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Granulocyte colony-stimulating factor induces *in vitro* lymphangiogenesis

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ABSTRACT

Granulocyte-colony stimulating factor (G-CSF) is reported to induce differentiation in cells of the monocyte lineage and angiogenesis in vascular endothelial cells, but its effects on lymphangiogenesis is uncertain. Here we examined the effects and the mechanisms of G-CSF-induced lymphangiogenesis using human lymphatic endothelial cells (hLECs). Our results showed that G-CSF induced capillary-like tube formation, migration and proliferation of hLECs in a dose- and time-dependent manner and enhanced sprouting of thoracic duct. G-CSF increased phosphorylation of Akt and ERK1/2 in hLECs. Supporting the observations, specific inhibitors of phosphatidylinositol 3′-kinase and MAPK suppressed the G-CSF-induced *in vitro* lymphangiogenesis and sprouting. Intraperitoneal administration of G-CSF to mice also stimulated peritoneal lymphangiogenesis. These findings suggest that G-CSF is a lymphangiogenic factor.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3, plays a critical role in differentiation of the precursor cells in the bone marrow into mature granulocyte and increases mobilization of hematopoietic stem cells from bone marrow [1,2]. Thus, a recombinant form of G-CSF has been used to treat the patient with anticancer chemotherapy-induced neutropenia [3]. In addition, G-CSF exhibits a protective effect on neuron cells, decreasing the lesion size in transient ischemic stroke [4–6]. The decreased volume of the ischemic lesion in G-CSF-treated patients has also been observed [7]. In fact, G-CSF receptor has been shown to be expressed in the penumbra area after an ischemic stroke [8] and spinal injury [9].

Accumulating evidence has indicated that G-CSF affects vascular endothelial cell functions and vascular angiogenesis [10]. Indeed, the G-CSF receptor expression on the surface of vascular endothelial cells has been demonstrated vascular endothelial cells express specific receptors for G-CSF on their surfaces [11,12]. G-CSF attenuates endothelial dysfunction after drug-eluting stent implantation [13]. G-CSF increases the number of proliferating and migratory murine and human microvascular endothelial cells [11,14]. Granulocyte–macrophage colony-stimulating factor (GM-CSF) also increases angiogenic activity in chick chorioallantoic membrane and sprouting of the aortic ring *in ex-vivo* [15,16]. In addition, the angiogiogenic effect of G-CSF appears to be linked to failure of anti-VEGF-A therapy; G-CSF-induced angiogenesis is associated with refractoriness to anti-VEGF-A therapy in a murine tumor model [17]. It has also been demonstrated that G-CSF treatment after myocardial ischemia increases new blood vessel formation while decreasing myocardial apoptosis [18].

Lymphangiogenesis, the increase of lymphatic vessels, is linked to several pathologic conditions including inflammation, tumor metastasis, fibrosis and lymphedema [19–22]. The increased body of evidence has suggested that the regulation of lymphangiogenesis opens a new avenue to ameliorate these conditions. Therefore, identifying new lymphangiogenic factor may provide new insight into lymphangiogenesis-associated conditions. Recently, it has been reported that human cytomegalovirus promotes lymphangiogenesis through secretion of GM-CSF from infected cells [23]. However, although G-CSF is known as an angiogenic factor, there are few reports on a lymphangiogenic effect of G-CSF.

In this study, we investigated whether G-CSF displays lymphangiogenesis using lymphatic endothelial cells. The results showed that treatment with G-CSF increases capillary-like tube formation, proliferation, and migration of the cells. Moreover, sprouting of mouse thoracic duct and peritoneal lymphangiogenesis were also stimulated by G-CSF.

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2. Materials and methods

2.1. Animal experiments: peritoneal lymphangiogenesis

The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chonbuk National University. For G-CSF-induced peritoneal lymphangiogenesis, the G-CSF (500 μg/kg in 200 μL of phosphate-buffered saline) was injected into the peritoneal cavity daily for 7 d [24].

2.2. Cell culture and chemicals

Human lymphatic endothelial cells (hLECs; Lonza, Basel, Switzerland) were incubated in EBM-2 medium (Lonza) with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS) at 37 °C in a 5% CO_2/95% O_2. The G-CSF (NEUPOGEN®) was purchased from Amgen Inc. (Thousand Oaks, CA). Recombinant human VEGF-A 165 was provided from R&D Systems (Minneapolis, MN). Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor, PD98059, the phosphatidylinositol 3′-kinase (PI3K) inhibitor LY294002, wortmannin, gelatin, antibiotics and antimycotics were purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Migration assay

The migration assay with hLECs was performed using a modified Boyden chamber (NeuroProbe, Cabin John, MD) [24].

2.4. Capillary-like tube formation assay

An *in vitro* capillary-like tube formation assay was performed using Matrigel (BD Biosciences, Bedford, MA) [19].

2.5. Cell proliferation by XTT assay

Proliferation of hLECs was evaluated using Cell Proliferation Kit II (XTT; Roche, Mannheim, Germany) after a 48 h-treatment with G-CSF at 10, 50 or 100 ng/mL according to the manufacturer's protocol.

2.6. Three-dimensional lymphatic ring assay

Thoracic ducts were harvested as described previously [24]. G-CSF (50 or 100 ng/mL), control buffer, or recombinant VEGF-A (R&D Systems) was added to the culture medium.

2.7. Reverse-transcription polymerase chain reaction (RT-PCR) of G-CSF receptor

The RT-PCR of total RNA isolated from hLEC was performed as described previously (Supplementary Table 1) [24].

2.8. Statistical analysis

Data were presented as the mean ± S.D. Groups were compared using Student's unpaired *t* test. A statistical value of $p < 0.05$ was considered significant.

3. Results

3.1. G-CSF increases capillary-like tube formation, migration, and proliferation of hLECs

Firstly, expression of G-CSF receptor on hLECs was examined by RT-PCR. As shown Fig. 1A, G-CSF receptor mRNA was expressed in

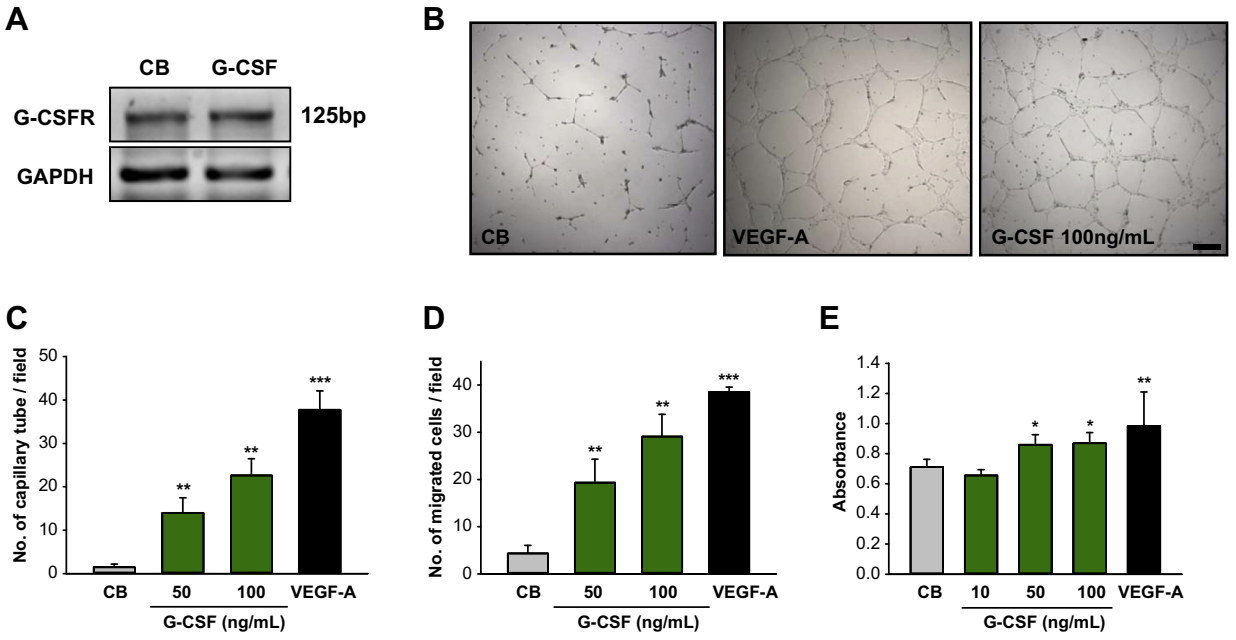

Fig. 1. G-CSF receptor is expressed on hLECs and G-CSF promotes the formation of capillary-like tube structures, migration and proliferation of hLECs. (A) RT-PCR analysis of mRNA expression of G-CSF receptor in hLECs with or without G-CSF treatment. (B) Representative phase-contrast photographs of capillary-like tube formation in Matrigel. Capillary-like tube formation was assayed in a 3-dimensional matrix Matrigel after incubation for 16 h. Bar scale = 50 μm. (C) Quantification of capillary-like tube formation. Tube formation was quantified by the number of tubes using phase-contrast microscope. (D) Numbers of migrated cells after stimulation with G-CSF. Control buffer (CB), G-CSF (50 and 100 ng/mL), or VEGF-A (30 ng/mL) in EBM-2 containing 0.5% bovine serum albumin was placed in the bottom wells of the chamber. Cells that migrated through to the lower chamber were stained with Diff-Quick solution and counted the number of migrated cells. Bars represent means ± S.D. from 4 independent experiments. (E) Quantification of proliferating cells after stimulation with G-CSF. G-CSF (50 and 100 ng/mL) or VEGF-A (30 ng/mL) was added to serum-starved hLECs for 48 h and hLEC proliferation was measured with an XTT assay. Bars represent means ± S.D. from 5 independent experiments. $^{*}P < 0.05$ versus CB; $^{**}P < 0.01$ versus CB; and $^{***}P < 0.001$ versus CB.

hLECs. To evaluate *in vitro* lymphangiogenic effect of G-CSF, we performed capillary-like tube formation, migration, and proliferation assay. Treatment of hLECs with G-CSF increased tube formation in a dose-dependent manner (Figs. 1B and C). At 100 ng/mL of G-CSF, the capillary-like tube formation was approximately 15.6-fold greater than that of control buffer-treated hLECs. G-CSF increased the number of migrating hLECs in a dose-dependent manner (Fig. 1D). At 100 ng/mL of G-CSF, the migration of hLECs was approximately 7.5-fold greater than that of control buffer-treated hLECs. In addition, G-CSF significantly increased proliferation of hLECs compared to that of control (Fig. 1E). VEGF-A was used as a positive control.

3.2. G-CSF increases phosphorylation of MAPK and Akt in hLECs

The MAPK- and Akt-dependent signaling pathways are involved in lymphangiogenesis [24], and G-CSF is known to activate Ras/(MAPK) [25] and PI3K/protein kinase B (Akt) [26]. Therefore, we examined the phosphorylation of ERK1/2 (p44/p42 MAPK) and Akt. G-CSF significantly increased ERK1/2 phosphorylation at 10 min after G-CSF treatment and then reduced up to the level of control buffer (Fig. 2A). G-CSF (10, 50 and 100 ng/mL) significantly increased ERK1/2 phosphorylation, reaching maximum phosphorylation at 10 ng/mL (Fig. 2B). Pretreatment with a MEK inhibitor, PD98059, blocked G-CSF-induced ERK1/2 phosphorylation. Treatment of hLECs with G-CSF (100 ng/mL) increased phosphorylation of Akt (1.3-fold over control) at 20 min and then reduced (Fig. 2C). G-CSF also increased Akt phosphorylation in a dose-dependent manner (Fig. 2D). Pretreatment with a PI3K inhibitor, LY294002 or wortmannin, significantly decreased the G-CSF-induced Akt phosphorylation.

3.3. PI3K- and MEK-dependent pathways are involved in G-CSF-induced in vitro lymphangiogenesis

We evaluated whether PI3K- and MEK-dependent pathways are linked to G-CSF-induced capillary tube formation, migration, and proliferation during *in vitro* lymphangiogenesis. Inhibition of PI3K or MEK pathway with LY294002, wortmannin or PD98059 significantly decreased G-CSF-induced capillary tube formation, migration, and proliferation of hLECs (Fig. 3A–C).

3.4. G-CSF increases sprouting of a lymphatic ring

To assess the spreading of mouse lymphatic endothelial cells (LEC) from mouse thoracic ducts, a lymphatic ring assay was performed. After 7 d of treatment with G-CSF, the number of LEC sprouts had increased approximately 21.6-fold (Fig. 4A and B).

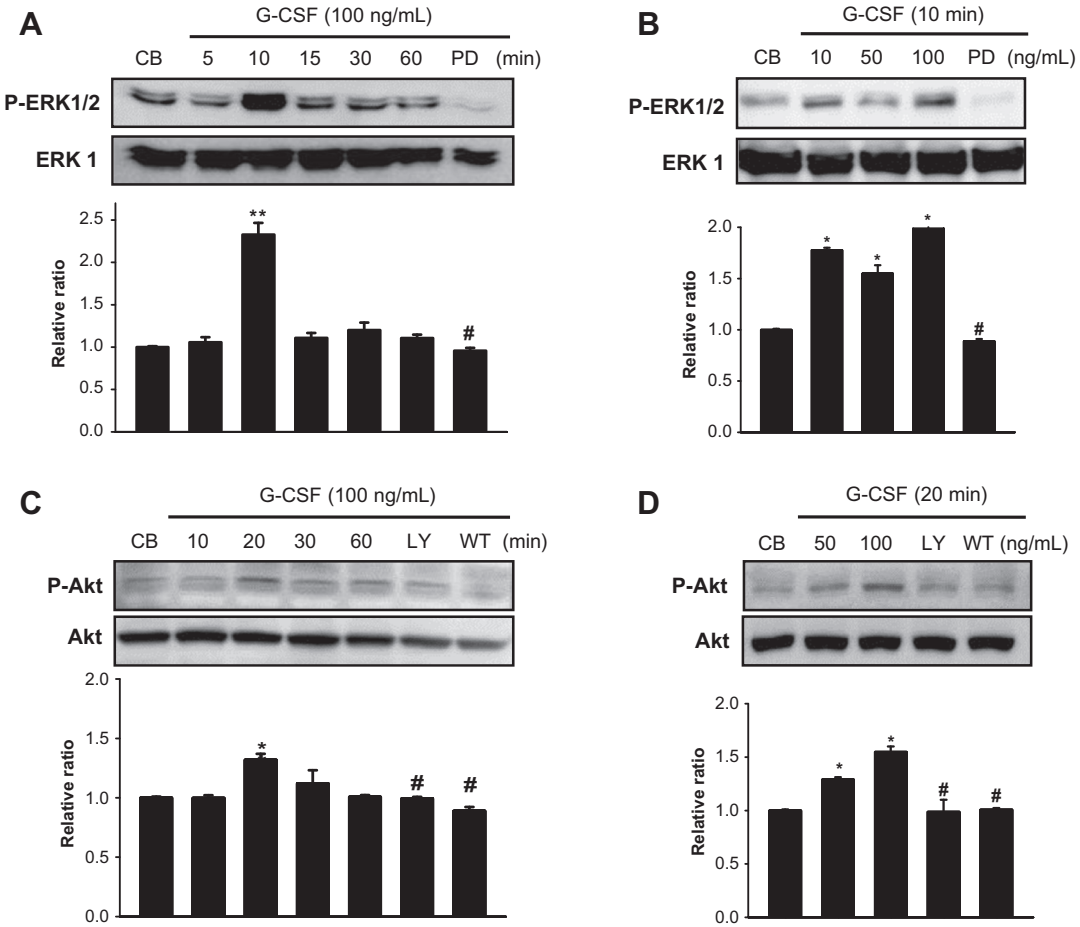

Fig. 2. G-CSF increases phosphorylation of ERK1/2 and Akt in hLECs. (A–D) Western blot analyses of phosphorylated ERK1/2 (P-ERK1/2) and phosphorylated Akt (P-Akt) in hLECs. Cells were incubated with G-CSF (100 ng/mL) in the presence or absence of PD98059 (50 mmol/L), LY294002 (10 mmol/L), or wortmannin (30 nmol/L) at the indicated times (A and C) and concentrations (B and D). Each lane contained 20 μg of total protein from the cell lysates. Blots were probed with an anti–phospho-ERK1/2 antibody or anti–phospho-Akt (Ser473) antibody. The membranes were stripped and reprobed with an anti–ERK1 or anti–Akt antibody, respectively. Densitometric analyses are presented as the relative ratio of phospho-ERK1/2 to ERK1 or phospho-Akt to Akt. The relative ratio to control buffer (CB) is arbitrarily presented as 1. Numbers represent the means ± S.D. from 3 independent experiments. Note that treatment with a MEK inhibitor, PD98059, blocked G-CSF-induced ERK1/2 phosphorylation at 10 min and 100 ng/mL, and treatment with a PI3K inhibitor LY294002 or wortmannin significantly decreased the G-CSF-induced Akt phosphorylation at 20 min in C and 100 ng/mL in D. $^{*}P < 0.05$ versus CB; $^{**}P < 0.01$ versus CB; $^{\#}P < 0.05$ versus the 10-min time point for p-ERK1/2 or the 20-min time point for p-Akt or 100 ng/mL of G-CSF.

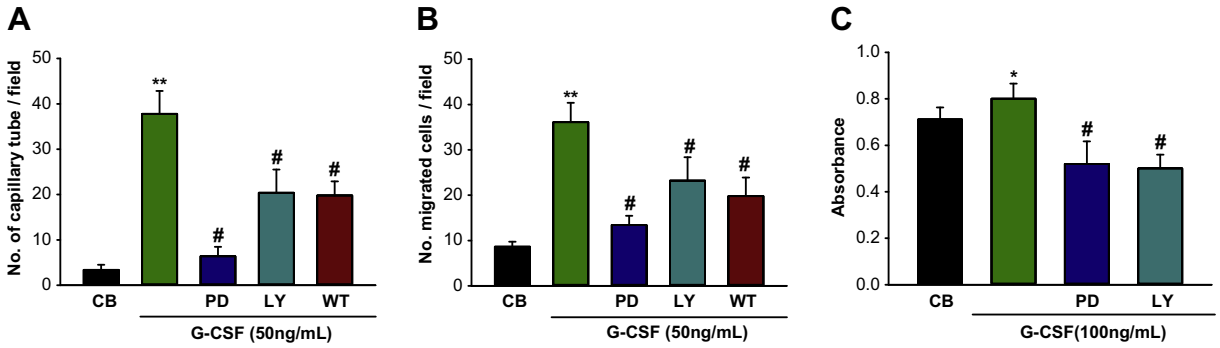

Fig. 3. G-CSF induces *in vitro* lymphangiogenesis through the PI3 K and ERK1/2 signal pathway. After incubation with control buffer (CB), G-CSF, G-CSF plus LY294002 (10 μmol/L), G-CSF plus wortmannin (30 nmol/L), and G-CSF plus PD98059 (50 μmol/L), the capillary-like tube formation (A), migration (B) and proliferation (C) of hLECs were assayed. Bars represent the means ± S.D. from 3 independent experiments. $^{*}P < 0.05$ versus CB; $^{**}P < 0.01$ versus CB; $^{\#}P < 0.05$ versus G-CSF+CB.

3.5. Intraperitoneal administration of G-CSF produces peritoneal lymphangiogenesis

To evaluate the effect of G-CSF on peritoneal lymphangiogenesis, we administered G-CSF to mice intraperitoneally once daily for up to 7 d and stained the diaphragm with an anti–LYVE-1 antibody after harvest of the diaphragm. Immunohistochemistry of LYVE-1 from mice treated with control buffer revealed the typical distribution of lymphatic vessels on the peritoneal side of the diaphragm. The densities of LYVE-1–positive lymphatic vessels on the

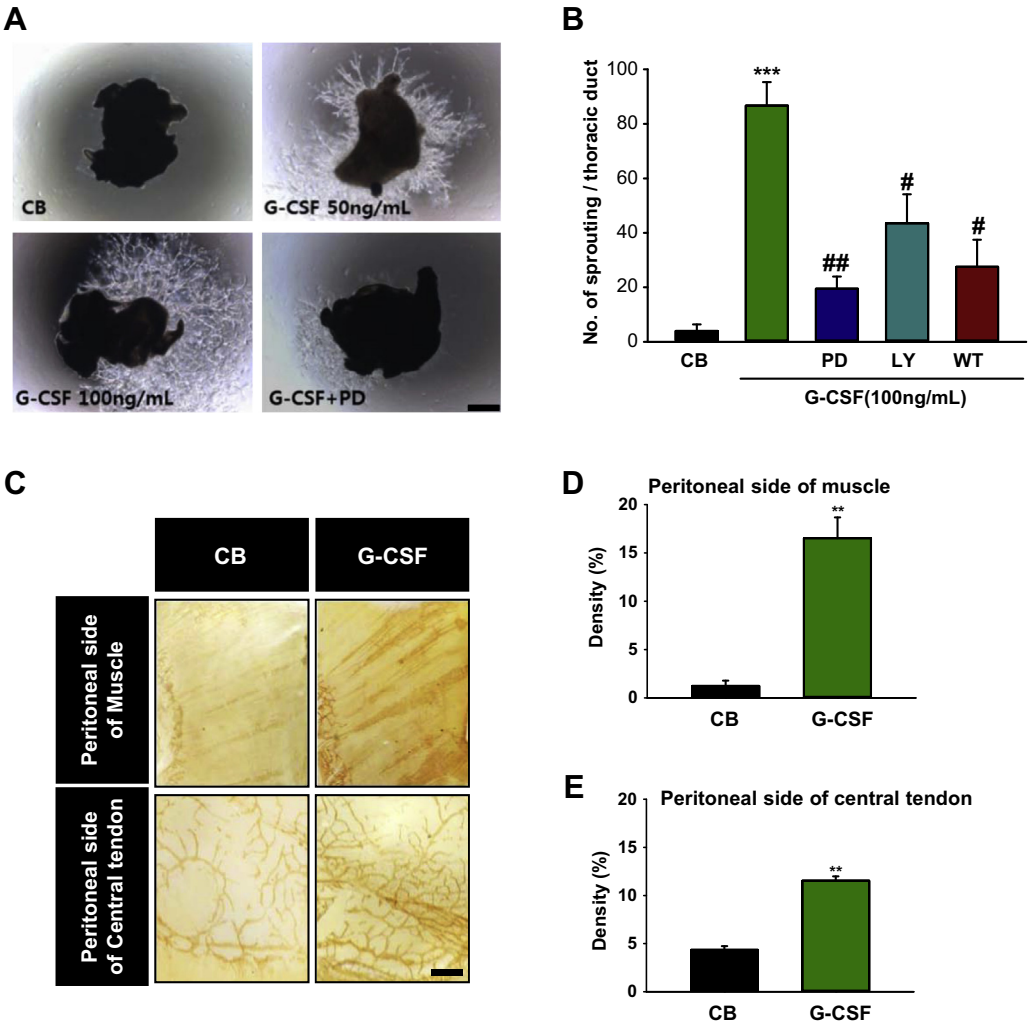

Fig. 4. G-CSF induces sprouting from thoracic duct and peritoneal lymphangiogenesis on the diaphragm side. (A) Phase-contrast photomicrographs of sprouting of mouse thoracic duct. Excised rings of mouse thoracic ducts were implanted into Matrigel and incubated with control buffer (CB) alone, G-CSF (50 or 100 ng/mL), G-CSF plus LY294002 (10 μmol/L), G-CSF plus wortmannin (30 nmol/L) and G-CSF plus PD98059 (50 μmol/L) as indicated. Bar scale = 50 μm. (B) Quantification of the sprouting of the thoracic duct. Total LEC sprouts from thoracic ducts were counted after 7 d. Data are means ± S.D. from 4 independent experiments. (C) Images of LYVE-1-positive lymphatic vessels on the peritoneal side of the diaphragm muscle and central tendon. Diaphragms were stained for LYVE-1 (brown). Bar scale = 50 μm. (D) The percentages of LYVE-1-positive lymphatic vessels in the diphragam muscle and central tendon were measured and the results are expressed as a percentage ($n = 4$). Bars represent the mean ± S.D. $^{**}P < 0.01$ versus CB; $^{***}P < 0.001$ versus CB; $^{\#}P < 0.05$ versus G-CSF; $^{\#\#}P < 0.01$ versus G-CSF.

peritoneal side of the diaphragm muscle and in the central tendon were higher in G-CSF-treated mice than that of the control mice (Fig. 4C–E).

4. Discussion

In this study, we have demonstrated that a G-CSF receptor is expressed in hLECs and that G-CSF induces lymphangiogenesis through the Akt- and ERK1/2-dependent signaling pathways.

The specific receptor for G-CSF is expressed in myeloid progenitor cells and is involved in the regulation of granulopoiesis [27,28]. The G-CSF receptor is also expressed on the surface of vascular endothelial cells and has an effect on vascular endothelial cell function and angiogenesis [11,12]. In this study, our data showed that hLECs express G-CSF receptor mRNA in and suggest that G-CS may play a role in hLECs through the G-CSF receptor (Fig. 1A).

G-CSF has an angiogenic effect in a rabbit model of chronic myocardial ischemia [18]. Immunohistochemistry data have shown that the density of von Willebrand factor-positive vascular endothelial cells in the G-CSF-treated group is significantly higher than the control group in ischemic myocardium. In addition, the apoptotic index in the ischemic myocardium is suppressed in the G-CSF group compared with the control group. These results have indicated that G-CSF may exhibit a direct angiogenic effect on microvascular endothelial cells in chronic ischemic myocardium. However, a lymphangiogenic role of G-CSF is unknown. In the present study, our results have shown that G-CSF increases capillary formation, proliferation, and migration of hLECs. Furthermore, G-CSF increased sprouting of mouse thoracic duct *in ex-vivo* and peritoneal lymphangiogenesis *in vivo*. To our best knowledge, our data may be the first finding that G-CSF acts as a novel lymphangiogenic factor.

The signaling pathways associated with the biological responses of G-CSF include those of Ras/(MAPK) [25], PI3K/Akt [26], and Janus kinase/signal transducer and activator of transcription [15,29]. Treatment with G-CSF increases dose-dependent mechanical hyperalgesia, and MAPK and PI3K inhibitors suppress G-CSF-induced pain [30]. GM-CSF also inhibits neutrophil apoptosis by activation of the ERK and PI3K pathway [31]. Previously, we have demonstrated that the signal pathways associated with erythropoietin-induced lymphangiogenesis were the Ras/(MAPK) and PI3K/Akt pathways in hLEC [24]. In same line with our previous findings, our present data indicated that G-CSF induces lymphangiogenesis through activation of ERK1/2 and Akt in hLECs.

In summary, our results have revealed that G-CSF increases migration, capillary-like tube formation, and proliferation of hLECs as well as sprouting of thoracic duct via PI3K/Akt and ERK1/2 signaling pathway. Administration of G-CSF also induces peritoneal lymphangiogenesis. These data strongly suggest that G-CSF can be a new lymphangiogenic factor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.062.

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