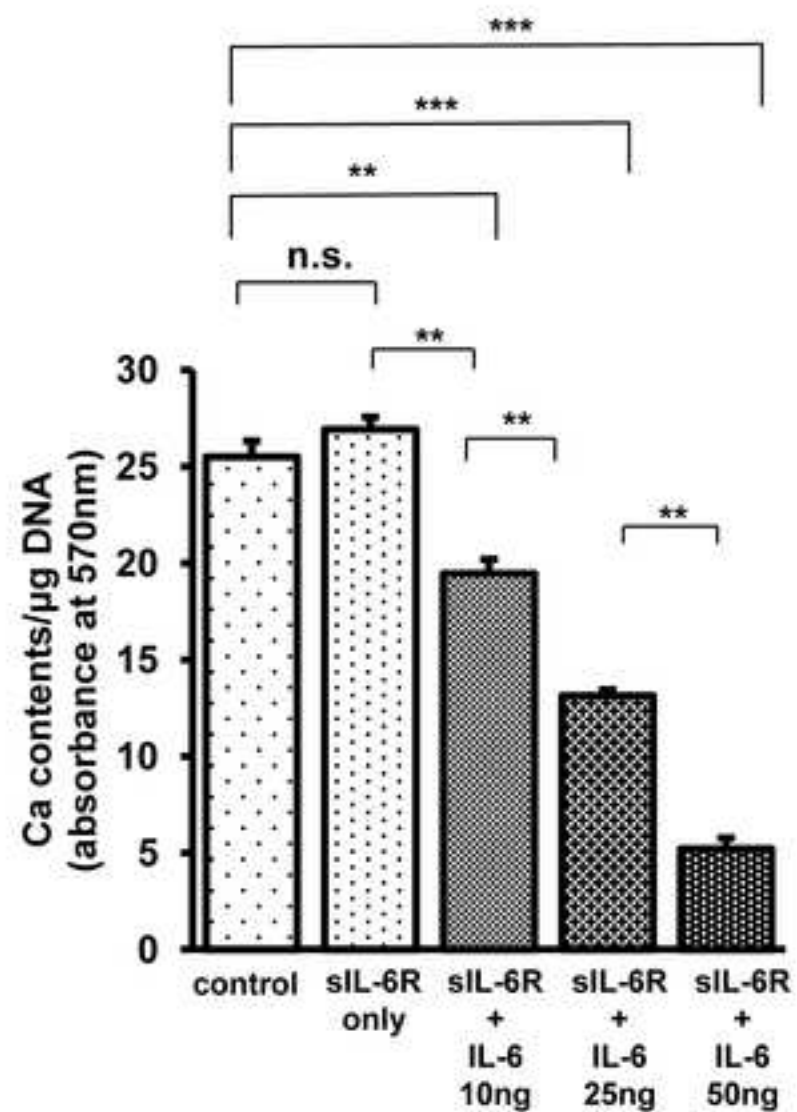


Figure3

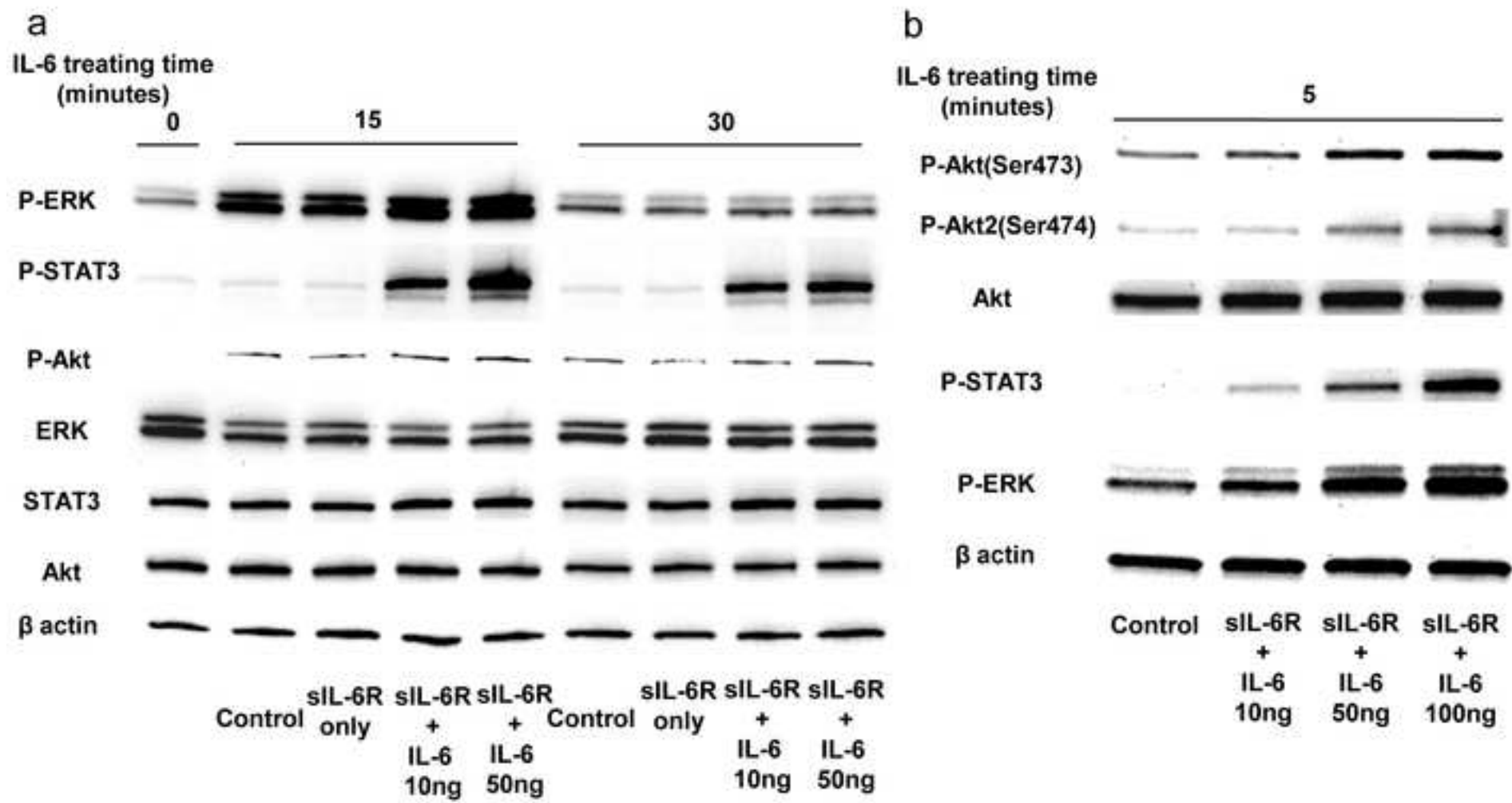
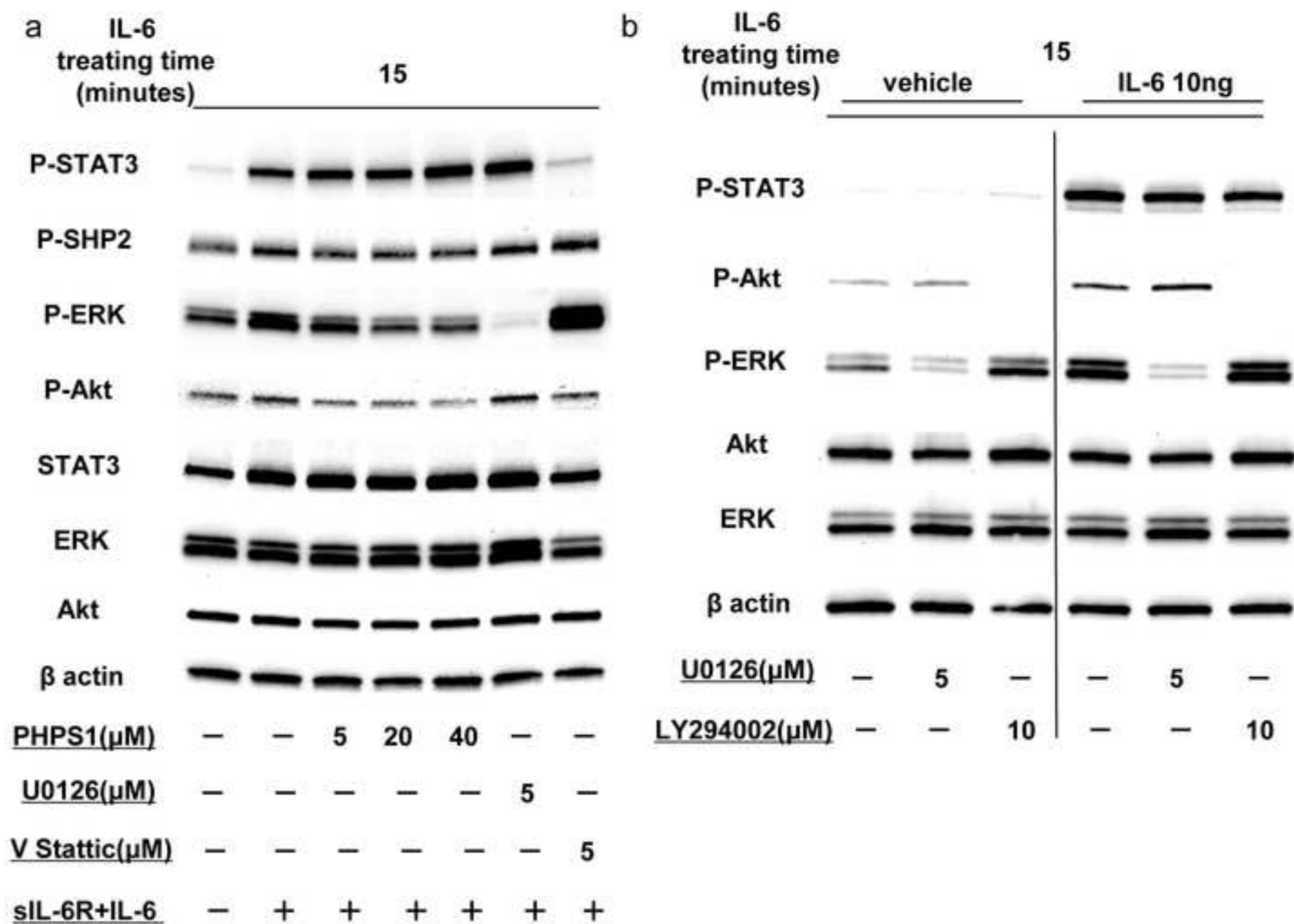
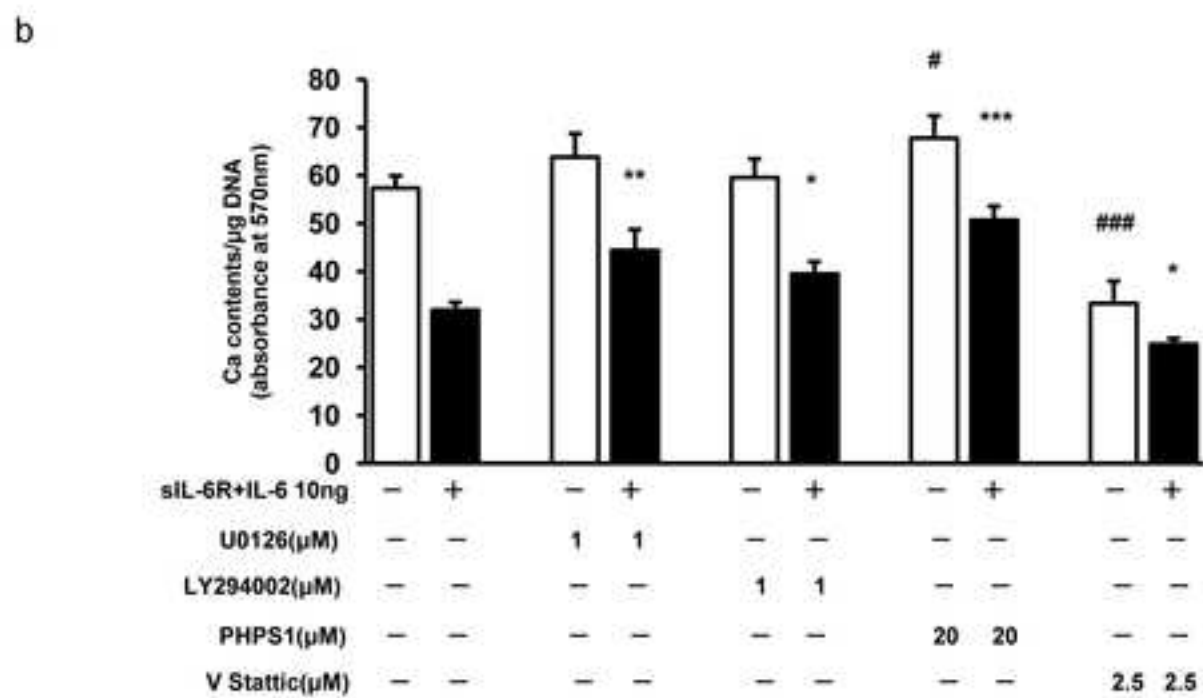
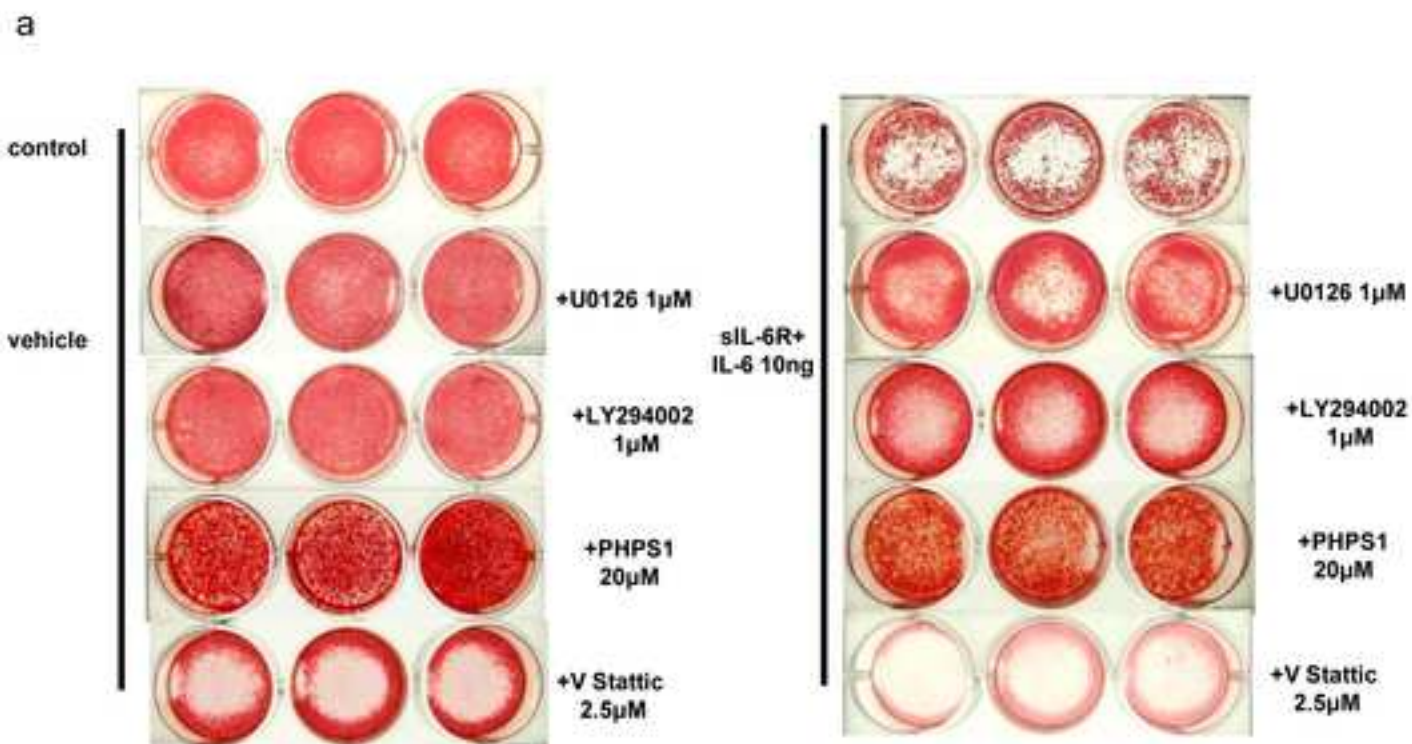


Figure 4





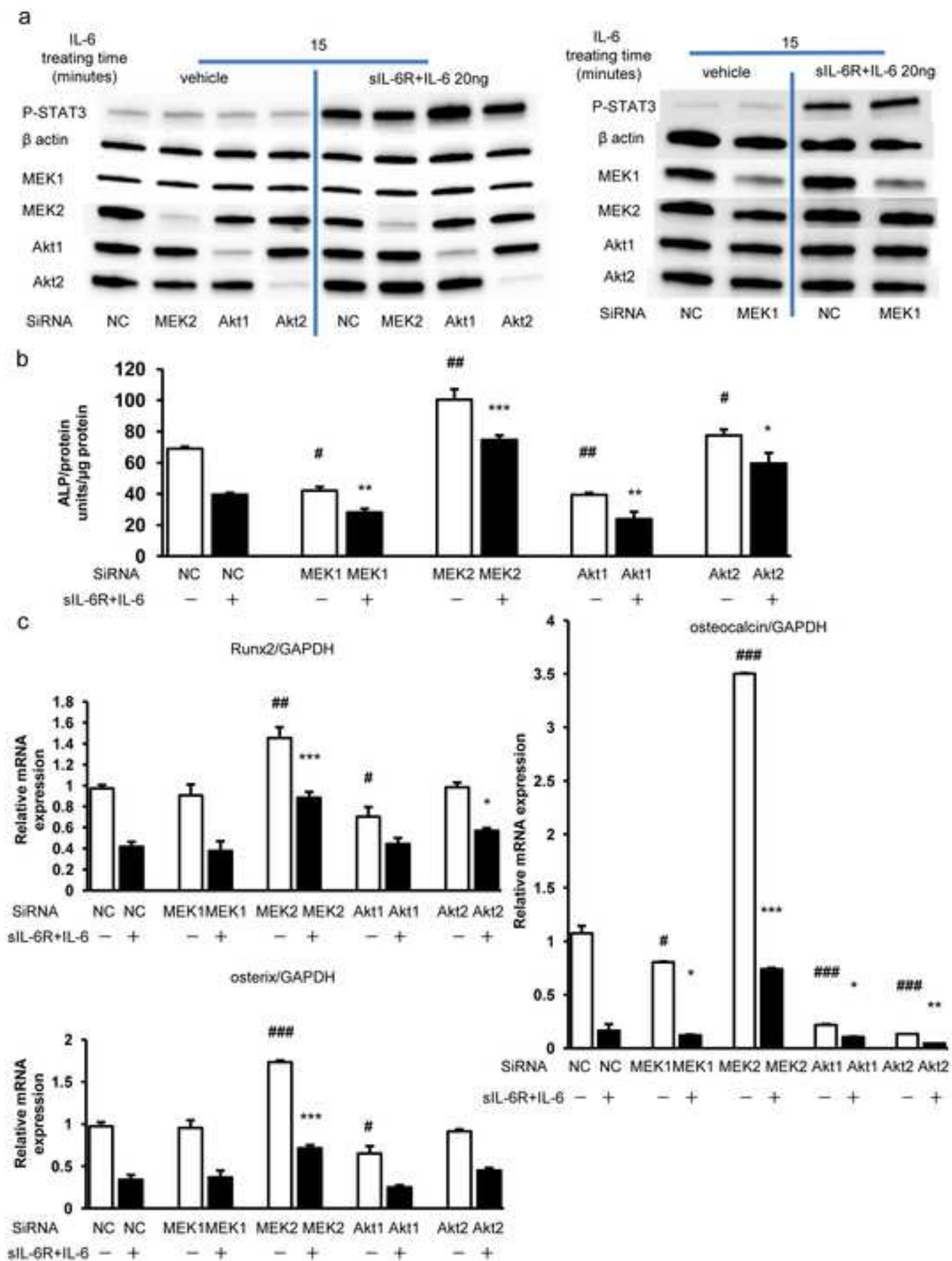


Figure 8

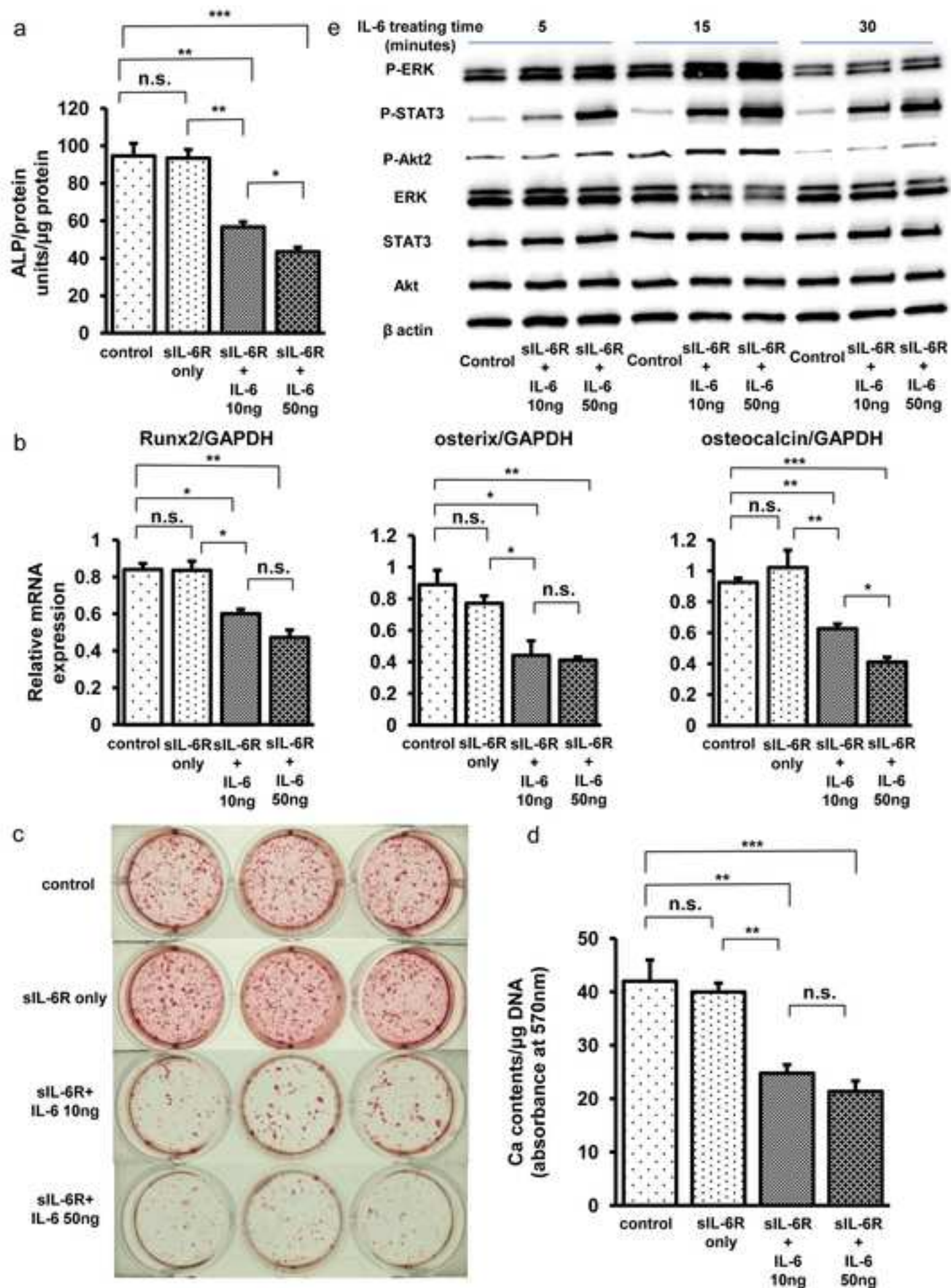


Figure9

