Ovarian cancer-derived lysophosphatidic acid stimulates secretion of VEGF and stromal cell-derived factor-1α from human mesenchymal stem cells

Eun Su Jeon[1,3]*, Soon Chul Heo[1,2]*, Il Hwan Lee[1,2], Yoon Ji Choi[1,2], Ji Hye Park[1,2], Kyung Un Choi[3], Do Youn Park[3], Dong-Soo Suh[4], Man-Soo Yoon[4] and Jae Ho Kim[1,2,5]

[1]Medical Research Center for Ischemic Tissue Regeneration
Medical Research Institute
[2]Department of Physiology
[3]Department of Pathology
[4]Department of Obstetrics and Gynecology
School of Medicine, Pusan National University
Yangsan 626-870, Korea
[5]Corresponding author: Tel, 82-51-510-8073;
Fax, 82-51-510-8076; E-mail, jhkimst@pusan.ac.kr
*These authors contributed equally to this work.
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Abstract

Lysophosphatidic acid (LPA) stimulates growth and invasion of ovarian cancer cells and tumor angiogenesis. Cancer-derived LPA induces differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs) to α-smooth muscle actin (α-SMA)-positive cancer-associated fibroblasts. Presently, we explored whether cancer-derived LPA regulates secretion of pro-angiogenic factors from hASCs. Conditioned medium (CM) from the OVCAR-3 and SKOV3 ovarian cancer cell lines stimulated secretion angiogenic factors such as stromal-derived factor-1α (SDF-1α) and VEGF from hASCs. Pretreatment with the LPA receptor inhibitor Ki16425 or short hairpin RNA lentiviral silencing of the LPA₁ receptor abrogated the cancer CM-stimulated expression of α-SMA, SDF-1, and VEGF from hASCs. LPA induced expression of myocardin

and myocardin-related transcription factor-A, transcription factors involved in smooth muscle differentiation, in hASCs. siRNA-mediated depletion of endogenous myocardin and MRTF-A abrogated the expression of α-SMA, but not SDF-1 and VEGF. LPA activated RhoA in hASCs and pretreatment with the Rho kinase inhibitor Y27632 completely abrogated the LPA-induced expression of α-SMA, SDF-1, and VEGF in hASCs. Moreover, LPA-induced α-SMA expression was abrogated by treatment with the ERK inhibitor U0126 or the phosphoinositide-3-kinase inhibitor LY294002, but not the PLC inhibitor U73122. LPA-induced VEGF secretion was inhibited by LY294002, whereas LPA-induced SDF-1 secretion was markedly attenuated by U0126, U73122, and LY294002. These results suggest that cancer-secreted LPA induces differentiation of hASCs to cancer-associated fibroblasts through multiple signaling pathways involving Rho kinase, ERK, PLC, and phosphoinositide-3-kinase.

Keywords: carcinoma; fibroblasts; lysophosphatidic acid; ovarian neoplasms; receptors, lysophosphatidic acid; rho-associated kinases; vascular endothelial growth factor A

Introduction

Tumors are composed of genetically altered malignant cells along with a heterogenous population of non-neoplastic stromal cell components, such as fibroblasts, myofibroblasts, endothelial cells, pericytes, and inflammatory cells, and tumor cells interact with the stromal cells via paracrine signaling (De Wever and Mareel, 2003). Carcinoma-associated fibroblasts (CAFs, also called as myofibroblasts or cancer stroma), which express α-smooth muscle actin (α-SMA), play important roles during cancer progression and metastasis (Bhowmick et al., 2004; Desmouliere et al., 2004; Orimo and Weinberg, 2006; De Wever et al., 2008). They stimulate tumorigenesis, angiogenesis, and invasion in a variety of solid tumors, including prostate, breast, and ovarian carcinomas (Sappino et al., 1988; Olumi et al., 1999; De Wever and Mareel, 2003; Orimo et al., 2005; Ganss, 2006) by secreting various extracellular matrix

proteins, proteases, chemokines, and angiogenic factors (Li *et al.*, 2007).

Mesenchymal stem cells (MSCs) have a self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types that include adipogenic, osteogenic, chondrogenic, and myogenic lineages (Prockop, 1997; Pittenger *et al.*, 1999; Short *et al.*, 2003; Barry and Murphy, 2004). MSCs are distributed throughout various tissues, and so may be local sources of tissue-resident stem cells (Crisan *et al.*, 2008). Moreover, MSCs are recruited into the stroma of developing tumors (Hall *et al.*, 2007) and constitute a large proportion of non-neoplastic stromal cells within the tumor microenvironment (De Wever *et al.*, 2008). Accumulating evidence suggests that MSCs could also have an adverse effect that favors tumor growth. When transplanted together with tumor cells, MSCs stimulate capability of proliferation of tumor cells and enhance angiogenesis in tumor tissues (Zhu *et al.*, 2006). Furthermore, MSCs stimulate metastatic potency of breast carcinoma when coinjected with human breast carcinoma cells into a subcutaneous site by xenograft transplantation (Karnoub *et al.*, 2007). Human bone marrow-derived MSCs exposed to tumor-conditioned medium (CM) exhibit phenotypic and functional characteristics of CAFs including sustained expression of α-SMA and stromal-derived factor-1 (SDF-1), a pro-angiogenic chemokine, and the ability to promote tumor cell growth *in vitro* and in an *in vivo* coimplantation model (Mishra *et al.*, 2008). Furthermore, it has been reported that VEGF secreted from MSCs induce sprouting of human umbilical vein endothelial cells (HUVECs) *in vitro* and stimulate *in vivo* tumor angiogenesis in a pancreatic carcinoma model (Beckermann *et al.*, 2008). These observations suggest that CAFs differentiated from MSCs plays a key role in the tumorigenesis and angiogenesis through production of SDF-1 and VEGF.

Lysophosphatidic acid (LPA) is a small bioactive phospholipid produced by activated platelets, mesothelial cells, fibroblasts, adipocytes, and some cancer cells (Gaits *et al.*, 1997; Mills and Moolenaar, 2003; Aoki, 2004). Accumulating evidence suggests that LPA is relevant to tumorigenesis and metastasis by stimulating proliferation, survival, migration, and invasion of cancer cells (Mills and Moolenaar, 2003). The biological functions of LPA are mediated through LPA receptors (Anliker and Chun, 2004; Moolenaar *et al.*, 2004; Tabata *et al.*, 2007; Murakami *et al.*, 2008). Activation of LPA receptors mediates the biological responses through activating multiple signaling pathways involving PLC, ERK, phosphatidylinositol-3-kinase, and Rho

kinase (Mills and Moolenaar, 2003; Birgbauer and Chun, 2006). We have previously reported that LPA induces migration of human adipose tissue-derived MSCs (hASCs) and stimulates expression of α-SMA and SDF-1 *in vitro* (Jeon *et al.*, 2008a; Lee *et al.*, 2008), suggesting a pivotal role of LPA in the generation of CAFs within the tumor microenvironment. LPA-induced expression of α-SMA is dependent on activation of an autocrine TGF-β1 signaling loop, whereas the stimulatory effect of LPA on SDF-1 expression is mediated in part by TGF-β1 (Jeon *et al.*, 2008a). Therefore, it is still unclear whether cancer-derived LPA can regulate expression of not only α-SMA and SDF-1, but also VEGF. Moreover, the molecular mechanisms by which LPA induces expression of α-SMA, SDF-1, and VEGF in hASCs remain elusive.

An increasing body of evidence supports the idea that α-SMA expression is regulated by serum response factor (SRF) and the myocardin family of SRF co-factors, i.e. myocardin, myocardin-related transcription factor-A (MRTF-A or MKL1), and myocardin-related transcription factor-B (MRTF-B or MKL2) (Owens *et al.*, 2004; Pipes *et al.*, 2006; Parmacek, 2007). SRF binds to the CArG boxes in the promoter of α-SMA and myocardin activates SRF-dependent transcription (Chen *et al.*, 2002; Wang *et al.*, 2002; Du *et al.*, 2003; Yoshida *et al.*, 2003). In unstimulated cells, MRTF-A/B are sequestered in the cytoplasm through direct interaction with G-actin, but RhoA-Rho kinase-mediated actin polymerization depletes the G-actin pool, which frees MRTF from G-actin to enter the nucleus where it can stimulate SRF-dependent transcription of α-SMA (Miralles *et al.*, 2003). Therefore, these results suggest that the RhoA-Rho kinase pathway plays a key role in the expression of α-SMA by regulating the integrity of the cytoskeleton and the cellular locale of MRTF (Miano, 2003; Cen *et al.*, 2004). However, the role of Rho kinase, myocardin, and MRTF in the LPA-induced expression of α-SMA has not been explored.

In the present study, we explored whether ovarian cancer-derived LPA induces expression of α-SMA, SDF-1, and VEGF, and characterized the signaling pathways involved in the LPA-induced gene expression.

Results

CM from ovarian cancer cells stimulates secretion of angiogenic factors from hASCs

In order to explore whether hASCs can regulate angiogenesis within tumor microenvironment,
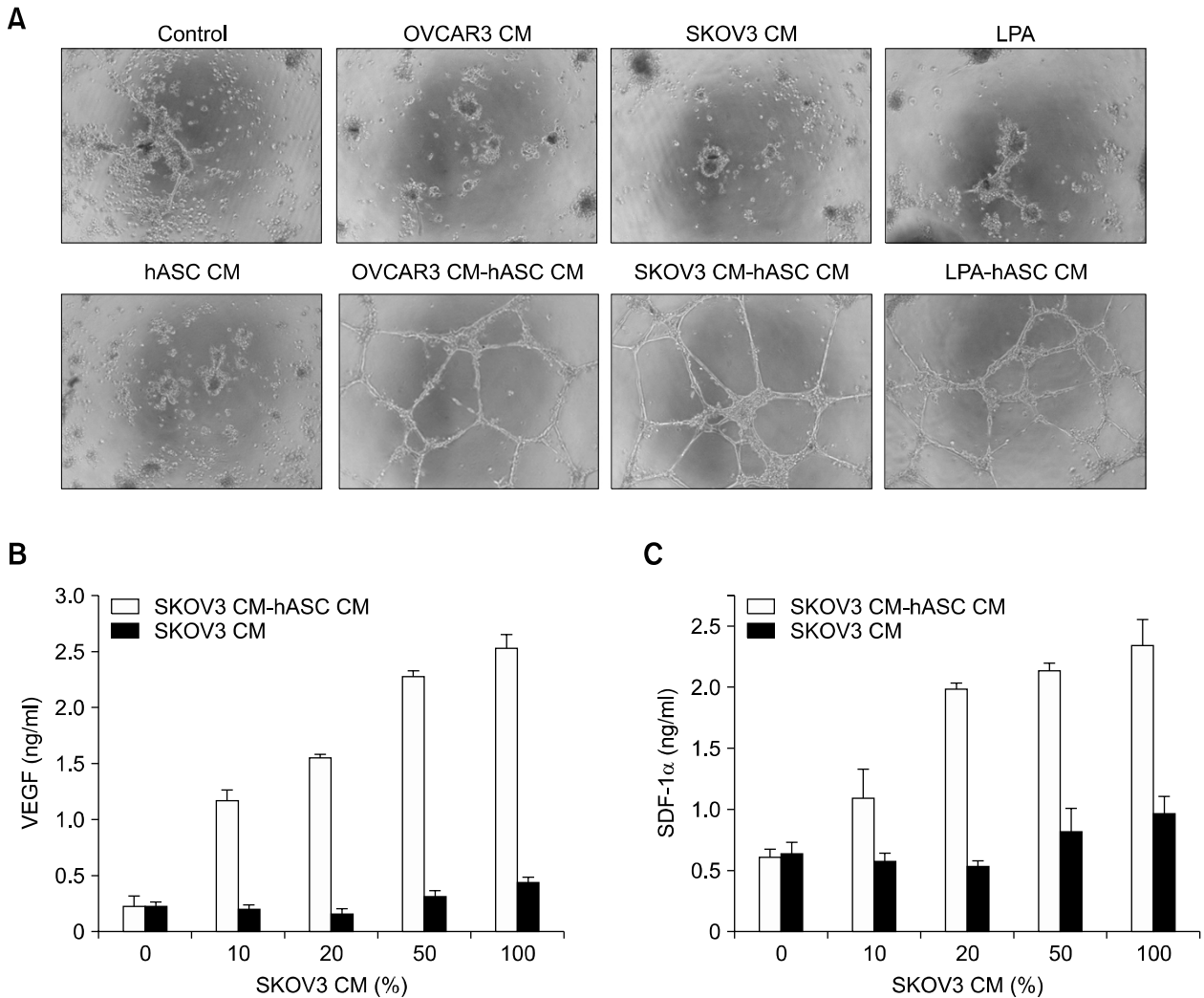
A

B

C

Figure 1. Cancer CM-induced secretion of VEGF and SDF-1 from hASCs. (A) hASCs were pretreated with SKOV3 CM, OVCAR3 CM, 5 μM LPA, or serum-free α-MEM for 48 h for collection of SKOV3 CM-hASC CM, OVCAR3 CM-hASC CM, LPA-hASC CM, or hASC CM, respectively. HUVECs were plated onto a layer of Matrigel, and treated with the culture supernatants from hASCs for 12 h. As control experiments, HUVECs were treated with α-MEM (control), SKOV3 CM, OVCAR3 CM, or 5 μM LPA for 12 h. The images were photographed using an inverted microscope with a digital camera. (B and C) hASCs were treated with the indicated concentrations of SKOV3 CM for 48 h for collection of SKOV3 CM-hASC CM. The concentrations of VEGF (B) and SDF-1 (C) in SKOV3 CM-hASC CM or SKOV3 CM were determined by ELISA.

hASCs were treated with CM from two different ovarian cancer cell types (OVCAR3 and SKOV3 cells) and the effects of culture supernatants from hASCs on tube formation of HUVECs were determined. As shown in Figure 1A, culture supernatants of hASCs (OVCAR3 CM-hASC CM or SKOV3 CM-hASC CM), which were prepared by pretreatment of hASCs with OVCAR3 CM or SKOV3 CM, greatly stimulated tube formation of HUVECs. Furthermore, culture supernatant from LPA-treated hASCs (LPA-hASC CM) also stimulated tube formation of HUVECs, whereas culture supernatant from serum-starved hASCs (hASC CM) had no significant impact on tube formation.

Moreover, exposure of HUVECs to OVCAR3 CM, SKOV3 CM, or LPA did not significantly influence tube formation. These results suggest that hASCs secrete pro-angiogenic factors in response to cancer CM or LPA. To explore whether cancer CM stimulates secretion of pro-angiogenic factors from hASCs, the protein levels of VEGF and SDF-1 in the culture supernatant from hASCs were determined using ELISA analysis. SKOV3 CM dose-dependently stimulated secretion of VEGF and SDF-1 from hASCs, whereas the endogenous levels of VEGF and SDF-1 included in SKOV3 CM were negligible compared to the levels in the culture supernatants of SKOV3 CM-treated hASCs
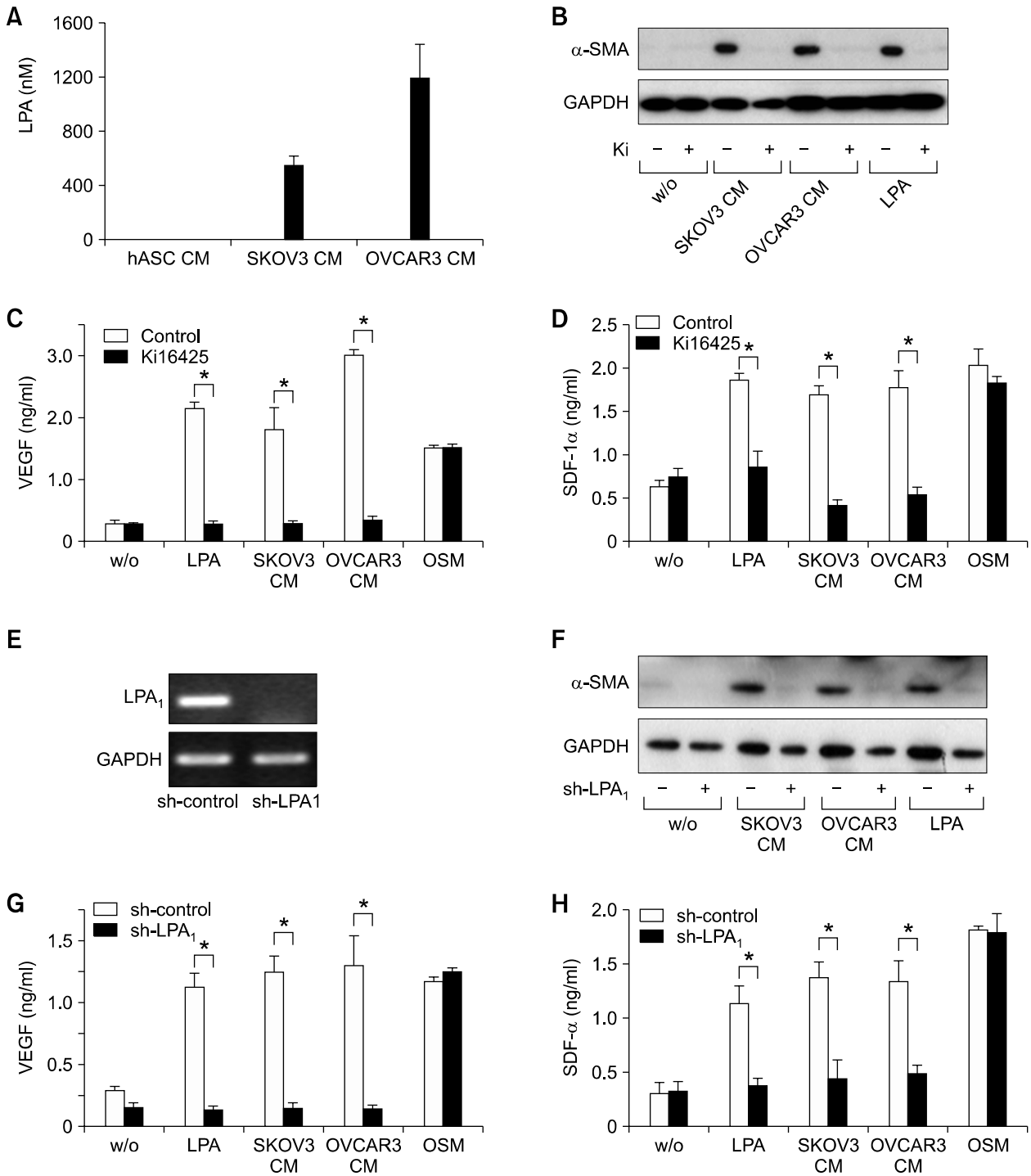
Figure 2. Role of LPA-LPA$_1$ signaling in the cancer CM-induced expression of α-SMA, VEGF, and SDF-1. (A) Concentrations of LPA in CM from OVCAR3, SKOV3, and hASCs were determined. (B) Serum-starved hASCs were exposed to OVCAR3 CM, SKOV3 CM, or 5 μM 1-oleoyl-LPA in the presence or absence of 1 μM Ki16425 for 4 days. The expression levels of α-SMA and GAPDH were determined by Western blotting. (C-D) hASCs were treated with serum-free medium containing OVCAR3 CM, SKOV3 CM, 5 αM LPA or 10 ng/ml OSM in the presence or absence of 1 αM Ki16425 for 48 h. (E) hASCs were infected with control (sh-control) or LPA$_1$ (sh-LPA$_1$) shRNA lentivirus (pLKO.1-puro, MISSION, Sigma). After puromycin selection, the expression levels of LPA$_1$ and GAPDH were determined by RT-PCR. (F) shRNA-infected cells were exposed to serum-free medium containing 5 μM LPA, OVCAR3 CM or SKOV3 CM for 4 days and the expression levels of α-SMA and GAPDH were determined by Western blotting. (G and H) hASCs were infected with control or LPA$_1$ shRNA lentivirus and treated with serum-free medium containing OVCAR3 CM, SKOV3 CM, 5 μM LPA or 10 ng/ml OSM for 48 h. The secreted levels of VEGF and SDF-1 were determined by ELISA. Data represent mean ± S.D. (n = 4). *, $P < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test.
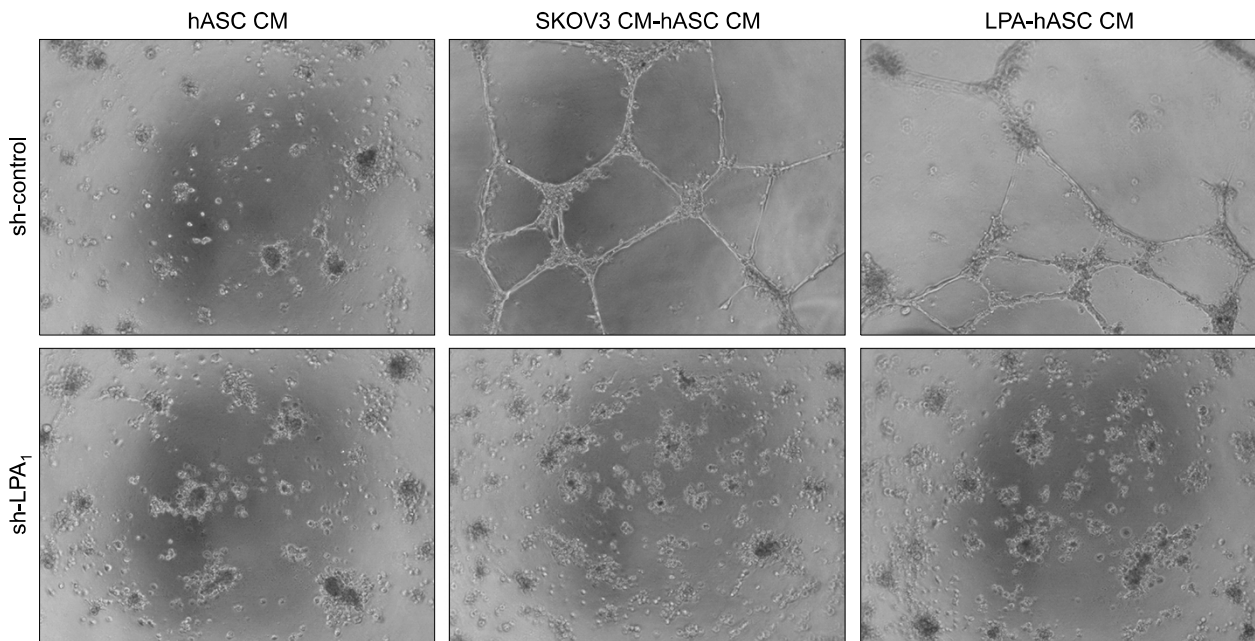
Figure 3. Role of LPA$_1$ in the cancer CM stimulation of hASCs-mediated paracrine function on endothelial tube formation. hASCs were infected with sh-control or sh-LPA$_1$ lentivirus and then treated with SKOV3 CM, 5 μM LPA, or serum-free α-MEM for 48 h for collection of SKOV3 CM-hASC CM, LPA-hASC CM, or hASC CM, respectively. HUVECs were plated onto a layer of Matrigel, and treated with the conditioned medium from hASCs for 12 h. The images were photographed using an inverted microscope with a digital camera. Representatives of three independent experiments are shown.

(Figures 1B and 1C). These results suggest that ovarian cancer cells stimulate secretion of VEGF and SDF-1 from hASCs through a paracrine mechanism.

Role of LPA-LPA$_1$ receptor signaling pathway in cancer CM-induced expression of α-SMA, SDF-1, and VEGF from hASCs

In order to explore the role of LPA in the cancer CM-stimulated gene expression, we determined the concentrations of LPA in OVCAR3 CM and SKOV3 CM. As shown in Figure 2A, LPA was detected in the CM from OVCAR3 and SKOV3 cells, but not CM from serum-starved hASCs. The concentrations of LPA in OVCAR3 CM and SKOV3 CM were 1,193 ± 245 and 545 ± 72 nM, respectively. Not only LPA but also CM from OVCAR3 and SKOV3 cells induced expression of α-SMA in hASCs, and the LPA receptor inhibitor Ki16425 abrogated the expression of α-SMA induced by LPA or cancer CM (Figure 2B). To assess whether cancer CM-induced secretion of VEGF and SDF-1 was dependent on the LPA-LPA$_1$ signaling pathway, we determined the effects of Ki16425 on the levels of SDF-1 and VEGF secreted from hASCs. As shown in Figures 2C and 2D, LPA elicited secretion of VEGF and SDF-1 from hASCs in a Ki16425-sensitive manner. Furthermore, CM from

OVCAR3 or SKOV3 cells elicited secretion of VEGF and SDF-1 from hASCs, and cancer CM-induced secretion of VEGF and SDF-1 was abrogated by treatment with Ki16425. Oncostatin M (OSM) has been reported to induce secretion of SDF-1 and VEGF from hASCs and adipocytes (Lee *et al.*, 2007; Rega *et al.*, 2007). OSM belongs to the interleukin (IL)-6 family of cytokines and it acts on target cells by binding to a heterodimeric membrane receptor composed of the leukemia inhibitory factor- or OSM-specific receptor and the gp130 receptor chain (Gomez-Lechon, 1999). In contrast to the inhibitory effect of Ki16425 on the LPA-stimulated secretion of SDF-1 and VEGF, OSM-induced secretion of SDF-1 and VEGF was not affected by Ki16425 (Figures 2C and 2D).

To confirm the involvement of LPA receptors in the cancer CM-induced expression of α-SMA and angiogenic factors, we examined the effect of shRNA-mediated silencing of LPA$_1$ expression. LPA$_1$ expression was down-regulated by lentiviral transduction of LPA$_1$-specific shRNA (Figure 2E). Depletion of endogenous LPA$_1$ expression blocked the α-SMA expression stimulated by LPA or cancer CM (Figure 2F), suggesting that the LPA-LPA$_1$ signaling axis plays a key role in ovarian cancer CM-induced expression of α-SMA in hASCs. We next clarified whether LPA$_1$ was involved in the cancer CM-induced secretion of VEGF and SDF-1
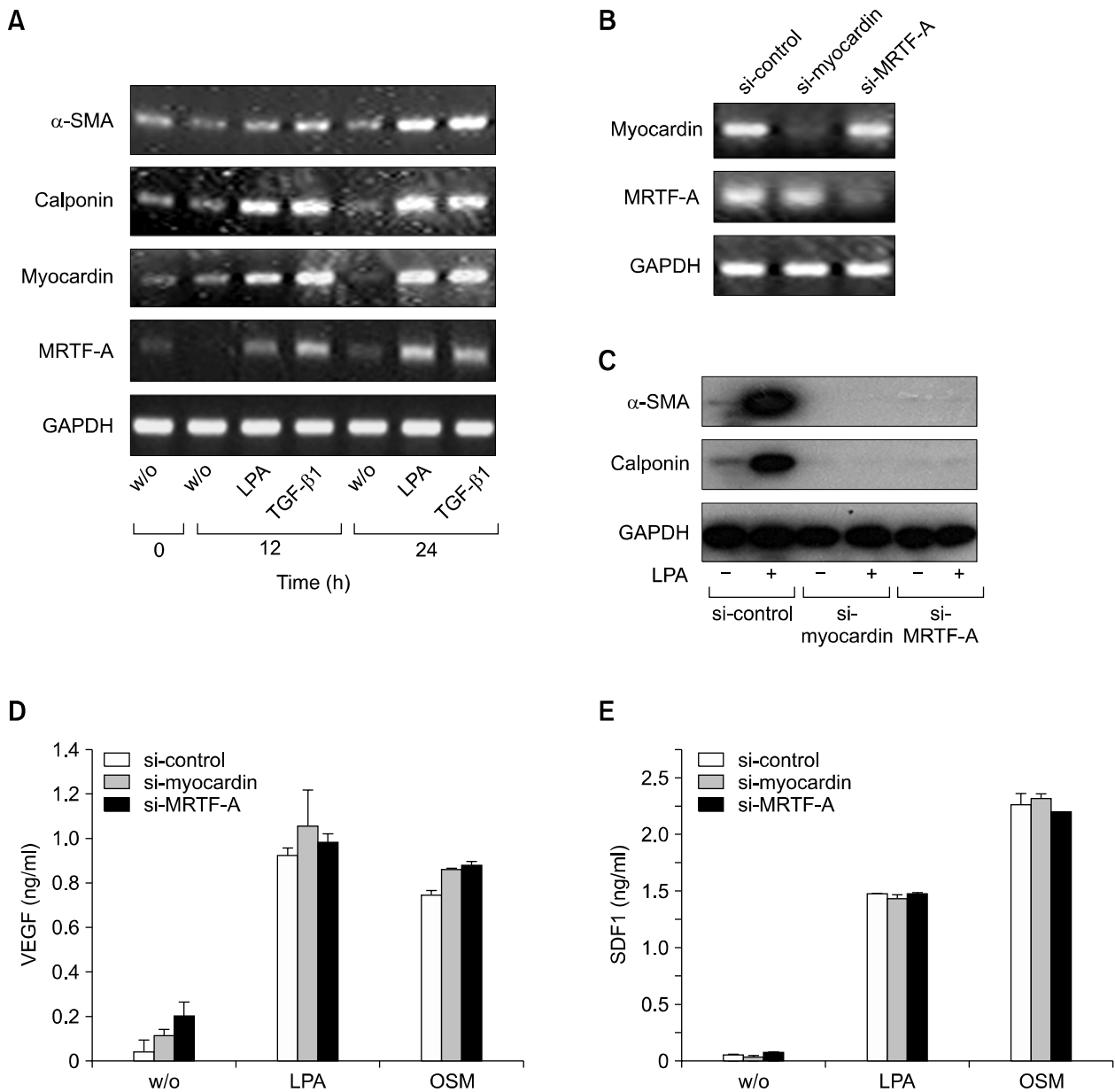
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E

Figure 4. Role of myocarin and MRTF-A in the LPA-induced expression of α-SMA, VEGF and SDF-1. (A) hASCs were treated with 5 μM LPA or 1 ng/ml TGF-β1 for the indicated times. (B) hASCs were transfected with control siRNA (si-control), siRNAs specific for myocardin (si-myocardin) or MRTF-A (si-MRTF-A). The mRNA levels of α-SMA, h1-calponin, myocardin, MRTF-A, and GAPDH were determined by RT-PCR. (C) The siRNA-transfected hASCs were treated with 5 μM LPA or vehicles for 4 days and the expression levels of α-SMA and GAPDH were determined by Western blotting. Representatives of three independent experiments are shown. (D and E). The siRNA-transfected hASCs were treated with serum-free media containing 5 μM LPA or 10 ng/ml OSM for 48 h and the CM from hASCs was subjected to ELISA for analysis of VEGF (D) and SDF-1 (E). Data represent mean $\pm$ S.D. (n = 4). *, $P < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test.

using sh-LPA$_1$ lentivirus. LPA- and cancer CM-induced secretion of VEGF and SDF-1 was abrogated by shRNA-mediated silencing of endogenous LPA$_1$ (Figures 2G and 2H). However, OSM-induced secretion of SDF-1 and VEGF was not affected by depletion of LPA$_1$ expression (Figures 2G and 2H). These results suggest that CM from OVCAR3 or SKOV3 cells induces expre-ssion of α-SMA, VEGF, and SDF-1 through a LPA-LPA$_1$-dependent mechanism.

Involvement of LPA$_1$ in the cancer CM stimulation of hASCs-mediated paracrine function on endothelial tube formation

To explore whether activation of LPA$_1$ is required

A

GTP-RhoA

Total RhoA

0   1   2   5   30   60
Time (min)

B

α-SMA

GAPDH

Y27632   –   +   –   +
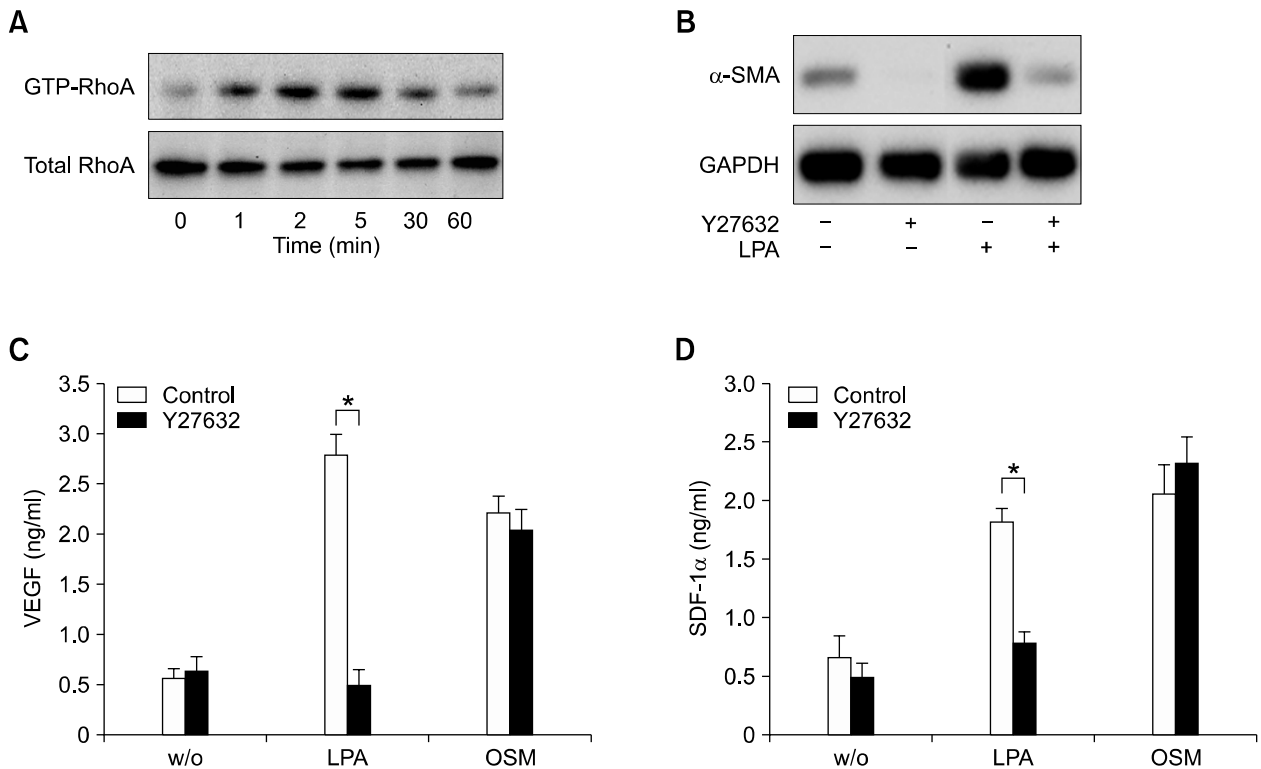LPA   –   –   +   +

C

D

Figure 5. Role of RhoA-Rho kinase-dependent pathway in the LPA-induced expression of α-SMA, VEGF and SDF-1. (A) Serum-starved hASCs were treated with 5 μM LPA for the indicated time periods. The amounts of RhoA in the whole cell lysates (total RhoA) or the GTP-bound RhoA precipitated from the lysates were revealed by Western blotting using anti-RhoA antibody. (B) Serum-starved hASCs were treated with 5 μM LPA in the absence or presence of 10 μM Y27632 for 4 days. The expression levels of α-SMA and GAPDH were determined by Western blotting. Representative data from three independent experiments are shown. (C and D) hASCs were treated with serum-free medium containing 5 μM LPA or 10 ng/ml OSM in the absence or presence of 10 μM Y27632 for 48 h and the CM from hASCs was subjected to ELISA for analysis of VEGF (C) and SDF-1 (D). Data represent mean $\pm$ S.D. ($n = 4$). *, $P < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test.

for the hASC-mediated stimulation of anigogene-sis, hASCs were infected with sh-control or sh-LPA$_1$ lentivirus and then treated with SKOV3 CM or 5μM LPA for collection of SKOV3 CM-hASC CM or LPA-hASC CM. As shown in Figure 3, SKOV3 CM-hASC CM or LPA-hASC CM, which were derived from sh-control-infected cells, stimu-lated endothelial tube formation, whereas those from sh-LPA$_1$-infected cells did not influence tube formation. These results suggest that LPA-LPA$_1$ signaling pathway plays a pivotal role in the paracrine stimulation of hASC on endothelial tube formation in response to cancer CM.

Role of myocardin and MRTF in LPA-induced expression of α-SMA, VEGF, and SDF-1

Recent reports suggest that the transcription factor SRF and its cofactors (myocardin, MRTF-A, and MRTF-B) regulate expression of α-SMA (Owens *et al.*, 2004; Pipes *et al.*, 2006; Parmacek, 2007). We have reported that TGF-β1 and sphingosylpho-

sphorylcholine induces expression of α-SMA through a myocardin-dependent mechanism (Jeon *et al.*, 2006). Furthermore, we demonstrated involvement of MRTF-A in the sphingosylphosphorylcho-line-induced α-SMA expression (Jeon *et al.*, 2008b). To explore the effects of LPA on the expression of myocardin and MRTF-A, we measured the mRNA levels of myocardin and MRTF-A after LPA treat-ment. As shown in Figure 4A, the expression levels of myocardin and MRTF-A were increased after treatment of hASCs with LPA or TGF-β1, suggesting a possible role of myocardin and MRTF-A in the LPA-induced α-SMA expression. We next examined the involvement of myocardin and MRTF-A in the LPA-stimulated α-SMA expression using siRNA-me-diated gene silencing. As shown in Figure 4B, si-myocardin and si-MRTF-A specifically down-re-gulated the mRNA levels of myocardin and MRTF-A, respectively. Furthermore, si-myocardin and si-MRTF-A abrogated the LPA-induced α-SMA expression (Figure 4C). These results suggest that both myocardin and MRTF-A play a key role in
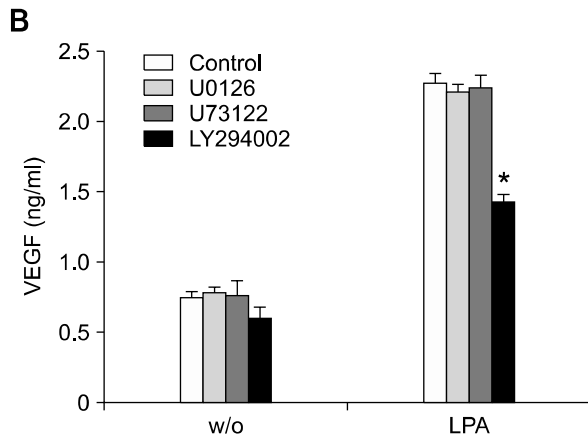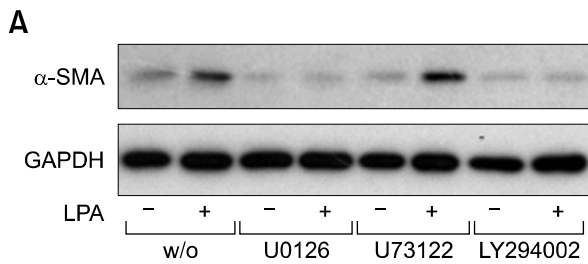
A

Figure 6. Signaling pathways involved in the LPA-induced expression of α-SMA, VEGF, and SDF-1. (A) hASCs were treated with 5 μM LPA or vehicles in the absence or presence of 10 μM U0126, 2.5 μM U73122, 10 μM LY294002 for 4 days and the expression levels of α-SMA and GAPDH were determined by Western blotting. (B and C) hASCs were treated with 5 μM LPA or vehicles in the absence or presence of 10 μM U0126, 2.5 μM U73122, 10 μM LY294002 for 2 days and the conditioned medium was subjected to ELISA for analysis of VEGF (B) and SDF-1 (C). Data represent mean ± S.D. (n = 4). *, $P < 0.01$ by two-way ANOVA and Scheffe's *post hoc* test.

B

C
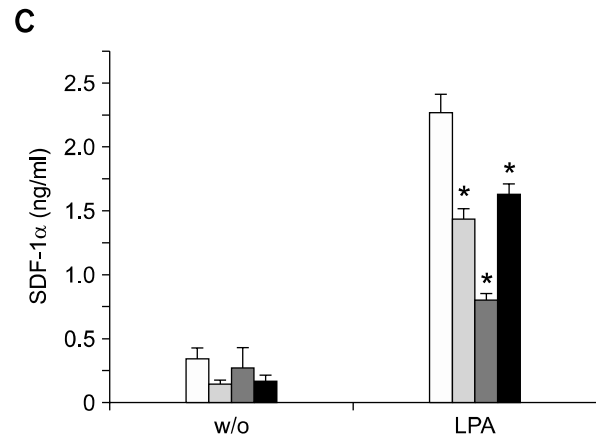
LPA-induced expression of α-SMA in hASCs.

To explore whether myocardin and MRTF-A are involved in the LPA-induced expression of SDF-1 and VEGF, we determined the effects of siRNA-mediated silencing of myocardin and MRTF-A on the secreted levels of SDF-1 and VEGF. As shown in Figures 4D and 4E, expression of VEGF and SDF-1, which were induced by LPA or OSM, was not abrogated by siRNA-mediated silencing of endogenous myocardin or MRTF-A. These results suggest that myocardin and MRTF-A play a key role in the LPA-induced expression of α-SMA, but not VEGF and SDF-1.

Role of RhoA-Rho kinase-dependent pathway in LPA-induced expression of α-SMA, SDF-1, and VEGF

Accumulating evidence suggests that RhoA-mediated rearrangement of actin cytoskeleton plays a pivotal role in the expression of α-SMA (Cen *et al.*, 2004). LPA regulates the actin cytoskeleton through a RhoA-dependent mechanism (Anliker and Chun, 2004; Moolenaar *et al.*, 2004). To explore whether LPA can activate RhoA in hASCs, we treated the cells with LPA and measured the resulting GTP-loaded RhoA levels. As shown in Figure 5A, GTP-RhoA was increased as early as 1 min following LPA treatment, decreasing to basal

levels by 60 min. To explore whether RhoA elicits LPA-induced α-SMA expression through a mechanism involving Rho kinase, one of its major downstream targets, we examined the effect of Y27632, a Rho kinase-specific inhibitor, on LPA induction of α-SMA expression. As shown in Figure 5B, pretreating cells with Y27632 abrogated their LPA-induced expression of α-SMA. To explore the involvement of Rho kinase in LPA-induced expression of SDF-1 and VEGF, we next determined the secreted levels of SDF-1 and VEGF using ELISA analysis. Y27632 blocked the LPA-induced secretion of VEGF and SDF-1 from hASCs, in contrast to the non-significant impact on the OSM-stimulated secretion of VEGF and SDF-1 (Figures 5C and 5D). These results indicate that Rho kinase pathway plays a key role in the LPA-induced expression of α-SMA and secretion of SDF-1 and VEGF in hASCs.

Signaling pathways implicated in LPA-induced expression of α-SMA, SDF-1, and VEGF

In addition to the RhoA pathway, LPA has been shown to activate diverse signaling pathways including PLC, phosphoinositide-3-kinase (PI3K), and ERK (Anliker and Chun, 2004; Moolenaar *et al.*, 2004). In order to explore the involvement of

these signaling enzymes in the LPA-induced α-SMA expression, we examined the effects of specific inhibitors against these signaling enzymes on the expression levels of α-SMA. As shown in Figure 6A, LPA-induced α-SMA expression was abrogated by the MEK-ERK inhibitor U0126 and the phosphoinositide-3-kinase inhibitor LY294002. However, the PLC inhibitor U73122 did not influence the LPA-stimulated α-SMA expression. These results indicate that phosphoinositide-3-kinase and ERK are involved in the LPA-stimulated α-SMA expression. To determine the involvement of PLC, PI3K, and ERK in the LPA-induced secretion of VEGF and SDF-1, we next examined the effects of the enzyme inhibitors on the secreted levels of VEGF and SDF-1. As shown in Figure 6B, LPA-induced VEGF secretion was inhibited by LY294002, but not by U73122 or U0126, whereas LPA-induced SDF-1 secretion was markedly attenuated by U0126, U73122, and LY294002 (Figure 6C). These results suggest that LPA regulates expression of α-SMA, VEGF, and SDF-1 through different signaling pathways involving PLC, PI3K, and ERK.

Discussion

Increasing evidence suggests that VEGF and SDF-1 are expressed in not only tumor cells but also CAFs within solid tumors (Allinen *et al.*, 2004; Orimo *et al.*, 2005; Hofmeister *et al.*, 2008). The expression of VEGF was found to be predominantly induced in the α-SMA-positive CAFs in a gastric cancer mouse model (Guo *et al.*, 2008) and in a xenograft ovarian cancer model (Spaeth *et al.*, 2009). It has been reported that stromal expression of VEGF is correlated with tumor grade and microvessel density in mammary phyllodes tumors (Tse *et al.*, 2004). In addition, SDF-1 expression is significantly up-regulated in CAFs isolated from breast cancer specimens compared to normal breast fibroblasts (Orimo *et al.*, 2005). CAF-derived SDF-1 not only increases carcinoma cell growth directly through the CXCR4 receptor displayed on tumor cells, but also serves to recruit endothelial progenitor cells into tumors, thereby stimulating neoangiogenesis (Orimo and Weinberg, 2006). CM from tumor cells promotes the production of SDF-1 in MSCs (Menon *et al.*, 2007; Mishra *et al.*, 2008). Furthermore, hASCs stimulate tumorigenesis of a murine breast cancer 4T1 cell line in a xenograft transplantation model, and breast cancer cells induce secretion of SDF-1 from hASCs (Muehlberg *et al.*, 2009). In spite of the functional importance of CAF-derived VEGF and SDF-1 in tumorigenesis,

the molecular mechanisms associated with the expression of pro-angiogenic factors in CAFs have not been clarified. In the present study, we demonstrate that LPA mediates the expression of VEGF and SDF-1 induced by CM from ovarian cancer cells. LPA stimulates VEGF secretion from MSCs *in vitro* and transplantation of LPA-treated MSCs enhances capillary density in ischemic myocardium (Liu *et al.*, 2009). Therefore, it is likely that LPA-induced secretion of VEGF and SDF-1 from MSCs may play a key role in the tumorigenesis and angiogenesis.

We demonstrated here that LPA-induced VEGF secretion was dependent on Rho kinase and PI3K in hASCs, while LPA-induced SDF-1 secretion was mediated by Rho kinase, ERK, PLC, and PI3K. LPA stimulates VEGF expression in ovarian cancer cells and colon carcinoma cells *in vitro* (Hu *et al.*, 2001; Shida *et al.*, 2003). In ovarian cancer cells, LPA induction of VEGF expression is dependent on PI3K and ERK pathways (Lee *et al.*, 2006). LPA stimulates VEGF expression through activation of c-Myc and SP-1 transcription factors in ovarian cancer cells, and $G_{12/13}$-RhoA-Rho kinase-dependent pathway is required for activation of c-Myc (Song *et al.*, 2009). These results suggest that multiple signaling pathways may regulate differentially the LPA-induced expression of VEGF and SDF-1 depending on cell types.

Presently, LPA treatment increased expression of MRTF-A and myocardin, and siRNA-mediated depletion of myocardin and MRTF-A abrogated the LPA-stimulated expression of α-SMA. It is well-established that expression of most SMC-specific differentiation marker genes including α-SMA relies on the SRF/myocardin-dependent pathway (Miano, 2003; Wang and Olson, 2004). In addition, mouse embryos lacking myocardin die during the early stage of smooth muscle development; they fail to express multiple smooth muscle marker genes in embryonic dorsal aorta and other vascular structures (Li *et al.*, 2003). Forced expression of myocardin is sufficient to induce the expression of smooth muscle-specific genes in human MSCs (van Tuyn *et al.*, 2005; Ventura, 2005), and dominant-negative myocardin mutants and myocardin-specific siRNAs inhibit SMC differentiation marker gene expression in a variety of smooth muscle cell types, including the primary rat aortic SMC, A7r5, Pac1, and A10 SMC lines (Du *et al.*, 2003; Wang *et al.*, 2003; Yoshida *et al.*, 2003). We have previously reported that LPA induces α-SMA expression via an autocrine TGF-β1 signaling pathway in part (Jeon *et al.*, 2008a) and that myocardin mediates the TGF-β-dependent expression of α-SMA (Jeon *et al.*, 2006). Taken together,

these results support the notion that myocardin plays a key role in the LPA-induced α-SMA expression. Furthermore, we have previously reported that RhoA-dependent activation of MRTF-A mediates α-SMA expression induced by sphingosylphosphorylcholine treatment in hASCs (Jeon *et al.*, 2008b). It has been amply documented that LPA induces RhoA activation via $G\alpha_{12/13}$-dependent mechanism, with the RhoA stimulating the downstream Rho kinase (Anliker and Chun, 2004; Moolenaar *et al.*, 2004; Meyer zu and Jakobs, 2007). Activation of the RhoA-Rho kinase pathway promotes the assembly of monomeric G-actin into F-actin filaments (Sotiropoulos *et al.*, 1999; Copeland and Treisman, 2002) and actin polymerization results in the translocation of MRTF-A from the cytoplasm to the nucleus (Cen *et al.*, 2003; Miralles *et al.*, 2003; Du *et al.*, 2004), suggesting that Rho kinase-dependent activation of MRTF-A may be associated with the LPA-stimulated α-SMA expression. Taken together, these results imply that myocardin and MRTF-A may play a key role in the differentiation of MSCs to α-SMA-positive CAFs.

The importance of reactive tumor microenvironments in tumorigenesis and metastasis is increasingly appreciated. LPA is an essential microenvironmental factor in ovarian cancer, and high levels of LPA in the ascitic fluid and sera of patients have been correlated with poor prognosis of the disease (Xu *et al.*, 1995; 1998). Moreover, LPA has been shown to be produced by ovarian cancer cells (Shen *et al.*, 1998; Eder *et al.*, 2000; Luquain *et al.*, 2003; Sengupta *et al.*, 2003). In the present study, we determined the LPA levels in ovarian cancer CM and the concentrations of LPA in OVCAR3 CM and SKOV3 CM were $1,193 \pm 245$ and 545 ± 72 nM, respectively. Furthermore, cancer CM induced expression of phenotypic and functional markers of CAFs (α-SMA, VEGF, and SDF-1) through Ki16425-sensitive mechanism. Although Ki16425 has been shown to inhibit both LPA_1 and LPA_3 (Ohta *et al.*, 2003), we have reported that LPA1 is a major LPA receptor isoform expressed in mesenchymal stem cells and that LPA_1 plays a key role in the LPA-induced cellular responses, such as α-SMA expression and cell migration (Jeon *et al.*, 2008a; Lee *et al.*, 2008; Song *et al.*, 2010). Taken together with the finding that shRNA-mediated silencing of LPA_1 completely abrogated the LPA-induced α-SMA expression, these results lead us to suggest that cancer-derived LPA promotes differentiation of tissue-resident MSCs toward CAFs in tumor microenvironment and that LPA may be a useful target for therapy of tumorigenesis and tumor angiogenesis.

Methods

Materials

α-Minimum essential medium (α-MEM), trypsin, and FBS were purchased from Invitrogen (Carlsbad, CA). Anti-GAPDH antibody was purchased from Millipore (Billerica, MA). Anti-RhoA antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). LY294002, Y27632, U0126, and U73122 were purchased from BIOMOL (Plymouth Meeting, PA). 1-Oleoyl-sn-glycero-3-phosphate (1-oleoyl-LPA), Ki16425, and anti-α-SMA antibody were from Sigma-Aldrich (St. Louis, MO). Human VEGF Duoset (DY293B), human SDF-1 Duoset (DY350), human recombinant oncostatin M, and human recombinant TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ). Culture plates were purchased from Nunc (Roskilde, Denmark). Peroxidase-labeled secondary antibodies and Enhanced Chemiluminescence Western blotting system were from Amersham Biosciences (Piscataway, NJ).

Cell culture

Subcutaneous adipose tissue was obtained from elective surgeries with the patient's consent as approved by the Institution Review Board of Pusan National University Hospital. For isolation of hASCs, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of collagenase type I suspension (1 g/liter of HBSS buffer with 1% bovine serum albumin) for 60 min at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugation at $300 \times g$ for 5 min. The cell pellet was resuspended in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cells were plated in tissue culture dishes at 3,500 cells/cm^2. The primary hASCs were cultured for 4-5 days until they reached confluence and were defined as passage "0". The passage number of hASCs used in these experiments was 3-10.

Tube formation assay

Human umbilical vein endothelial cells (HUVECs, Lonza, Basel, Switzerland) were maintained in a 37°C, 5% CO_2 humidified environmental chamber. The cells were grown in an Endothelial Growth Medium-2 bullet kit (Lonza) according to the manufacturer's instructions. For tube formation assay of HUVECs, aliquots (250 μl) of growth factor-reduced Matrigel (10 mg protein/ml) were added into 24-well culture dishes and polymerized for 30 min at 37°C. HUVECs were trypsinized, resuspended in EGM-2 basal medium supplanted with 1% FBS, and plated onto a layer of Matrigel at a density of 1×10^5 cells/well. The cells were then exposed to cancer CM or culture supernatants from cancer CM-pretreated hASCs. After incubation of the Matrigel cultures at 37°C for 12 h, the cultures were photographed using an inverted microscope with a digital camera.

Enzyme-linked immunosorbent assay (ELISA)

Commercially available sandwich ELISA kits were used to evaluate the protein levels of SDF-1 and VEGF in the CM derived from hASCs. In brief, cells were seeded in wells of a 24-well culture plate at a density of 1×10^4 cells/well and cultured for 48 h to confluence in the growth medium. After treatment of the cells with serum-free medium supplemented with appropriate reagents, CM was collected and centrifuged at $15,000 \times g$ for 30 min to remove particulates. ELISA of the CM was carried out according to the manufacturer's protocol. The absorbance (450 nm) for each sample was analyzed by an ELISA reader and was interpolated with a standard curve.

Western blot analysis

Serum-starved hASCs were treated with appropriate conditions, washed with ice-cold PBS, and then lysed in lysis buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM PMSF, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1% Triton X-100, pH 7.4). Lysates were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and then stained with 0.1% Ponceau S solution (Sigma-Aldrich). After blocking with 5% nonfat milk, the membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with HRP-conjugated secondary antibodies using the Enhanced Chemiluminescence Western blotting system (Amersham Biosciences).

RhoA activation assay

A commercially available pull-down assay kit (Rho activation assay kit, Upstate Biotechnology, Lake Placid, NY) was used to measure the effect of LPA on Rho activity in hASCs. The cells were washed twice with α-MEM and incubated in fresh modified α-MEM without serum for 24 h. After treatment of the cells with LPA for the indicated times, the cells were lysed, and the activated Rho pull-down assay was performed according to the manufacturer's protocol. Protein concentration was determined prior to pull-down assay to equalize the total protein concentration of each treatment group.

Preparation of CM

Cells were seeded on 100 mm-diameter cell culture dishes and cultured in growth medium until reaching confluence. The cells were briefly rinsed twice with PBS, and then incubated with 10 ml of fresh, serum-free α-MEM for 48 h before collection of medium. The CM was centrifuged at 2,000 rpm for 10 min to remove cell debris, filtrated using 0.45-μm Millipore Ultrafree centrifugal filters (Millipore, Bedford, MA), and stored at -70°C for subsequent use.

Measurement of LPA concentration in CM

The LPA level was determined by a commercially available LPA assay kit (Universal LPA assay kit, Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions. In brief, each well of coated microtiter plate was blocked by adding blocking solution and washed with ice-cold PBS four times. Samples or LPA standards were mixed with biotinylated anti-LPA antibody and the mixtures (100 μl/well) were transferred to the microtiter plate. After incubation for 1 h at 4°C, the plate was washed with ice-cold PBS and streptavidin HRP solution was added to each well of the microtiter plate. After 1 h, the plate was washed, and then tetramethyl-benzidine substrate were added to each well and incubated for 4 min at room temperature. H_2SO_4 (1N) was added to each well to stop the reaction and the absorbance of the solution at 450 nm was determined by using a PowerWavex microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT).

RT-PCR analysis

Cells were treated as indicated, and total cellular RNA was extracted by the Trizol method (Invitrogen). For RT-PCR analysis, aliquots of 2 μg RNA were subjected to cDNA synthesis with 200 U of M-MLV reverse transcriptase (Invitrogen) and 0.5 μg of oligo (dT) 15 primer (Promega, Madison, WI). The cDNA in 2 μl of the reaction mixture was amplified with 0.5 U of GoTaq DNA polymerase (Promega) and 10 pmol each of sense and antisense primers as follows: GAPDH 5'-TCCATGACAACTTTGG-TATCG-3' (sense), 5'-TGTAGCCAAATTCGTTGTCA-3' (anti-sense); LPA_1 5'-TCTTCTGGGCCATTTTCAAC-3' (sense), 5'-TGCCTRAAGGTGGCGCTCAT-3' (antisense); myocardin 5'-TCCAACGGCTTCTACCACTT-3' (sense), 5'-CACCTTCTGCTTCTCCACCA-3' (anti-sense); MRTF-A 5'-ACCGTGACCAATAAGAATGC-3' (sense), 5'-CATCTGC-TGGCTTGAGGAAC-3' (anti-sense); α-SMA 5'-CCTGAC-TGAGCGTGGCTATT-3' (sense), 5'-GATGAAGGATGGCT-GGAACA-3' (anti-sense); h1-calponin 5'-GCCCAGAAGT-ATGACCACCA-3' (sense), 5'-TGATGAAGTTGCCGATGT-TC-3' (anti-sense). The thermal cycle profile was as follows: denaturation for 30 s at 95°C, annealing for 45 s at 52-58°C depending on the primers used, and extension for 45 s at 72°C. For semi-quantitative assessment of expression levels, each PCR reaction was carried out for 30 cycles. PCR products were size fractionated on 1.2% ethidium bromide/agarose gel and quantified under ultra-violet transillumination.

Transfection with siRNA

For siRNA experiments, hASCs were plated on 60 mm-diameter dishes. At 70% confluence, the cells were transfected with appropriate siRNAs using Lipofectamine™ 2000 reagent according to manufacturer's instructions (Invitrogen). LPA_1 siRNA duplexes were synthesized, desalted, and purified by Samchully Pharm. Co. Ltd. (Siheung, GyeongGi, Korea) as follows: LPA_1 5'-GGACU-UGGAAUCACUGUUUUU-3' (sense) and 5'-AAACAGUG-AUUCCAAGUCCUU-3'. Myocardin siRNA, MRTF-A siRNA, and nonspecific control siRNA were purchased from Dharmacon (Lafayette, CO). Briefly, LipofectamineTM 2000 reagent was incubated with serum-free medium for 10 min and respective siRNAs were then added into the mixtures. After incubation for 15 min at room temperature, the mixtures were diluted with serum-free medium and

added to each well to a final concentration of 100 nM. After incubation of hASCs with siRNAs for 6 h, the cells were cultured in growth medium for 24 h, and the expression levels of target genes were determined by Western blotting or RT-PCR analysis.

Short hairpin RNA (shRNA) lentivirus-mediated depletion of endogenous LPA$_1$

pLKO.1-puro lentiviral vectors expressing LPA$_1$ shRNA (TRCN0000011368) or nontarget control shRNA (SHC002) were purchased from Sigma-Aldrich. The functional sequence in the LPA$_1$ shRNA lentiviral vector is "CCGGCCTTCTGAAGACTGTGGTCATCTCGAGATGACC ACAGTCTTCAGAAGGTTTTT" to target the LPA$_1$ gene sequence (CCTTCTGAAGACTGTGGTCAT). To generate lentiviral particles, HEK293FT cells were co-transfected with the shRNA lentiviral plasmid (pLKO.1-puro) and ViraPower Lentiviral packaging mix (pLP1, pLP2, pLP-VSV-G; Invitrogen) using Lipofectamine 2000 (Invitrogen) and the culture supernatants containing lentivirus were harvested at 48 h after transfection. For lentiviral transduction, hASCs were treated with the shRNA-expressing lentivirus in the presence of 5 μg/ml polybrene (Sigma-Aldrich) and stable cell lines expressing shRNA were generated by selection with puromycin (5 μg/ml). To ensure shRNA-mediated silencing of LPA$_1$ expression, the mRNA levels of LPA$_1$ and GAPDH were determined by RT-PCR analysis.

Statistical analyses

The results of multiple observations are presented as mean $\pm$ SE. Student's *t* test were used to analyze differences between two groups. For multivariate data analysis, group differences were assessed with two-way ANOVA, followed by *post hoc* comparisons tested with Scheffes method.

Acknowledgments

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