

Fig. 4 IL-6-induced activation of ERK was enhanced by blocking the STAT3 signaling pathway, and IL-6-induced ERK and Akt signaling pathways negatively regulated each other reciprocally. **a** MC3T3-E1 cells were stimulated with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (15 min) after pretreatment either with PHPS1 (5, 20, 40 μM; 1 h), with U0126 (5 μM; 1 h), or with V Stattic (5 μM; 1 h), and the cell lysates were subjected to Western blotting. PHPS1 inhibited IL-6-induced phosphorylation of ERK and Akt to the constitutive level, but

not of STAT3. IL-6-induced activation of ERK was enhanced by V Stattic. **b** MC3T3-E1 cells were treated with vehicle or with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (15 min) after pretreatment either with U0126 (5 μM; 1 h) or with LY294002 (10 μM; 1 h), and the cell lysates were subjected to Western blotting. Both constitutive and IL-6-induced phosphorylation of Akt and ERK were enhanced by treatment with U0126 and LY294002, respectively. Representative data from at least three independent experiments are shown

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

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

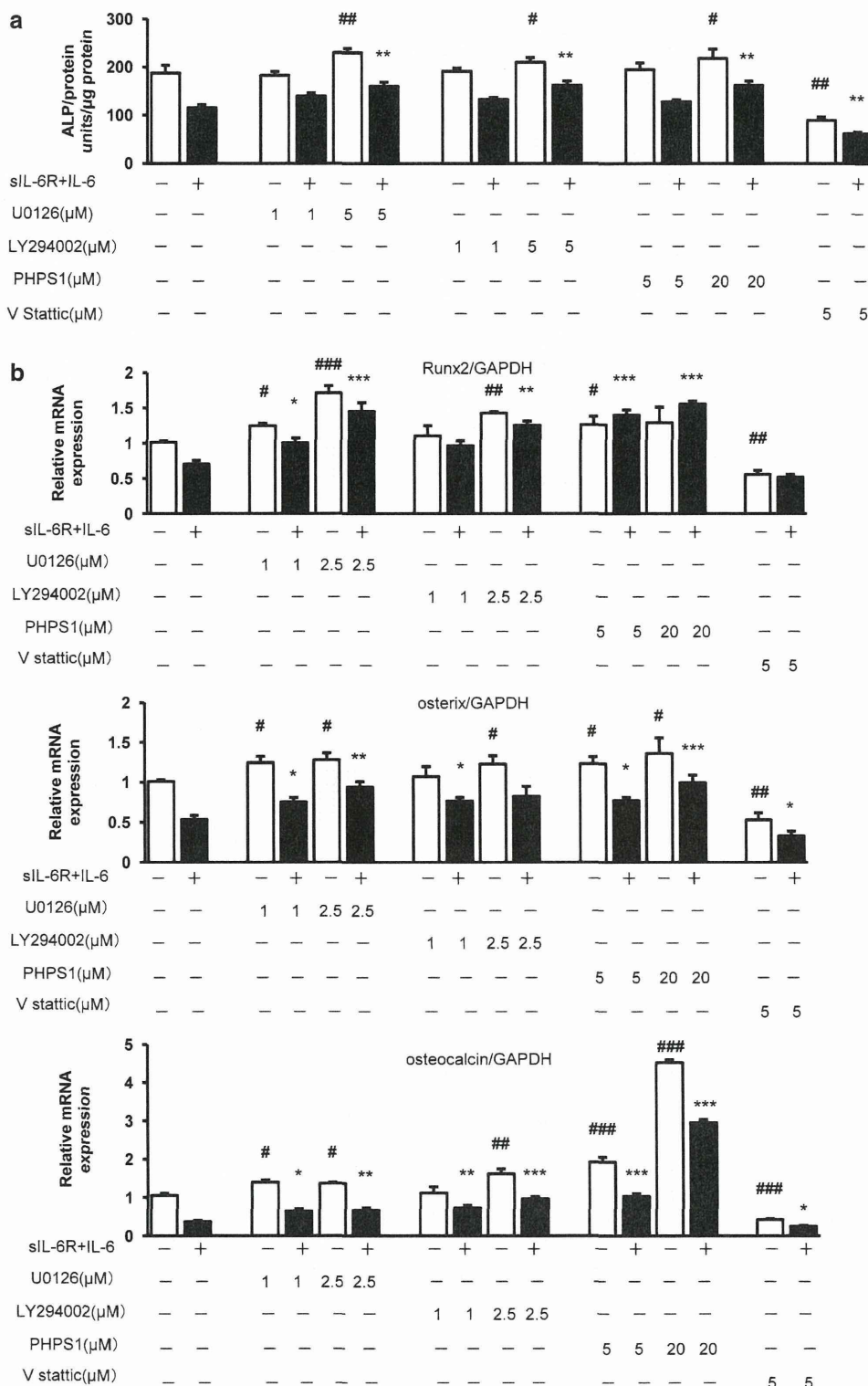
Furthermore, the negative effects of ALP activity, osteoblastic gene expression and mineralization of ECM by stimulation with IL-6/sIL-6R were compared for levels in the presence and in the absence of each inhibitor. The

negative effects on osteoblast differentiation by IL-6/sIL-6R showed a tendency to decrease in the presence of each inhibitor, as compared to the absence of inhibitors (Figs. 5, 6). The negative effects were decreased by 15–44, 20–61, 7–140, and 21–80 % in the presence of U0126, LY294002, PHPS1 and V Stattic, respectively, as compared to the absence of inhibitors. These results indicate that the effects of IL-6/sIL-6R on osteoblast differentiation might be mediated either by MEK/ERK, PI3K/Akt, or JAK/STAT3 pathways.

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

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

Fig. 5 The negative effects of IL-6 on ALP activity and the expression of osteoblastic genes were restored by inhibition of MEK, PI3K, and SHP2, while they were enhanced by inhibition of STAT3. MC3T3-E1 cells were pretreated either with U0126 (1, 2.5, 5 μ M; 1 h), LY294002 (1, 2.5, 5 μ M; 1 h), PHPS1 (5, 20 μ M; 1 h), or V Static (5 μ M; 1 h), then stimulated either with 10 ng/ml IL-6 and 100 ng/ml sIL-6R or with vehicle and incubated for 6 days. **(a)** ALP activity of the cell lysates was measured using p-nitrophenylphosphate as a substrate. The negative effect of IL-6 on ALP activity was restored by treatment with either U0126, LY294002, or PHPS1 in a dose-dependent manner, while it was enhanced by treatment with V Static. **(b)** Total RNA was extracted and real-time PCR for Runx2, osterix, and osteocalcin was performed. Data were normalized to GAPDH expression and are shown as the ratio of gene expression compared to control cells treated with vehicle. The negative effect of IL-6 on expression of osteoblastic genes was restored by treatment either with U0126, LY294002, or PHPS1 in a dose-dependent manner, while it was enhanced by treatment with V Static. Representative data from at least three independent experiments are shown. Data are shown as mean \pm SE. *n.s.* not significant; $\#P < 0.05$; $\#\#P < 0.001$; $\#\#\#P < 0.001$, compared to the group treated with vehicle. $*P < 0.05$; $**P < 0.001$; $***P < 0.001$, compared to group treated with IL-6/sIL-6R



The protein expression level of each molecule was found to be diminished selectively at 48 h after transfection of the respective siRNAs (Fig. 7a). The ALP activity in MC3T3-E1 cells treated with IL-6/sIL-6R was restored by knockdown of MEK2 and Akt2 as compared to that in cells transfected with negative control siRNA.

On the other hand, knockdown of MEK1 and Akt1 enhanced the negative effects of IL-6/sIL-6R on ALP activity (Fig. 7b) (ALP activity after transfection with each siRNA without IL-6/sIL-6R demonstrated the same behavior; Fig. 7b) Quantitative real-time PCR analysis revealed that the gene expressions of Runx2, osterix, and

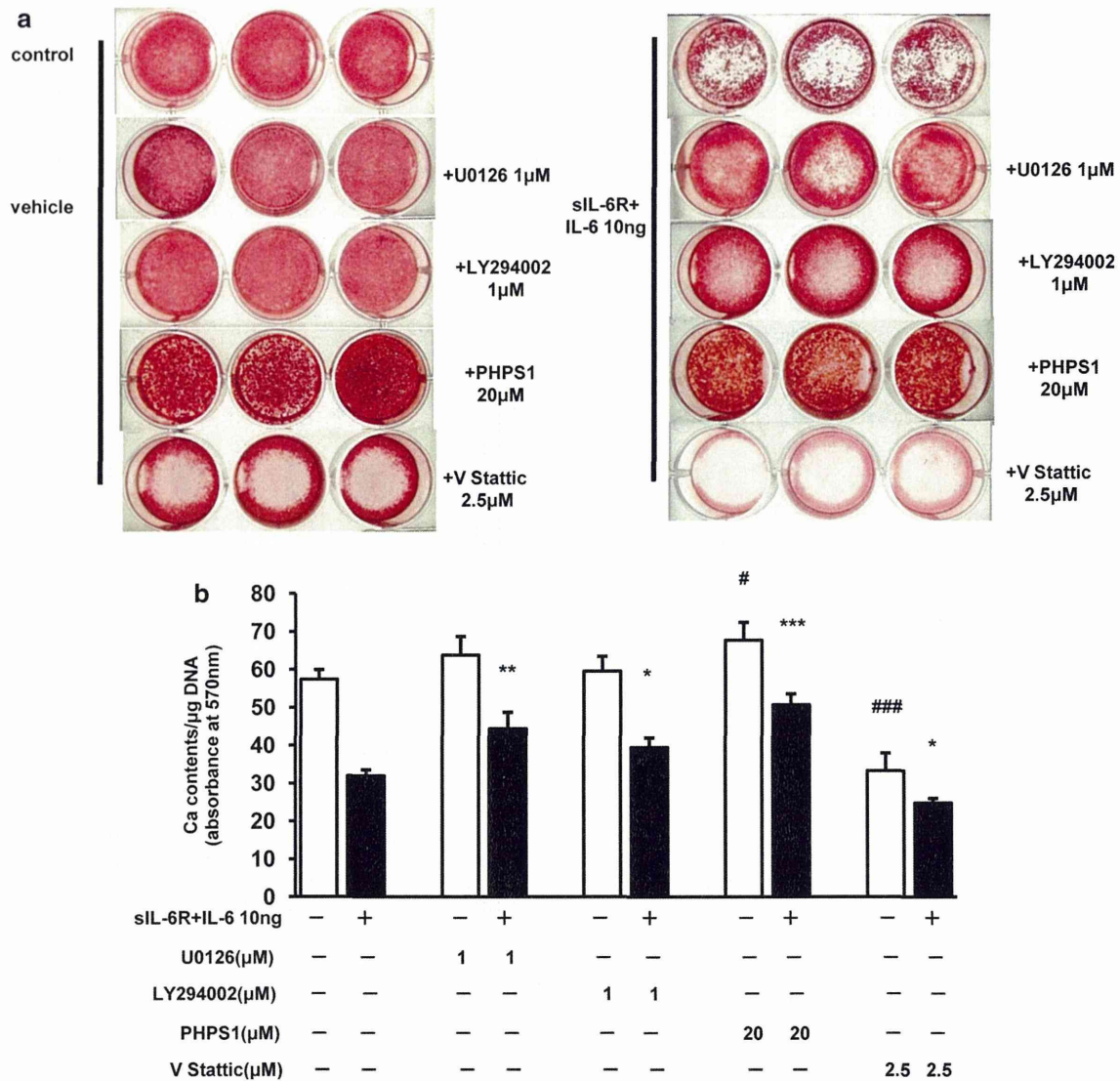


Fig. 6 The negative effect of IL-6 on mineralization of ECM was restored by inhibition of MEK, PI3K, and SHP2, while it was enhanced by inhibition of STAT3. MC3T3-E1 cell were pretreated either with U0126 (1 μM; 1 h), LY294002 (1 μM; 1 h), PHPS1 (20 μM; 1 h), or V Stattic (2.5 μM; 1 h), then stimulated with either 10 ng/ml IL-6 and 100 ng/ml sIL-6R or with vehicle and incubated for 21 days. **a** After fixation, the cells were stained with alizarin red solution. The reduction of alizarin red staining by IL-6/sIL-6R was restored in cells treated with either U0126, LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. **b** Quantification

of matrix mineralization was by measurement of absorbance for alizarin red normalized by total DNA content. The reduction of matrix mineralization by IL-6/sIL-6R was restored in cells treated with either U0126, LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. Representative data from at least three independent experiments are shown. Data are shown as mean ± SE. *n.s.* not significant; #*P* < 0.05; ##*P* < 0.001; ###*P* < 0.001, compared to the group treated with vehicle. **P* < 0.05; ***P* < 0.001; ****P* < 0.001, compared to group treated with IL-6/sIL-6R

osteocalcin were restored by knockdown of MEK2. On the other hand, knockdown of Akt2 also restored Runx2, but decreased osteocalcin expression (Fig. 7c), while knockdown of Akt2 without IL-6/sIL-6R caused no significant difference in Runx2 expression (Fig. 7b). As was recognized for ALP activity, knockdown of MEK1 and Akt1 enhanced the downregulation of osteocalcin expression (Fig. 7b, c). Also, the negative effects of IL-6/

sIL-6R on osteoblast differentiation showed some tendency to decrease with each knockdown compared to those without knockdown. The negative effects were decreased by 2–24, 4–27, 7–43, and 21–26 % with knockdown of MEK1, MEK2, Akt1, and Akt2, respectively, as compared to those without knockdown. These results indicate that IL-6 may suppress osteoblast differentiation through MEK2 and Akt2.

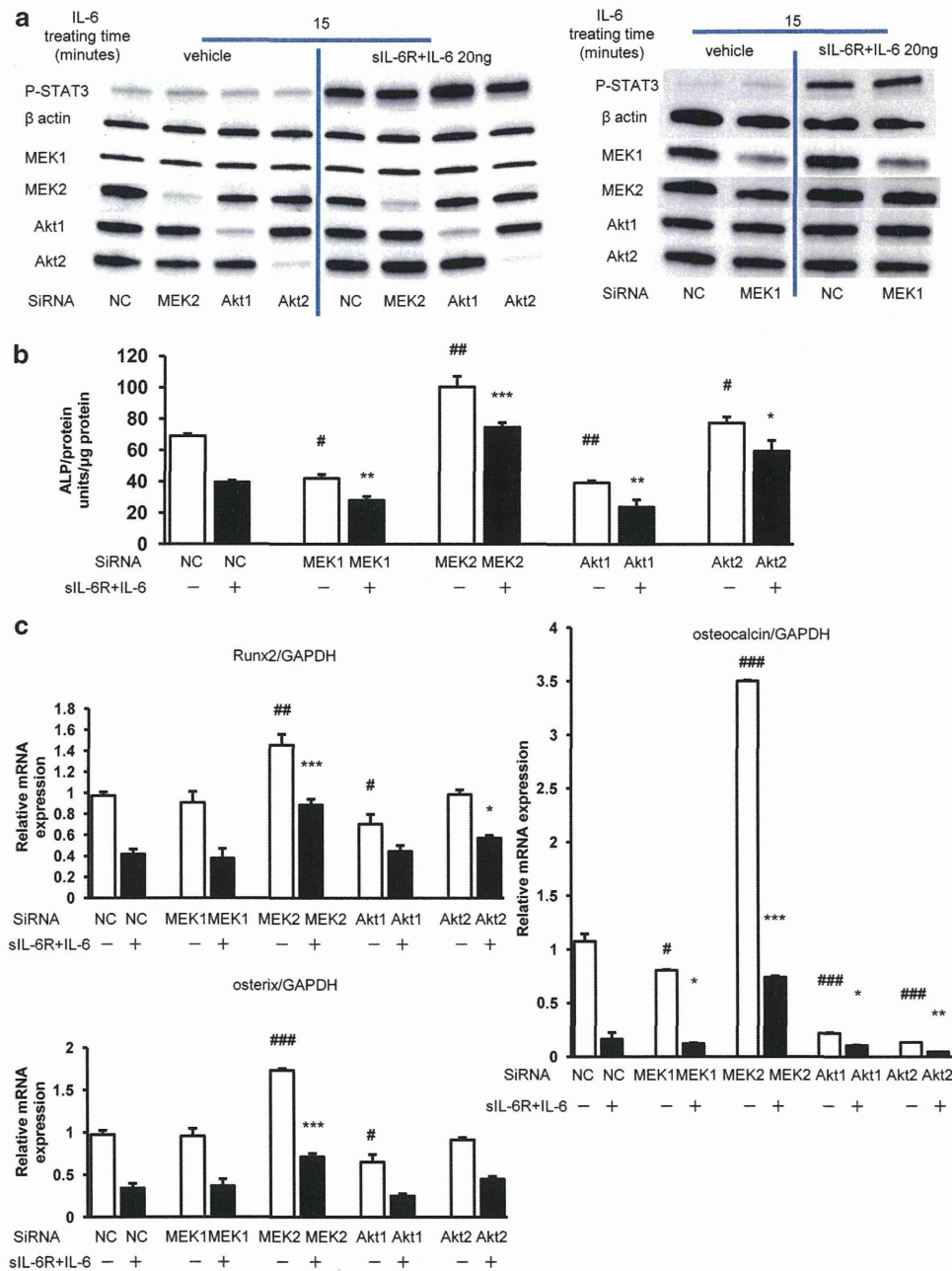


Fig. 7 Knockdown of MEK2 and Akt2 in cells transfected with siRNA restored ALP activity and Runx2 gene expression. **a** MC3T3-E1 cells transfected with respective siRNAs were cultured for 48 h. Western blotting was performed using cell lysates stimulated with vehicle or with 20 ng/ml IL-6 and 100 ng/ml sIL-6R (15 min). Expression levels of each protein, MEK1, MEK2, Akt1, and Akt2, were selectively diminished at 48 h after transfection with respective siRNAs. **b** MC3T3-E1 cells transfected with respective siRNAs were incubated for 48 h after which the medium was changed to differentiation medium with vehicle or with 20 ng/ml IL-6 and 100 ng/ml sIL-6R. The cells were then incubated for 3 days to evaluate osteoblast differentiation. ALP activity in MC3T3-E1 cells treated with IL-6/sIL-6R was restored by knockdown of MEK2 and

Akt2 as compared to that in cells transfected with negative control siRNA. **c** Expression of osteoblastic genes in MC3T3-E1 cells transfected with respective siRNAs was assessed by real-time PCR. The expression of each gene was normalized against GAPDH expression. The gene expressions of Runx2, osterix, and osteocalcin were restored by knockdown of MEK2. Knockdown of Akt2 also restored Runx2, but decreased osteocalcin. Representative data from at least three independent experiments are shown. Data are shown as mean ± SE. *n.s.* not significant; #*P* < 0.05; ##*P* < 0.001; ###*P* < 0.001, compared to negative control group treated with vehicle. **P* < 0.05; ***P* < 0.001; ****P* < 0.001, compared to negative control group treated with IL-6/sIL-6R

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

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

Discussion

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

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

IL-6 activates another major intracellular signaling pathway, SHP2/ERK, and can also lead to the activation of an additional signaling cascade involving SHP2/PI3K/Akt. IL-6-induced activation of PI3K and downstream protein kinase Akt/PKB has been reported to play important roles in the proliferation of prostate cancer cells [30, 31], hepatoma cells [32], and multiple myeloma cells [29]. They were also reported to associate with neuroendocrine differentiation of prostate cancer cells induced by IL-6 [32]. In this study, we focused on the PI3K/Akt pathway triggered by IL-6, because no reports have demonstrated the role of IL-6 in the activation of PI3K/Akt signaling pathway in osteoblasts. We have demonstrated for the first time that IL-6-induced activation of Akt2, one of the downstream pathways of SHP2, may be a key player in the negative regulation of osteoblast differentiation induced by IL-6. Among the three isoforms of Akt, Akt1 and Akt2 are highly expressed in osteoblasts [39]. Mice lacking Akt1, the major isoform in bone tissue, exhibit osteopenia [40, 41], and the impact of Akt1 deficiency in osteoblast differentiation and bone development have also been

published [39, 42–44], all of which are consistent with our results showing that knockdown of Akt1 signaling by siRNA inhibited osteoblast differentiation. In contrast, Mukherjee et al. [44] reported enhanced osteogenic differentiation in the absence of Akt1 in cell lines. Moreover, they reported that Akt2 was required for BMP2-initiated osteoblast differentiation of cultured murine mesenchymal stem cells, but that Akt1 was dispensable in this assay [45], which is inconsistent with our results showing that knockdown of Akt2 signaling by siRNA promoted osteoblast differentiation. These discrepancies might be due to the difference between cell types, i.e. intramembranous (calvariae) cells and endochondral (long bones) cells.

In this study, gene expression of osteocalcin, a late osteoblastic differentiation marker, was upregulated by treatment with a PI3K/Akt inhibitor, but was downregulated by knockdown of both Akt1 and Akt2. Moreover, a complete blockade with a high dose (more than 10 μ M) of the PI3K/Akt inhibitor conversely downregulated the expression of osteocalcin (data not shown). This discrepancy may be due to the difference between the temporary or partial blockade by the inhibitor and constitutive knockdown by siRNA. Since bone formation has been reported to increase without impairment of mineralization and resorption even in osteocalcin-deficient mice [46], the expression of osteocalcin may not directly affect bone formation.

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

With respect to intracellular signaling pathways, our results showed that IL-6 triggers three signaling pathways, one of which has a conflicting function with the others.

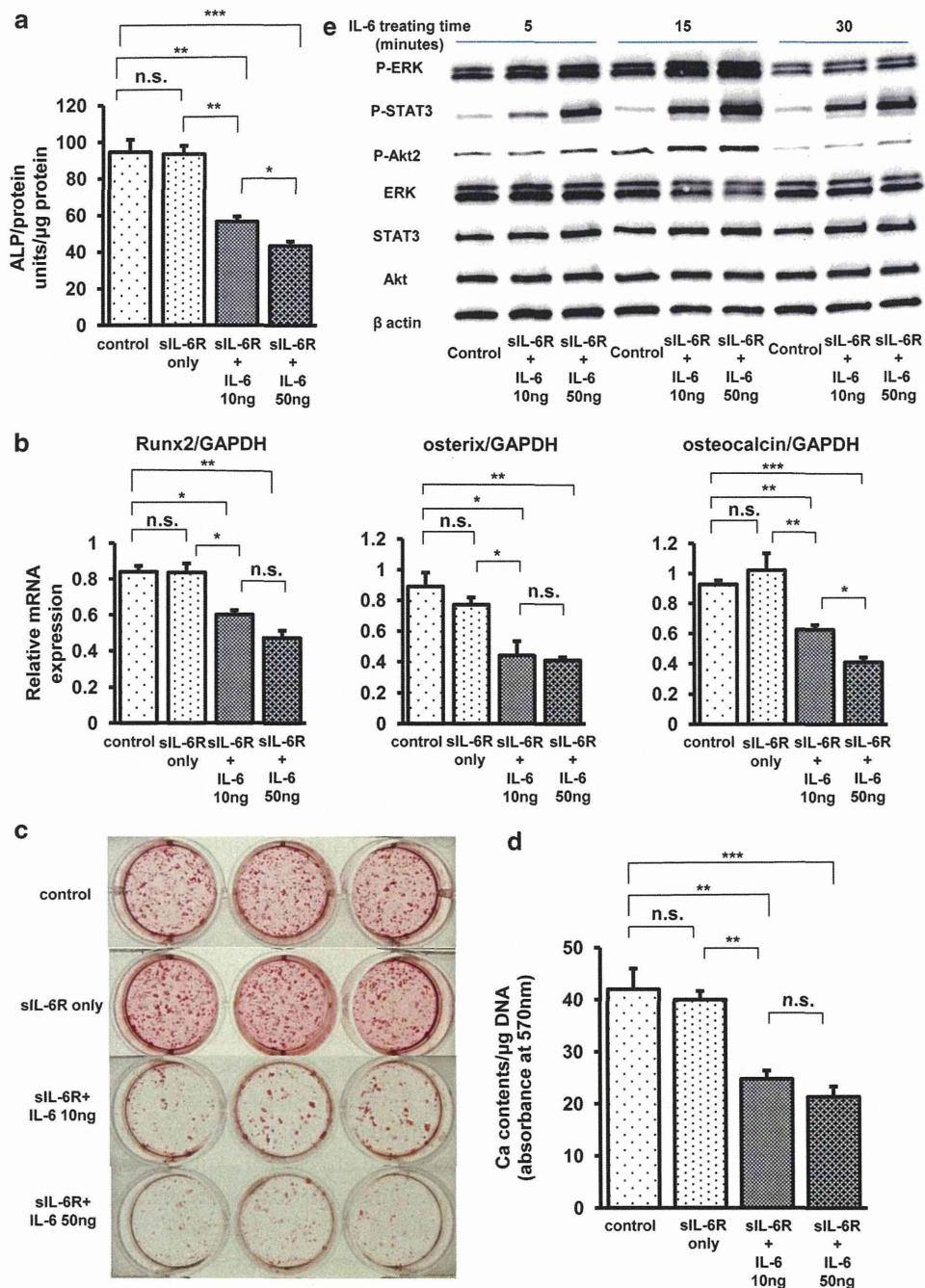
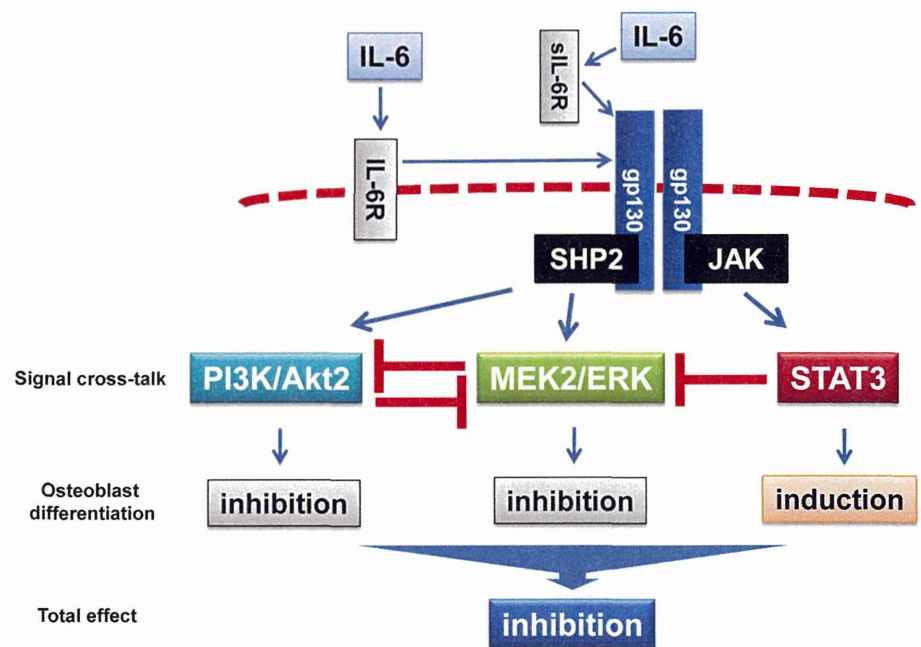


Fig. 8 IL-6/siL-6R inhibited the differentiation of primary murine calvarial osteoblasts with the activated phosphorylation of ERK, Akt2, and STAT3. **a** ALP activity of lysates of murine calvarial osteoblasts treated with or without IL-6/siL-6R for 6 days was measured using p-nitrophenylphosphate as a substrate. IL-6/siL-6R significantly reduced ALP activity in a dose-dependent manner. **b** Total RNA was extracted from murine calvarial osteoblasts treated with or without IL-6/siL-6R for 6 days, and real-time PCR for Runx2, osterix, and osteocalcin was performed. Data were normalized to GAPDH expression and are shown as the ratio of gene expression as compared to control cells treated with vehicle. The expression of osteoblastic genes was significantly downregulated by IL-6/siL-6R in a dose-dependent manner. **c** Murine calvarial osteoblasts were treated with or without IL-6/siL-6R and were cultured for 21 days. After

fixation, the cells were stained with alizarin red solution. Apparently significant reduction of alizarin red staining was recognized in cells treated with either 10 or 50 ng/ml IL-6. **d** Matrix mineralization was quantified by measurement of absorbance for alizarin red normalized by total DNA content. IL-6/siL-6R significantly inhibited mineralization of ECM in a dose-dependent manner. **e** Primary murine calvarial osteoblasts were treated with vehicle or 10 or 50 ng/ml IL-6 and 100 ng/ml siL-6R in a time-course experiment (5, 15, and 30 min). Western blotting was performed using cell lysates. IL-6 significantly induced the phosphorylation of ERK, Akt2, and STAT3 in a dose-dependent manner. Representative data from at least three independent experiments are shown. Data are shown as mean ± SE. n.s. not significant; **P* < 0.05; ***P* < 0.001; ****P* < 0.001

Fig. 9 Schematic presentation of signaling pathways involved in osteoblast differentiation induced by IL-6. IL-6-induced novel SHP2/MEK2/ERK and SHP2/PI3K/Akt2 signal crosstalk in osteoblastic cells; ERK and Akt signaling pathways, both of which are downstream of SHP2, negatively regulate each other reciprocally. On the other hand, the STAT3 signaling pathway negatively regulates the ERK signaling pathway. MEK2/ERK and PI3K/Akt2 have negative effects on osteoblast differentiation, whereas STAT3 has a positive effect. Overall, IL-6 inhibits osteoblast differentiation through MEK2 and Akt2 signaling pathways



SHP2/ERK and SHP2/Akt2 negatively affects osteoblast differentiation, whereas JAK/STAT3 positively affects it (Fig. 9). In other cells, it is often that simultaneous activation of the SHP2/ERK and JAK/STAT3 cascades generate opposing, or at least different signals. In osteoclasts, for example, SHP2/ERK activation inhibits osteoclastogenesis [53], whereas STAT3 is a pro-osteoclastic molecule after phosphorylation on serine727 [54]. In myeloid leukemic M1 cells, STAT3 induces differentiation *in vitro* [55], whereas the SHP2/ERK pathway promotes their proliferation [56]. These examples suggest that the integration of opposing activities transduced by more than one pathway could provide a biologically balanced state in the end, leaving availability to respond to another physiological situation. Indeed, Hirano and colleagues [57] have proposed a “signaling orchestration” model in a single cell, where the balance or interplay of simultaneously generated contradictory signals eventually determines the biological outcome. Thus, the inconsistent results regarding the effects of IL-6 on osteoblast differentiation in previous reports could be explained by which intracellular signaling pathway was predominantly activated in each study. The balance of three signaling pathways could be influenced by such conditions as the variety of cultured cells, the stage of cell differentiation, and the employed culture conditions.

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

STAT3 are all critical pathways in osteoblast differentiation even in the absence of IL-6/sIL-6R, and even though one pathway is blocked, another pathway is enhanced by reciprocal regulation in the crosstalk between IL-6-activated signaling pathways (Fig. 9). Our results demonstrated that a STAT3 inhibitor significantly enhanced IL-6-induced activation of ERK and SHP2, but not of Akt (Fig. 4a). SHP2 could predominantly lead to the activation of the ERK signaling pathway as compared to Akt, and the enhanced signaling of ERK may restrain the enhancement of the Akt signaling pathway in a negative feedback manner.

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

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

Conflict of interest All authors have no conflicts of interest.

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RESEARCH ARTICLE

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Venous thromboembolism after total joint arthroplasty: results from a Japanese multicenter cohort study

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Abstract

Introduction: Real-world evidence of the effectiveness of pharmacological thromboprophylaxis for venous thromboembolism (VTE) is limited. Our objective was to assess the effectiveness and safety of thromboprophylactic regimens in Japanese patients undergoing joint replacement in a real-world setting.

Method: Overall, 1,294 patients (1,073 females and 221 males) who underwent total knee arthroplasty (TKA) and 868 patients (740 females and 128 males) who underwent total hip arthroplasty (THA) in 34 Japanese national hospital organization (NHO) hospitals were enrolled. The primary efficacy outcome was the incidence of deep vein thrombosis (DVT) detected by mandatory bilateral ultrasonography up to post-operative day (POD) 10 and pulmonary embolism (PE) up to POD28. The main safety outcomes were bleeding (major or minor) and death from any cause up to POD28.

Results: Patients undergoing TKA (n = 1,294) received fondaparinux (n = 360), enoxaparin (n = 223), unfractionated heparin (n = 72), anti-platelet agents (n = 45), or no medication (n = 594). Patients undergoing THA (n = 868) received fondaparinux (n = 261), enoxaparin (n = 148), unfractionated heparin (n = 32), anti-platelet agents (n = 44), or no medication (n = 383). The incidence rates of sonographically diagnosed DVTs up to POD10 were 24.3% in patients undergoing TKA and 12.6% in patients undergoing THA, and the incidence rates of major bleeding up to POD28 were 1.2% and 2.3%, respectively. Neither fatal bleeding nor fatal pulmonary embolism occurred. Significant risk factors for postoperative VTE identified by multivariate analysis included gender (female) in both TKA and THA groups and use of a foot pump in the TKA group. Only prophylaxis with fondaparinux reduced the occurrence of VTE significantly in both groups. Propensity score matching analysis (fondaparinux versus enoxaparin) showed that the incidence of DVT was lower (relative risk 0.70, 95% confidence interval (CI) 0.58 to 0.85, $P = 0.002$ in TKA and relative risk 0.73, 95% CI 0.53 to 0.99, $P = 0.134$ in THA) but that the incidence of major bleeding was higher in the fondaparinux than in the enoxaparin group (3.4% versus 0.5%, $P = 0.062$ in TKA and 4.9% versus 0%, $P = 0.022$ in THA).

Conclusions: These findings indicate that prophylaxis with fondaparinux, not enoxaparin, reduces the risk of DVT but increases bleeding tendency in patients undergoing TKA and THA.

Trial registration: University Hospital Medical Information Network Clinical Trials Registry: UMIN000001366. Registered 11 September 2008.

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