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Oncostatin M Promotes Osteoblastic Differentiation of Human Vascular Smooth Muscle Cells Through JAK3-STAT3 Pathway

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ABSTRACT

Vascular calcification is a clinically significant component of atherosclerosis and arises from chronic vascular inflammation. Oncostatin M (OSM) derived from plaque macrophages may contribute to the development of atherosclerotic calcification. Here, we investigated the stimulatory effects of OSM on osteoblastic differentiation of human vascular smooth muscle cells (HVSMC) derived from various arteries including umbilical artery, aorta, and coronary artery and its signaling pathway. Osteoblastic differentiation was induced by exposure of HVSMC to osteogenic differentiation medium (ODM) (10% fetal bovine serum, 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid 2-phosphate in Dulbecco's modified Eagle's medium [DMEM]). OSM significantly increased alkaline phosphate (ALP) activity and matrix mineralization in HVSMC from all sources. Osteoblast marker genes such as ALP and Runx2 were also up-regulated by OSM in these cells. OSM treatment induced activation of STAT3 in HVSMC from umbilical artery as evidenced by immunoblot. Moreover, not only a JAK3 inhibitor, WHI-P154, but also knockdown of JAK3 by siRNA prevented the OSM-induced ALP activity and matrix mineralization in umbilical artery HVSMC. On the other hand, silencing of STAT3 almost completely suppressed OSM-induced ALP expression and matrix mineralization in HVSMC from all sources. These data suggest that OSM promotes osteoblastic differentiation of vascular smooth muscle cells through JAK3/STAT3 pathway and may contribute to the development of atherosclerotic calcification. J. Cell. Biochem. 116: 1325–1333, 2015.

KEY WORDS: ONCOSTATIN M; OSTEOBLASTIC DIFFERENTIATION; VASCULAR SMOOTH MUSCLE CELLS; JAK3; STAT3

Vascular calcification is a common feature of atherosclerosis, especially in advanced plaque lesions and is an independent risk factor for cardiovascular disease. Coronary artery calcification (CAC) occurs almost exclusively in atherosclerotic arteries and is absent in the normal vessel wall [Greenland et al., 2007]. CAC score measured with cardiac computed tomography (CT) has been shown to predict future coronary event [LaMonte et al., 2005; Vliegenthart et al., 2005]. In addition, microcalcification in the thin fibrous cap overlying the necrotic core of atherosclerotic plaques may cause microfractures that lead to plaque rupture and acute coronary events [Vengrenyuk et al., 2006].

Vascular inflammation and calcification are closely related phenomena occurring within the vessel wall during progression of atherosclerosis. Non-invasive imaging studies of human atherosclerosis using positron emission tomography (PET)/CT revealed that atherosclerotic plaque inflammation ([^{18}F]fluorodeoxyglucose (FDG) uptake) precedes active calcification as evidenced by the uptake of [^{18}F]sodium fluoride (NaF) [Derlin et al., 2011; [Cocker et al., 2012].

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Similar evidence suggestive of inflammation-dependent atherosclerotic calcification has been provided by the serial imaging studies in apolipoprotein (apo) E-deficient mice [Aikawa et al., 2007]. Histopathologically, active inflammation within plaque mainly represents macrophage infiltration in the above-mentioned imaging studies [New and Aikawa, 2011]. Potential roles of macrophages in plaque calcification have been suggested by in vitro studies [Watson et al., 1994; Tintut et al., 2000; Parhami et al., 2002; Shioi et al., 2002]. Macrophage-derived cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) induce osteogenic differentiation and mineralization of vascular smooth muscle cells (VSMC). In addition, by using a coculture model, we have identified oncostatin M (OSM) as an essential component derived from macrophages of inflammatory mediators to induce calcifying phenotype in human vascular smooth muscle cells (HVSMC) [Shioi et al., 2002]. Although OSM facilitates induction of HVSMC calcification only in combination with other mediators including TNF-α, interferon-γ and 1α, 25-dihydroxyvitamin D_3, the precise roles of OSM alone in osteogenic differentiation of VSMC remain incompletely understood.

OSM is an inflammatory cytokine that belongs to the IL-6 class of cytokines. It is mainly produced by macrophages, neutrophils, and T lymphocytes and exerts many unique biological activities in inflammation, remodeling of extracellular matrix and modulation of cell growth and differentiation [Tanaka and Miyajima, 2003]. OSM also plays a crucial role in bone remodeling, especially osteoclastogenesis and bone formation. OSM stimulates osteoclast formation by enhancing expression of receptor activator of nuclear factor-κB ligand (RANKL) in osteoblast-lineage cells [Palmqvist et al., 2002; Walker et al., 2010]. Osteoblastic differentiation of adipose tissue-derived mesenchymal stem cells is induced by OSM [Song et al., 2007]. Therefore, macrophage-derived OSM per se may facilitate osteogenic differentiation of VSMC and contribute to atherosclerotic calcification.

In this study, we investigated the stimulatory effects of OSM on osteoblastic differentiation of HVSMC derived from various sites of arterial vessels including umbilical artery, aorta, and coronary artery. OSM significantly stimulated expression of osteoblastic differentiation markers such as alkaline phosphate (ALP) and Runx2 and mineralization in HVSMC. The cytokine exerted its effects through the JAK3/STAT3 signaling pathway as evidenced by the experiments using a specific inhibitor reagent and siRNAs. These data suggest that OSM accelerates osteoblastic differentiation of HVSMC through JAK3/STAT3 pathway.

MATERIALS AND METHODS

CELL CULTURE

Primary HVSMC derived from neonatal umbilical arteries were obtained from Cell Systems (Kirkland, WA), maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. Normal human aortic smooth muscle cells (HAoSMC) and normal human coronary smooth muscle cells (HCASMC) were purchased from Lonza (Walkersville, MD), and cultured in SMC growth medium (Smooth Muscle Cell Basal Medium [SmBM™] supplemented with growth factors [SingleQuots™] containing FBS, insulin, hFGF-B, 50 μg/ml of gentamicin and 50 μg/ml of amphotericin, and hEGF).

INDUCTION OF HVSMC CALCIFICATION BY OSM

The cells were seeded at 2×10^5 cells/well in 6-well plates. After confluency, HVSMC were cultured for the indicated period of time with osteogenic differentiation medium (ODM) containing 10% FBS, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate in DMEM. Calcium deposits of cell layer were assessed by methylxylenol blue (MXB) method (Calcium E-test Wako; Wako, Osaka, Japan) and normalized by protein content as previously described [Shioi et al., 2002].

ALKALINE PHOSPHATASE (ALP) ASSAY

After the cells were washed twice with PBS, the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were assayed for ALP activity as described previously [Shioi et al., 2002]. One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30 min. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL).

WESTERN BLOT ANALYSIS

HVSMC lysates were prepared and analyzed as previously described [Shioi et al., 2002]. Anti-phospho-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-STAT3 (Santa Cruz Biotechnology), anti-JAK3 (Santa Cruz Biotechnology), anti-α-smooth muscle actin (Dako, Glostrup, Denmark) antibodies were used as primary antibodies at dilutions with 1:1,000, 1:1,000, 1:200, and 1:500, respectively. The signal was detected by enhanced chemiluminescence detection Select Western Blotting System (GE Healthcare, Buckinghamshire, UK).

REAL-TIME QUANTITATIVE RT-PCR

Total RNA was extracted from HVSMC with Trizol (Ambion, Carlsbad, CA) reagent according to the manufacturer's instruction. cDNAs were reverse transcribed from 1 μg of isolated total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) to determine the relative abundance of assayed mRNAs. Samples were normalized by determining the relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The 5′ terminus of fluorogenic probes was labeled with FAM (6-carboxy-fluorescein) or VIC and the 3′ terminus contained the quenching dye TAMRA (6-carboxytetramethyl-rhodamine). Taqman primer-probe sets (Taqman Assay-on-Demand Gene Expression Products) for human ALP (Hs00758162_m1), human Runx2 (Hs00231692_m1), and human GAPDH (GAPDH control reagents #402869) were obtained from Applied Biosystems. PCR reactions contained Universal Master Mix (Applied Biosystems), specific primers, Taqman probe, and cDNA. Two-step PCR cycling was carried out as follows: 50°C 2 min for 1 cycle, 95°C 10 min for 1 cycle, and 95°C 15 s, 60°C 1 min for 40 cycles. The relative levels of mRNA of a specific gene were calculated using standard curve generated from cDNA of HVSMC.

ASSESSMENT OF CELL DEATH

Cell death of HUASMC during mineralization was evaluated morphologically by fluorescence microscopy after labeling with propidium iodide (Invitrogen) and Hoechst33342 (Invitrogen). HUASMC were cultured for 14 days in the presence of ODM and OSM, washed twice with PBS and stained with 1 μg/ml propidium iodide and 1 μg/ml Hoechst33342 for 30 min at room temperature without fixation. Dual-stained cells were examined using an inverted fluorescence microscope (IX70 Olympus, Tokyo, Japan).

SMALL INTERFERING RNA AND NUCLEOFECTIONS

JAK3 and STAT3 siRNAs were obtained from Darmacon (Lafayette, CO). Nucleofection was performed on HVSMC using the Amaxa nucleofector device and the Human AoSMC Nucleofector Kit (Lonza, Cologne, Germany). For the optimized reaction, 5×10^5 cells were resuspended in 100 μl nucleofector solution with 300 nM either STAT3 siRNA or JAK3 siRNA. Program U-25 was used for the electrical settings. The cells were used for the experiments 24 h after nucleofection.

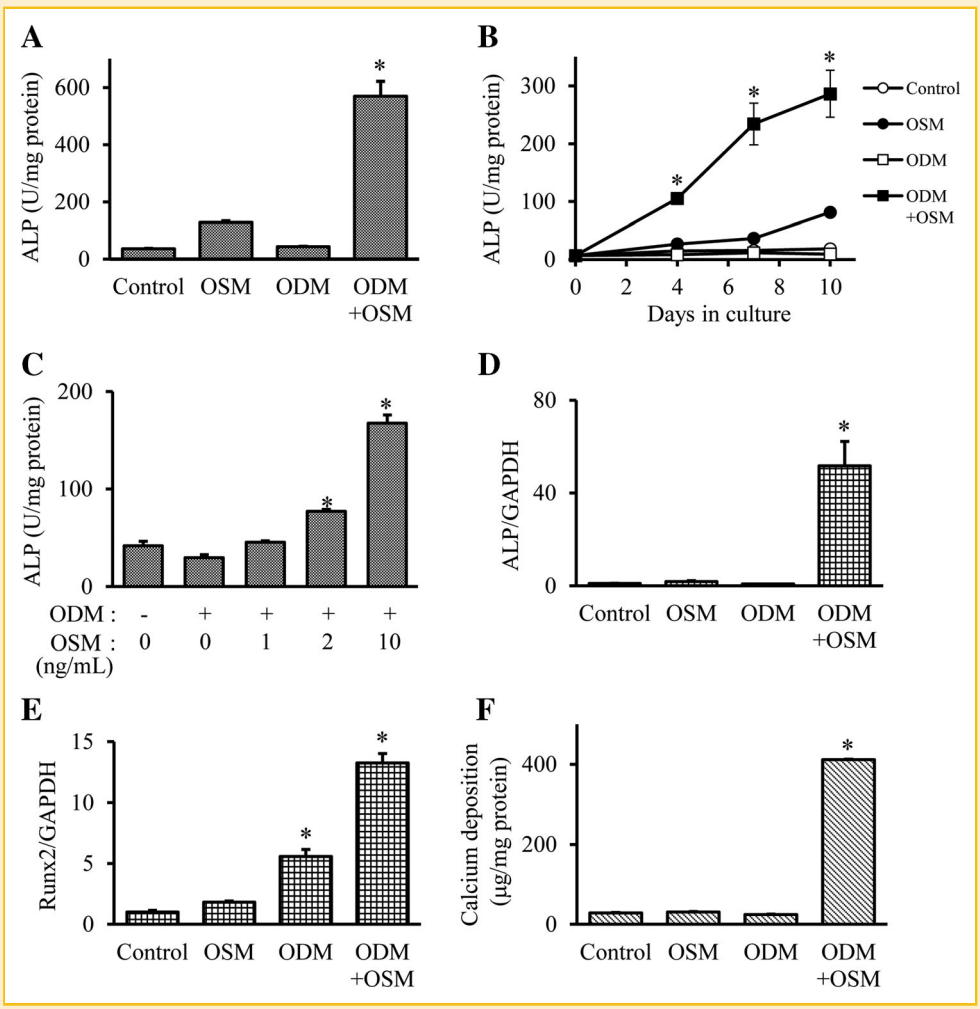

Fig. 1. Oncostatin M (OSM) induces osteoblastic differentiation of human umbilical artery smooth muscle cells (HUSMC). A: Effects of OSM on ALP activity in HUASMC. HUASMC were cultured for 4 days in the presence or absence of osteoblast differentiation medium (ODM) with or without OSM (10 ng/ml). ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3 for each group). $^*P < 0.05$ versus the control cultures. B: Time course effects of OSM on ALP activity in HUASMC. HUASMC were cultured for 10 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured at the indicated time points, normalized by cellular protein content, and are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus each control culture. C: The dose-dependent effect of OSM on ALP activity in HUASMC. HUASMC were cultured for 4 days under the indicated conditions. ALP activities were measured, normalized, and are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures without both ODM and OSM. D, E: Real-time quantitative RT-PCR analysis of mRNA expression of ALP (D) and Runx2 (E) in HUASMC. HUASMC were cultured for 4 days under the same conditions as those of (A) and mRNA expression of these genes was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the control cultures. F: Effect of OSM on in vitro calcification of HUASMC. HUASMC were incubated for 14 days under the same conditions as those of (A). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the control cultures.

STATISTICS

Data were presented as mean ± SEM and analyzed for statistical significance by ANOVA with post hoc analysis (Scheffe′s test). These analyses were performed with the statistics add-in software Statcel 2 (OMS, Tokorozawa, Japan) for Microsoft Excel. *P*-values less than 0.05 were considered statistically significant.

RESULTS

OSM PROMOTES OSTEOBLASTIC DIFFERENTIATION OF HUMAN UMBILICAL ARTERY SMOOTH MUSCLE CELLS

In the previous study, we utilized human umbilical artery smooth muscle cells (HUASMC) to examine the effects of osteogenic activity of macrophages treated with interferon-γ and 1,25-dihydroxyvitamin D_3 [Shioi et al., 2002]. Therefore, we first examined the effects of OSM on osteoblastic differentiation of HUASMC. OSM significantly increased ALP activities of HUASMC only in the presence of ODM (Fig. 1A) and its stimulatory effects on ALP activities were time- and dose-dependent (Fig. 1B,C, respectively). Moreover, mRNA expression of ALP and Runx2 was significantly induced by OSM (Fig. 1D,E, respectively). Mineralization of HUASMC was increased by OSM treatment only in the presence of ODM (Fig. 1F). The possibility that the OSM-induced mineralization of HUASMC may be mediated through cell death was evaluated not only by a double staining method with propidium iodide and Hoechest33342, but also examining the effect of a pan-caspase inhibitor, zVAD-FMK on OSM-induced in vitro mineralization. Dead cells were not increased in the culture of HUASMC treated with OSM and ODM compared with the control cultures without any stimulation (data not shown). OSM-induced in vitro mineralization was not affected by the treatment with zVAD-FMK at 10 μM (Supplementary Fig. S1). These data suggest that OSM promote osteoblastic differentiation but not dystrophic mineralization of HUASMC.

OSM INDUCES OSTEOBLASTIC DIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS DERIVED FROM AORTA AND CORONARY ARTERY

We next examined whether OSM may exert its osteogenic effects on VSMC derived from adult tissues by utilizing human aortic and coronary artery smooth muscle cells (HAoSMC and HCASMC, respectively). OSM significantly increased ALP activities of HAoSMC only in the presence of ODM (Fig. 2A) and this effect was also dose-dependent in HAoSMC treated with ODM (Supplementary Fig. S2). Mineralization of HAoSMC was induced by 14-day treatment of OSM only in the presence of ODM (Fig. 2B). Osteoblast-specific marker genes including ALP and Runx2 were up-regulated by OSM in a dose-dependent manner (Fig. 2C,D, respectively). Furthermore, HCASMC

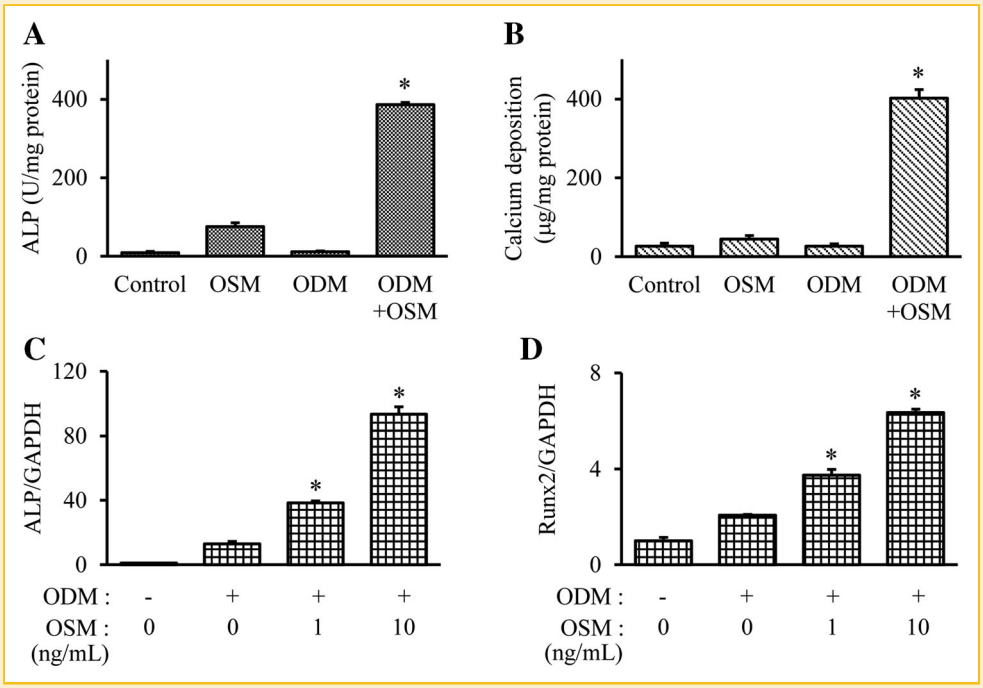

Fig. 2. OSM enhances osteoblastic differentiation of human vascular smooth muscle cells derived from aorta (HAoSMC). A: Effects of OSM on ALP activity in HAoSMC. HAoSMC were cultured for 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the control cultures. B: Effect of OSM (10 ng/ml) on in vitro calcification of HAoSMC. HAoSMC were treated for 14 days under the same conditions as those of (A). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the control cultures. C, D: Real-time quantitative RT-PCR analysis of mRNA expression of ALP (C) and Runx2 (D) in HAoSMC. HAoSMC were cultured for 4 days under the indicated conditions and mRNA expression of these genes was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean ± SEM (n = 3). $^*P < 0.05$ versus the cultures without both of OSM and ODM.

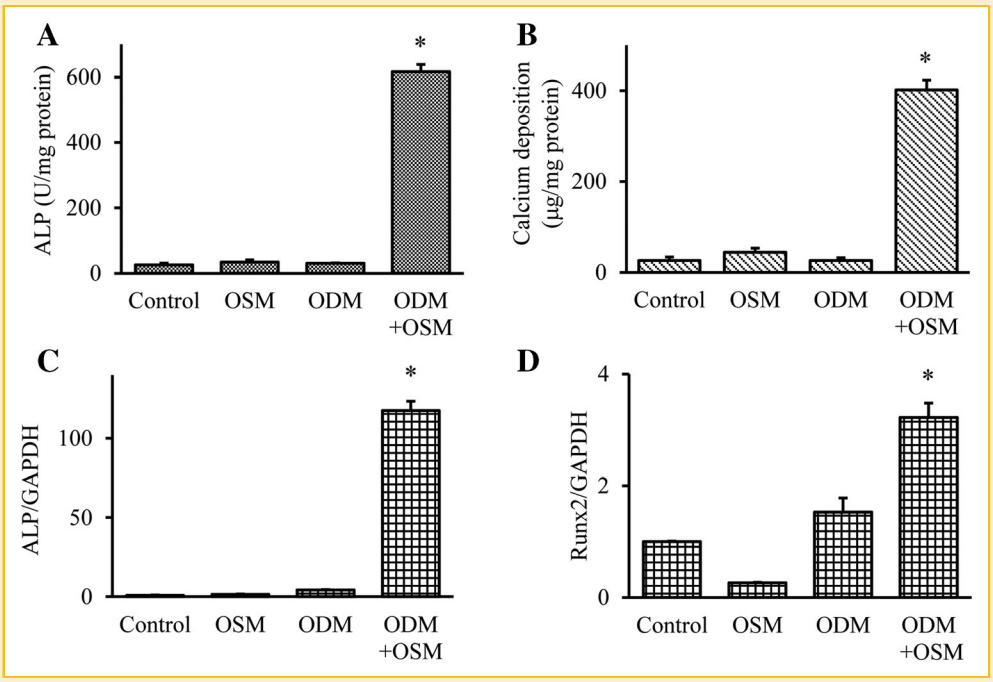

Fig. 3. OSM enhances osteoblastic differentiation of human vascular smooth muscle cells derived from coronary artery (HCASMC). A: Effects of OSM on ALP activity in HCASMC. HCASMC were cultured for 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control cultures. B: Effect of OSM on in vitro calcification of HCASMC. HCASMC were treated for 14 days under the same conditions as those of (A). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control cultures. C, D: Real-time quantitative RT-PCR analysis of mRNA expression of ALP (C) and Runx2 (D) in HCASMC. HCASMC were cultured for 4 days under the same conditions as those of (A) and mRNA expression of these genes was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control cultures.

also exhibited similar responses to OSM. ALP activities and mineralization of HCASMC were significantly increased only in the presence of ODM (Fig. 3A,B, respectively). Gene expression of ALP and Runx2 was also stimulated by the treatment with OSM and ODM (Fig. 3C,D, respectively). These data suggest that OSM facilitates osteoblastic differentiation of HVSMC derived from adult tissues such as aorta and coronary artery.

JAK3/STAT3 SIGNALING PATHWAY MEDIATES THE EFFECTS OF OSM ON OSTEOBLASTIC DIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS

The previous studies have demonstrated that the effect of OSM on osteogenic differentiation of mesenchymal stem cells is mediated through activation of JAK3/STAT3 signaling pathway [Song et al., 2007]. Therefore, we further investigated the roles of JAK3/STAT3 signaling pathway in promotion of osteoblastic differentiation of HUASMC by OSM. OSM induced the activation of STAT3 within 15 min in HUASMC and this activation was dose-dependently inhibited by the treatment with WHI-P154, a specific inhibitor of JAK3 (Fig. 4A). WHI-P154 also inhibited OSM-induced ALP activities in a dose-dependent manner (Fig. 4B). Mineralization of HUASMC by OSM was almost completely inhibited by 10 μM WHI-P154 (Fig. 4C). Moreover, inhibition of JAK3 by WHI-P154 significantly suppressed the induction of ALP mRNA in HUASMC by OSM, while OSM-enhanced expression of Runx2 was not affected by the JAK3 inhibitor (Fig. 4D,E, respectively). Knockdown of JAK3 using siRNA (Fig. 5A) significantly inhibited OSM-induced ALP activities and mineralization of HUASMC (Fig. 5B,C, respectively). OSM-induced gene expression of ALP and Runx2 was not inhibited by knockdown of JAK3 (Fig. 5D,E, respectively). On the other hand, knockdown of STAT3 using siRNA (Fig. 5A) almost completely suppressed ALP activities, its mRNA expression, and mineralization of HUASMC (Fig. 5B–D, respectively). However, Runx2 expression was not inhibited by knockdown of STAT3 (Fig. 5E). Furthermore, pharmacological inhibition of JAK3 or knockdown of JAK3 also exhibited significant inhibition of ALP activities, but not mineralization of HAoSMC and HCASMC (Supplementary Fig. S3A–D), while STAT3 knockdown almost completely blocked OSM-induced osteogenic differentiation in these cells (Fig. 6A–D). These data suggest that JAK3-STAT3 signaling pathway plays an important role in OSM-induced osteoblastic differentiation of VSMC.

DISCUSSION

Recently, the stimulatory effect of monocyte/macrophage-derived OSM on osteoblast commitment has been shown in bone marrow-derived mesenchymal stem cells [Guihard et al., 2012; Nicolaidou et al.,

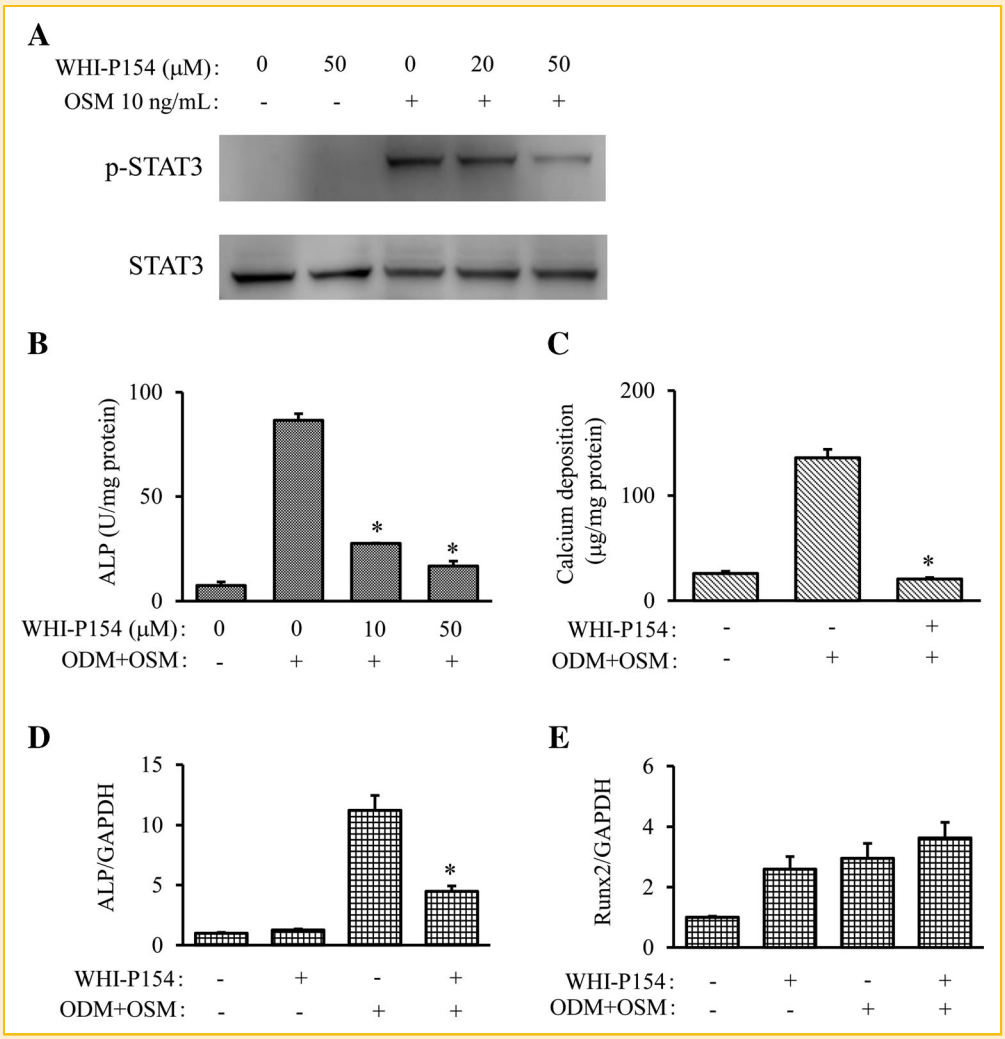

Fig. 4. A specific JAK3 inhibitor (WHI-P154) suppresses OSM-induced osteoblastic differentiation of HUSMCs. A: HUASMC were cultured with or without WHI-P154 at the indicated concentrations for 5 h and then treated for 15 min with OSM (10 ng/ml). Cell lysates were analyzed with Western blot using antibodies recognizing phospho-STAT3 (p-STAT3) and total STAT3 as described. B: Effects of WHI-P154 on OSM-induced ALP activities in HUASMC. HUASMC were cultured for 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the cultures in the presence of ODM + OSM without WHI-P154. C: Effects of WHI-P154 (10 μM) on OSM-induced in vitro calcification in HUASMC. HUASMC were cultured for 14 days under the indicated conditions. OSM was utilized at 10 ng/ml. Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the cultures in the presence of ODM + OSM without WHI-P154. D, E: Effects of WHI-P154 (20 μM) on real time quantitative RT-PCR analysis of mRNA expression of ALP (D) and Runx2 (E) in HUASMC. HUASMC were cultured for 4 days under the indicated conditions and mRNA expression of these genes was quantified. OSM was utilized at 10 ng/ml. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the cultures in the presence of ODM + OSM without WHI-P154.

2012]. Although we have previously identified OSM as an essential component of inflammatory mediators derived from macrophages to induce calcifying phenotype in HVSMC, the osteoblastogenic action of OSM per se on HVSMC remained to be determined [Shioi et al., 2002]. In the present study, we clearly demonstrated that OSM promotes osteoblastic differentiation of HVSMC derived from various sites of arterial vessels, suggesting that OSM plays an important role in the development of vascular calcification.

In this study, we examined the stimulatory effect of OSM on osteogenic differentiation of HVSMC by the cultures of HVSMC in the presence of ascorbic acid 2-phosphate, β-glycerophosphate, and dexamethasone, namely ODM, compounds known to favor the expression of the osteoblastic phenotype in several bone cell systems [Coelho and Fernandes, 2000]. Among these osteogenic supplements, β-glycerophosphate, which is widely used in osteoblast cultures as an inorganic phosphate source for mineralization, has been shown to reduce cell viability and cause widespread, non-specific ("dystrophic") mineralization, when utilized at higher concentrations (≥5 mM) [Orriss et al., 2012]. Therefore, we investigated whether cell death is involved in the calcification by HVSMC under osteogenic culture. There was no significant increase of cell death in the osteogenic cultures of HVSMC in the presence of OSM compared with

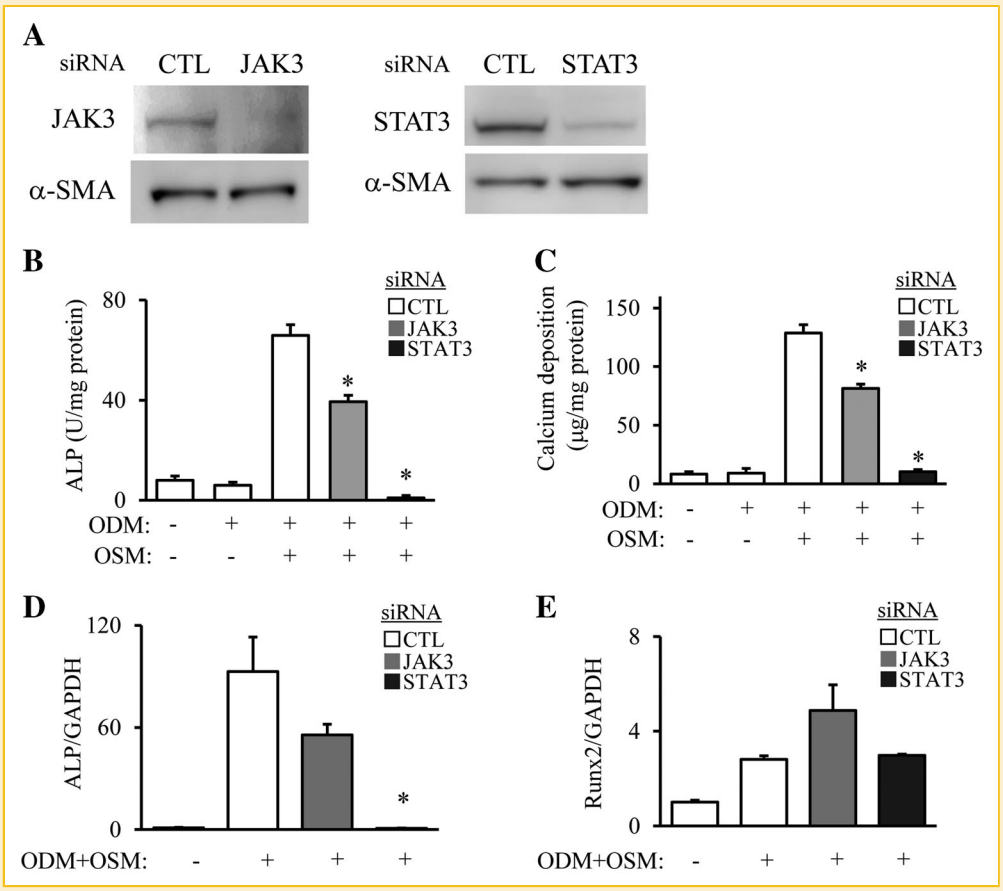

Fig. 5. JAK3/STAT3 knockdown inhibits OSM-induced osteoblastic differentiation of HUASMC. A: Suppression of JAK3/STAT3 expression by siRNA. After 24 h of siRNA introduction, expression of JAK3, STAT3, and α-smooth muscle actin (α-SMA) was analyzed by Western blot. B: Effects of knockdown of JAK3 or STAT3 on OSM-induced ALP activities in HUASMC. After 24 h of siRNA introduction, HUASMC were cultured for additional 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM ($n = 3$). $^*P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM. C: Effects of knockdown of JAK3 or STAT3 on in vitro calcification in HUASMC. After 24 h of siRNA introduction, HUASMCs were cultured for additional 14 days under the same conditions as those of (B). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM ($n = 3$). $^*P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM. D, E: Effects of knockdown of JAK3 or STAT3 on real-time quantitative RT-PCR analysis of mRNA expression of ALP (D) and Runx2 (E) in HUASMC. Twenty-four hours after siRNA introduction, HUASMC were cultured for additional 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. Messenger RNA expression of these genes was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize RNA input. The data are presented as mean ± SEM ($n = 3$). $^*P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM.

the control cultures. Furthermore, a pan-caspase inhibitor (zVAD-FMK) did not inhibit OSM-induced HVSMC calcification. These data suggest that OSM-induced HVSMC calcification under the cultures with ODM is not dependent on cell death.

IL-6-type cytokines including OSM exert their effects through activation of JAK/STAT signaling cascade [Heinrich et al., 2003]. Although it has been documented that OSM induces phosphorylation of JAK1 and JAK2 and activation of STAT1 and STAT3 in VSMC, the role of JAK3 in signaling pathway of OSM in VSMC remains to be determined [Bernard et al., 1999; Nagata et al., 2003; Demyanets et al., 2011]. The involvement of JAK3 in OSM-induced osteogenesis of adipose tissue-derived mesenchymal stem cells has been demonstrated by using a specific inhibitor of JAK3 [Song et al., 2007]. In the present study, we showed for the first time the significant role of JAK3 in OSM-induced osteoblastic differentiation of VSMC including HUASMC by utilizing a JAK3 inhibitor, WHI-P154 and JAK3 siRNA. Therefore, JAK3 in VSMC contributes to OSM-dependent progression of vascular calcification. However, JAK3 seems to have a minor contribution to OSM-induced osteogenesis of HAoSMC and HCASMC.

On the other hand, STAT3 is the most important transcription factor mediating anabolic signals in osteoblasts and regulates bone formation [Li, 2013]. Activation of the JAK/STAT3 signal transduction pathway by IL-6 type cytokines including OSM promotes osteoblast differentiation, as evidenced by increased ALP activity and osteocalcin expression in vitro [Bellido et al., 1997]. Moreover, STAT3 activation contributes to OSM-induced osteogenic differentiation of human mesenchymal stem cells derived from bone marrow and adipose tissue [Song et al., 2007; Guihard et al., 2012; Nicolaidou et al., 2012]. In this study, we evidently demonstrated that STAT3

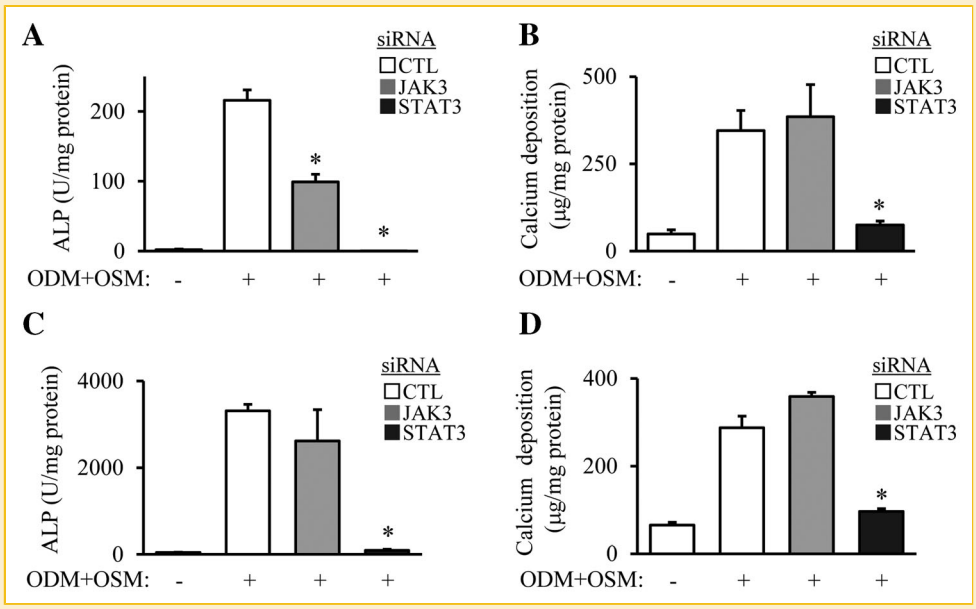

Fig. 6. Knockdown of JAK3/STAT3 modulates OSM-induced osteoblastic differentiation of HAoSMC and HCASMC. A: Effects of knockdown of JAK3 or STAT3 on OSM-induced ALP activities in HAoSMC. Twenty-four hours after siRNA introduction, HAoSMC were cultured for additional 4 days under the indicated conditions. OSM was utilized at 10 ng/ml. ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM. B: Effects of knockdown of JAK3 or STAT3 on in vitro calcification in HAoSMC. Twenty-four hours after siRNA introduction, HAoSMC were cultured for additional 14 days under the same conditions as those of (A). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM. C: Effects of knockdown of JAK3 or STAT3 on OSM-induced ALP activities in HCASMC. Twenty-four hours after siRNA introduction, HCASMC were cultured for additional 4 days under the same conditions as those of (A). ALP activities were measured, normalized to cellar protein contents, and are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM. D: Effects of knockdown of JAK3 or STAT3 on in vitro calcification in HCASMC. Twenty-four hours after siRNA introduction, HCASMC were cultured for additional 14 days under the same conditions as those of (A). Calcium deposition was quantified by MXB method. The data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ versus the control siRNA-transfected cultures with ODM and OSM.

signaling pathway mediates OSM-induced osteoblastic differentiation of all three kinds of HVSMC. These results suggest that STAT3 plays a crucial role in the development of vascular calcification.

In this study, we also showed that inhibition of JAK3/STAT3 signaling by pharmacological agents or siRNA does not suppress Runx2 mRNA expression induced by OSM in HUASMC. These data suggest that STAT3 signaling pathway modulates downstream of Runx2 and that OSM may up-regulate Runx2 expression through a STAT3-independent mechanism. However, microarray analysis has revealed that overexpression of STAT3 by adenovirus in mesenchymal stem cells significantly increased Runx2 expression [Nicolaidou et al., 2012]. Additionally, STAT3 silencing has been recently reported to reduce IL-6/sIL-6R-induced Runx2 expression in human adipose tissue-derived mesenchymal stem cells [Fukuyo et al., 2014]. Therefore, it remains to be determined whether OSM-induced STAT3 transcription factor may regulate Runx2 expression in HVSMC.

OSM is produced by monocyte/macrophage-lineage cells derived from various sites and pathological conditions such as chronic wound macrophages, microglia, tumor-associated macrophages, peripheral blood monocytes, and atherosclerotic plaque macrophages [Repovic and Benveniste, 2002; Albasanz-Puig et al., 2011; Ganesh et al., 2012; Nicolaidou et al., 2012; Vlaicu et al., 2013]. Our preliminary studies by immunohistochemistry also demonstrated that Mac-3-positive macrophages express OSM protein in atherosclerotic plaque lesions in apoE-deficient mice (data not shown). Therefore, it is likely that OSM derived from plaque macrophages may contribute to the development of atherosclerotic calcification.

In conclusion, OSM promotes osteoblastic differentiation of HVSMC mainly through JAK3/STAT3 signaling pathway. Therefore, OSM derived from plaque macrophages may play an important role in the development of atherosclerotic calcification. Furthermore, pharmacological inhibition of OSM in plaque macrophages may be a possible therapeutic target for prevention of atherosclerotic calcification.

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