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Research Article

The cooperative role of S1P3 with LYVE-1 in LMW-HA-induced lymphangiogenesis

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ABSTRACT

Lymphangiogenesis, the formation of new lymph vessels, plays a significant role in the development and metastasis of various cancers. We and others have demonstrated that low molecular weight hyaluronan (LMW-HA) promotes lymphangiogenesis. However, the underlying mechanisms are poorly defined. In this study, using immunofluorescence and co-immunoprecipitation, we found that LMW-HA increased the colocalization of lymphatic vessel endothelial HA receptor (LYVE-1) and sphingosine 1-phosphate receptor (S1P3) at the cell surface. Silencing of either LYVE-1 or S1P3 decreased LMW-HA-mediated tube formation in lymphatic endothelial cells (LECs). Furthermore, silencing of either LYVE-1 or S1P3 significantly inhibited LMW-HA-induced tyrosine phosphorylation of Src kinase and extracellular signal-regulated kinase (ERK1/2). In summary, these results suggest that S1P3 and LYVE-1 may cooperate to play a role in LMW-HA-mediated lymphangiogenesis. This interaction may provide a useful target for the intervention of lymphangiogenesis-associated tumor progression.

1. Introduction

Lymphangiogenesis, the generation of new lymphatic vessels, is considered an important step in cancer dissemination and metastasis [1,2]. This process involves extracellular interactions between the inner endothelial layer and the surrounding matrix. Clinical and experimental evidence suggest that lymphangiogenesis is associated with poor prognosis in cancer patients [3]. Understanding the molecular mechanisms that regulate lymphangiogenesis could have a significant impact on the development of novel treatment strategies.

Hyaluronan (HA) is a linear polymer that belongs to the glycosaminoglycan (GAG) family, which comprises the major fraction of carbohydrates in the extracellular matrix (ECM) [4]. Studies on histological sections from various types of tumors revealed that tumor ECM is rich in HA. Ectopic expression of HA is associated with cancer malignancy and aggressiveness in breast, stomach and colon carcinomas [5]. HA molecules have a large variability in length and can reach up to 10^7 Da in size. The molecular weight of HA can affect its biological characteristics. High molecular weight HA (HMW-HA) exerts anti-angiogenic and immunosuppressive effects. By contrast, low molecular weight HA (LMW-HA), which is synthesized or generated by either hyaluronidase-mediated degradation or hydrolysis of native HA under pathological conditions, plays a more active biological role and is involved in cell growth, angiogenesis and migration [6,7]. Recently, increasing evidence has shown that LMW-HA plays a significant role in lymphangiogenesis [8,9]; however, the precise molecular mechanism remains unclear. Therefore, it is of great significance to explore the exact molecular mechanism underlying LMW-HA-mediated lymphangiogenesis.

Sphingosine 1-phosphate receptor (S1P3), formerly known as endothelial differentiation gene-3 (Edg-3), regulates endothelial cell functions, such as angiogenesis and barrier function [10]. S1P3-mediated activation of extracellular signal-regulated kinase (ERK1/2) is involved in S1P3 ligand-induced angiogenesis [11–13]. As reported previously, the interaction between S1P3 and CD44 (vascular endothelial HA receptor) plays a role in LMW-HA-mediated barrier disruption in vascular endothelial cells [14]. Moreover, the barrier function of vascular endothelial cells was

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shown to be related to angiogenesis [15]. LYVE-1, a recently discovered homolog of the CD44 glycoprotein, has an overall homology of 41% with CD44 and is largely restricted to the endothelial cells that line lymphatic vessels as a lymph-specific receptor for HA [17]. This discovery raised the interesting possibility that S1P3 may interact with LYVE-1 to play a role in LMW-HA-mediated lymphangiogenesis.

In this study, we examined the different effects of HMW-HA and LMW-HA on lymphangiogenesis, including the proliferation, migration and tube formation of lymphatic endothelial cells (LECs). To further clarify the mechanism underlying LMW-HA induced lymphangiogenesis, we explored the association of S1P3 with LYVE-1 in LECs and silenced LYVE-1 and S1P3 to investigate their roles in LMW-HA-induced LEC tube formation and intercellular signaling transduction.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit polyclonal anti-LYVE-1 receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal anti-S1P3 receptor was purchased from Abcam (Cambridge, MA, USA). Rabbit monoclonal anti-phospho-Src and anti-phospho-ERK1/2 (Tyr202/Tyr204) were obtained from Cell Signaling Technology (Beverly, MA, USA). Native HMW-HA was obtained from Sigma-Aldrich (St. Louis, MO). LMW-HA was prepared as described previously [16]. LMW-HA was prepared as a mixed fraction with an average molecular weight of 2.5×10^6 and was composed of 3–10 disaccharide units that were fractionated from testicular hyaluronidase type 1-S (Sigma-Aldrich) digests of hyaluronan sodium salt (Sigma-Aldrich).

2.2. Cell cultures

The murine endothelial cell line SVEC4-10 (American Type Culture Collection), which was recently characterized as lymphatic endothelial cells [17], was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) in a humid atmosphere at 37 °C under 5% CO_2.

2.3. Cell proliferation assay

SVEC4-10 cells were seeded in 96-well plates at a density of 4×10^3 cells/well and cultured in 100 μL of DMEM with 10% FBS overnight at 37 °C under 5% CO_2. After the medium was removed, the cells were treated with DMEM plus 10 μg/ml HMW-HA, 10 μg/ml LMW-HA, or medium only for 48 h at 37 °C under 5% CO_2, then 20 μl methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent was added to each well and the plates were incubated for 4 h at 37 °C. The absorbance was measured using a microplate reader (Bio-Rad, Model 550) at a wavelength of 492 nm.

2.4. Transwell monolayer permeability assay

The migration activity of SVEC4-10 cells was evaluated using a transwell system (Corning Costar, MA, USA) that was composed of 8 μm polycarbonate filter inserts in 24-well plates. Briefly, serum-starved cells were trypsin-harvested in DMEM. Then, 1×10^4 SVEC4-10 cells were suspended in 200 μL of serum-free DMEM and seeded in the upper chambers, and the lower chambers were filled with 700 μl of DMEM with 10% FBS containing 10 μg/ml HMW-HA, 10 μg/ml LMW-HA, or medium only. After incubation for 24 h at 37 °C under 5% CO_2, cells on the upper surface of the filter were removed using a cotton swab and the membrane between the two compartments was fixed and stained with 1% crystal violet. Cells on the lower surface of the filter were photographed using brightfield optics with a × 100 objective lens (Nikon, Tokyo, Japan) and counted.

2.5. Tube formation assay

A matrigel-based tube formation assay was performed as previously described. Each well of a 96-well plate was coated with 100 μl of Matrigel (BD Biosciences, Bedford, MA, USA), which was allowed to polymerize for 30 min at 37 °C. Serum-starved SVEC4-10 cells (2×10^4) were dispensed into each well in 100 μL of EBM-2, with 10 μg/ml HMW-HA, 10 μg/ml LMW-HA, or medium only. After incubation for 6 h at 37 °C under 5% CO_2, images of the tube formation were captured using microscopy, and the relative tube length was calculated by normalizing the results to the control group. Each treatment was performed in triplicate and experiments were repeated three times. The results are expressed as the relative tube length per treatment.

2.6. Immunofluorescence staining

SVEC4-10 cells grown on coverslips for 2 days (50–60% confluence) were treated with 10 μg/ml HMW-HA, 10 μg/ml LMW-HA or medium only for 30 min in serum-free DMEM. After treatment, cells were washed with PBS twice and fixed with methanol at −20 °C for 10 min. Then, cells were washed with PBS and blocked with 1% bovine serum albumin in PBS at room temperature for 1 h. Next, cells were incubated overnight with anti-LYVE-1 antibody and anti-S1P3 antibody at 1:100 and 1:200 dilution, respectively. After extensive washing, cells were incubated with Alexa Fluxo 488-conjugated donkey anti-rabbit IgG and Alexa Fluxo 594-conjugated donkey anti-goat IgG secondary antibodies at 1:100 dilution for 2 h at room temperature. Nuclei were stained with DAPI for 10 min at room temperature. Images were acquired using a confocal microscope (Nikon, Tokyo, Japan).

2.7. Immunoprecipitation and immunoblotting

Cells were lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, 5 mM NaF and 5 mM Na_3VO_4. The samples were then either immunoprecipitated with anti-LYVE-1 or not immunoprecipitated followed by SDS-PAGE in 12% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with specific primary and secondary antibodies. Immunoreactive bands were developed using a chemiluminescence assay (Pierce) and visualized using Image pro-Plus 6.0 software.

2.8. Sirna transfection

Transfections of cells with S1P3, LYVE-1, and scramble siRNA were performed according to the manufacturer's protocol (Santa Cruz Biotechnology). Briefly, SVEC4-10 cells were grown on six-well tissue culture plates to 60–80% confluence. Cells were then washed twice with transfection medium (sc-36868). Aliquots of 0.2 ml of transfection medium containing scramble siRNA (sc-37007), S1P3 siRNA (sc-35262), and LYVE-1 siRNA (sc-42902) and 8 μL of transfection reagent (sc-29528) were incubated at room temperature for 30 min before being added to the cells. Cells were used for experiments 48 h after transfection.

2.9. Statistical analysis

Analyses were performed using the statistical software SPSS13.0. Differences were considered statistically significant at

$P < 0.01$. At least three independent experiments were performed for each assay.

3. Results

3.1. LMW-HA increased LEC proliferation

LECs were treated with HMW-HA or LMW-HA at various concentrations, ranging from 0 to 40 μg/ml. The most significant effect of LMW-HA on proliferation occurred at 10 μg/ml, and there was no significant effect of HMW-HA on LEC proliferation compared with control (Fig. 1).

3.2. LMW-HA promoted LEC migration

Based on the optimal concentration in promoting SVEC4-10 proliferation, 10 μg/ml LMW-HA was used for further experiments. The number of migrated cells in groups treated with 10 μg/ml LMW-HA was significantly increased ($P < 0.01$), but HMW-HA had no significant effect on cell migration (Fig. 2).

3.3. LMW-HA enhanced LEC tube formation

Tube formation of LECs was significantly enhanced by 10 μg/ml LMW-HA ($P < 0.01$). This is consistent with previous reports [5] that found LMW-HA could stimulate lymphangiogenesis. HMW-HA did not exert significant effects on tube formation (Fig. 3).

3.4. Effects of HMW-HA and LMW-HA on the phosphorylation of Src kinase and ERK1/2 in LECs

Src kinase and its downstream molecular mediator ERK1/2 are involved in regulating cell growth, differentiation, and migration [18]. The phosphorylation of Src kinase and ERK1/2 is involved in LMW-HA-induced angiogenesis [19]. To understand the intracellular mechanism through which LMW-HA promoted lymphangiogenesis, we studied the effect of LMW-HA on the activation of Src kinase and ERK1/2 signaling in LECs. The results of western blotting showed that LMW-HA induced the phosphorylation of Src kinase and ERK1/2 in a time-dependent manner in LECs. Typically, LMW-HA stimulated a strong and sustained phosphorylation of both Src kinase and ERK1/2 with 30 min of incubation in LECs. In the meanwhile, the expressions of total Src kinase and ERK1/2 remained unaltered from 0 to 60 min (Fig. 4A). We further analyzed the effects of HMW-HA and LMW-HA on phosphorylation of Src kinase and ERK1/2 with 30 min of incubation in LECs. We found that LMW-HA significantly increased the protein levels of phosphorylated Src kinase and ERK1/2 compared with control ($P < 0.01$). In contrast, HMW-HA presented no significant effects on phosphorylation of Src kinase and ERK1/2 (Fig. 4B).

3.5. LMW-HA increased S1P3 and LYVE-1 colocalization in LECs

A previous study showed that LMW-HA interacted with CD44 to induce angiogenesis [20,21]. Moreover, LMW-HA induced an association of S1P3 with CD44 in lipid raft microdomains [21]. Thus, we explored the effects of LMW-HA on the localization of S1P3 and LYVE-1 in LECs. Using immunofluorescence assays, we showed that in the absence of treatment, LYVE-1 colocalized with S1P3 on the cell membrane and in the cytoplasm (Fig. 5A–E). Treatment with LMW-HA resulted in an increase in the colocalization of LYVE-1 and S1P3 (Fig. 5A–O). Using co-immunoprecipitation, we found that the association between LYVE-1 and S1P3 was significantly increased upon LMW-HA treatment, whereas the association remained unchanged with HMW-HA treatment (Fig. 5A).

3.6. Role of LYVE-1 and S1P3 in LMW-HA-induced lymphangiogenesis

Previous studies have shown that the interaction of LMW-HA and its receptor induces tube formation and tyrosine phosphorylation of Src kinase and ERK1/2 in vascular endothelial cells [22]. Therefore, we hypothesized that LYVE-1 and S1P3 may have roles in LMW-HA-induced tube formation and activation of Src kinase and ERK1/2 signaling pathways. Using western blot analysis, we found that treating LECs with either LYVE-1 or S1P3 siRNA significantly reduced the protein levels of LYVE-1 and S1P3, respectively (Fig. 7A and C). Silencing of either LYVE-1 or S1P3 significantly inhibited LMW-HA-mediated LEC tube formation (Fig. 6A) and the activation of Src kinase and ERK1/2 signaling (Fig. 7B and D).

4. Discussion

There is growing evidence that lymphangiogenesis is one of the most important prognostic factors in various types of cancer [23]. Clinical evidence suggests that the dissemination of malignant tumor cells to regional lymph nodes via lymphangiogenesis is important in tumor metastasis; however, the molecular mechanisms regulating lymphangiogenesis are largely unclear [24].

Although previous studies have reported that VEGF-C and PDGF-BB are the most potent lymphangiogenic growth factors,

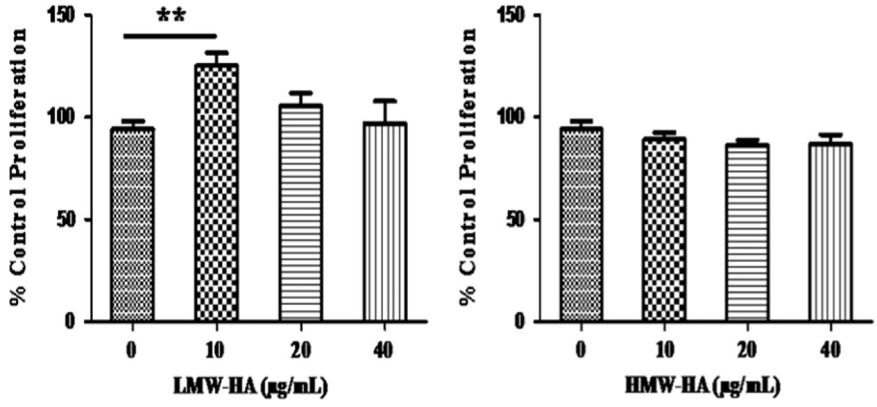

Fig. 1. Effects of HMW-HA and LMW-HA on LEC proliferation. SVEC4-10 cells were treated with 10, 20, and 40 μg/ml HMW-HA, LMW-HA or medium only for 48 h at 37 °C and 5% CO_2 in 96-well culture plates. Cell proliferation was examined using MTT assays. Each assay was prepared in triplicate and repeated at least five times. (**$P < 0.01$).

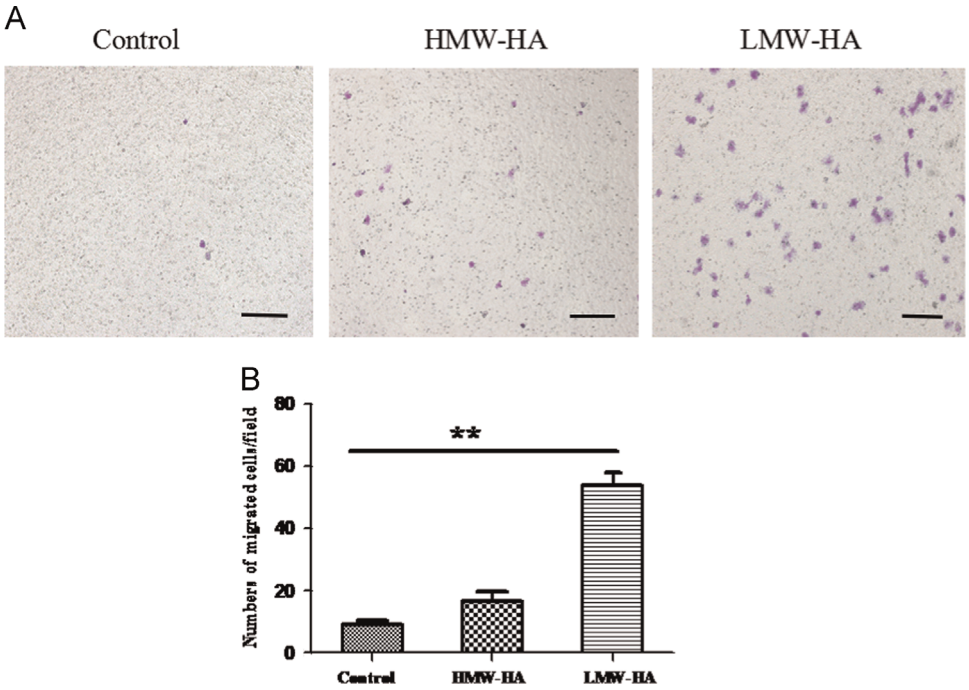

Fig. 2. LMW-HA stimulated LEC migration. SVEC4-10 cell migration was evaluated using transwell assays. Cells were seeded on the upper chambers, and medium containing 10 μg/ml HMW-HA, 10 μg/ml LMW-HA or medium only was added to the lower chambers. After 24 h of incubation, cells attached to the lower membrane were stained with 0.1% crystal violet (× 100 magnification). A, Representative photographs of SVEC4-10 migration (scale bar, 100 μm). B, The migrated cells in each field were counted (× 100 magnification, 10 random fields). Each assay was prepared in triplicate and repeated at least five times. (**$P < 0.01$).

recent studies showed that the polysaccharide fragment LMW-HA, found in the extracellular matrix, could also promote lymphangiogenesis [25,26]. Under various pathologic conditions, LMW-HA rapidly accumulates in the affected interstitial space and appears to function as an endogenous "danger signal" related to tumor metastasis [27–29]. LMW-HA-induced lymphangiogenesis may be an important mechanism in tumor metastasis. Therefore, knowledge of the molecular mechanisms underlying LMW-HA-

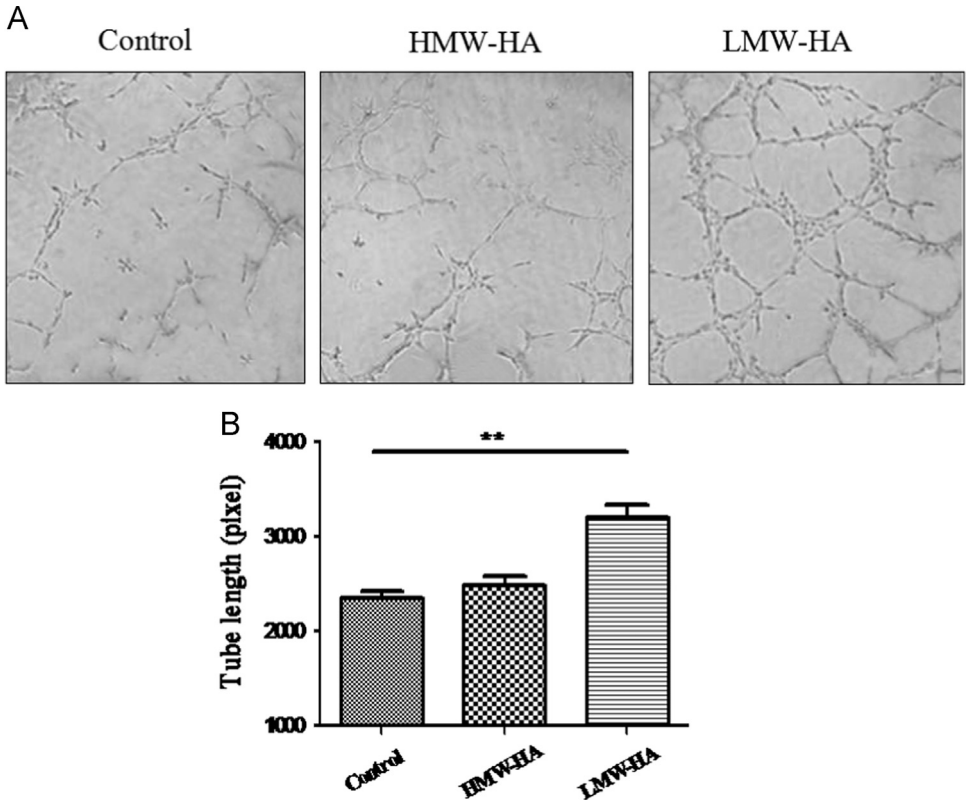

Fig. 3. LMW-HA promoted tube formation of LECs. Tube formation assays were performed as described in the materials and methods section. A, Representative photographs of SVEC4-10 cell tube formation on Matrigel after 6 h of treatment with 10 μg/ml HMW-HA or 10 μg/ml LMW-HA. B, The total tube formation length was measured and is shown as pixel length per field. Each treatment was performed in triplicate and experiments were repeated three times. (**$P < 0.01$).

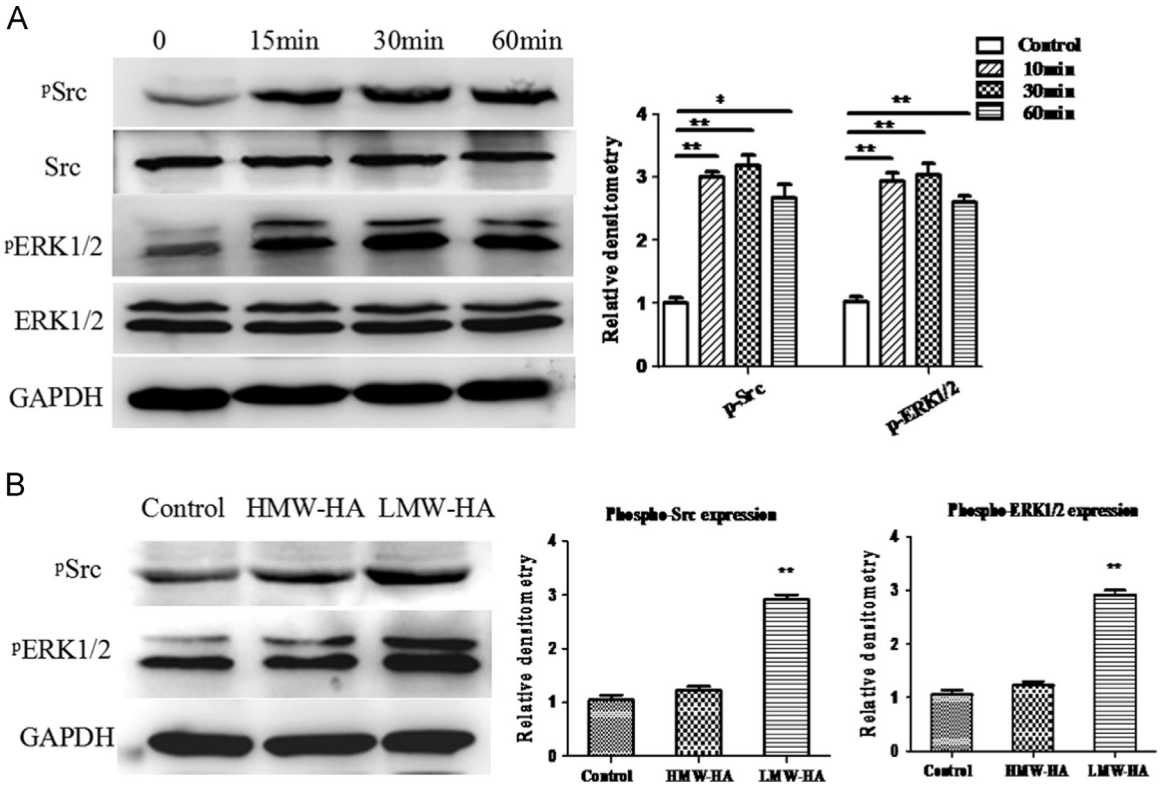

Fig. 4. Src kinase and ERK1/2 were activated following LMW-HA treatment of LECs. A, SVEC4-10 cells were stimulated with 10 μg/ml of LMW-HA from 0 to 60 min. Protein samples were immunoblotted for total and phosphorylated Src kinase and ERK1/2. B, SVEC4-10 cells were stimulated with 10 μg/ml of HMW-HA or 10 μg/ml of LMW-HA for 30 min and phosphorylations of Src kinase and ERK1/2 were analyzed by western blotting. Relative densitometry was analyzed using Image Pro Plus 6.0 software. Data were representative of three independent experiments. ($^{**}P < 0.01$).

induced lymphangiogenesis could help in the discovery of novel treatments for the modulation of lymphangiogenic diseases.

During lymphangiogenesis, the proliferation, migration and tube formation of LECs are important processes that maintain the net vascular structure by replenishing new endothelial cells in the empty space [30]. In this study, we showed that 10 μg/ml of LMW-HA induced LEC proliferation, migration and tube formation, whereas HMW-HA at the same concentration had no significant effect on lymphangiogenesis (Figs. 1–3). Consistent with a previous report [6], our results suggest that LMW-HA can induce lymphangiogenesis. To further explore the intracellular mechanism underlying LMW-HA-mediated lymphangiogenesis, we determined the effect of LMW-HA on the activation of Src kinase and the ERK1/2 signal transduction pathway. Src kinase is a regulatory protein that participates in cell differentiation, migration and proliferation [31,32]. ERK1/2, a member of the mitogen-activated protein kinase (MAPK) family, contributes to cell proliferation, migration, differentiation and apoptosis [33]. Inhibition of Src kinase and ERK1/2 can suppress lymphangiogenesis both in vitro and in vivo [34,35]. A previous study reported that LMW-HA promoted the phosphorylation of Src kinase and ERK1/2 and increased the expression of cyclin D1, resulting in enhanced PIEC proliferation [36]. Our results showed that LMW-HA significantly induced phosphorylation of Src kinase and its downstream signal ERK1/2 (Fig. 4), which suggested that activation of Src kinase and ERK1/2 may be involved in LMW-HA-induced lymphangiogenesis.

S1P3, a lipid-activated G protein-couple receptor, is expressed in many cell types, including endothelial cells [37]. The S1P receptor was originally identified as an immediate early gene product that was abundantly induced during the activation of endothelial cells, suggesting that S1P3 may participate in the (patho) physiological regulation of endothelial cell functions [38,39]. Moreover, S1P3 could play a vital role in processes that regulate ligand-mediated angiogenesis and endothelium permeability, including cell–cell junction assembly, morphogenesis, cell survival, and vessel formation [40–42]. It has been reported that S1P3 and CD44 colocalized and interacted in vascular endothelial cell lipid rafts and that this interaction played an important role on LMW-HA induced barrier regulation [15]. During vascular sprouting, junctions are partially disorganized and barrier function is disrupted by increasing vascular permeability, which allows endothelial cells to migrate, proliferate and undergo angiogenesis [14]. LYVE-1, a major receptor for HA on LECs, is a member of the Link protein superfamily and is similar to CD44 with significant sequence homology [43]. Based on the colocalization of S1P3 and CD44 in vascular endothelial cells and its role in LMW-HA-mediated EC biology, it could be speculated that S1P3 may colocalize with LYVE-1 in LECs. Consistent with this hypothesis, we determined using immunofluorescence and coimmunoprecipitation assays that native S1P3 and LYVE-1 colocalized with each other. (Fig. 5A and B).

A previous study found that LYVE-1 played a significant role in LMW-HA-induced lymphangiogenesis [5]. Our results showed that accompanying LMW-HA-induced lymphangiogenesis, LMW-HA also induced an increase in the colocalization of S1P3 and LYVE-1 (Fig. 5A–O), raising the possibility that S1P3 may interact with LYVE-1 and participate in LMW-HA-mediated lymphangiogenesis. Further experiments showed that silencing either LYVE-1 or S1P3 significantly inhibited LMW-HA-induced tube formation (Fig. 6). In addition, when either LYVE-1 or S1P3 was silenced, the LMW-HA-induced phosphorylation of Src kinase and ERK1/2 was significantly decreased (Fig. 7B and D). Overall, our results revealed that S1P3 and LYVE-1 may cooperate to play an important role in LMW-HA-induced lymphangiogenesis.

In conclusion, this study demonstrated for the first time that LMW-HA can increase the colocalization of LYVE-1 and S1P3 receptors in LECs. We also corroborated the crucial involvement of

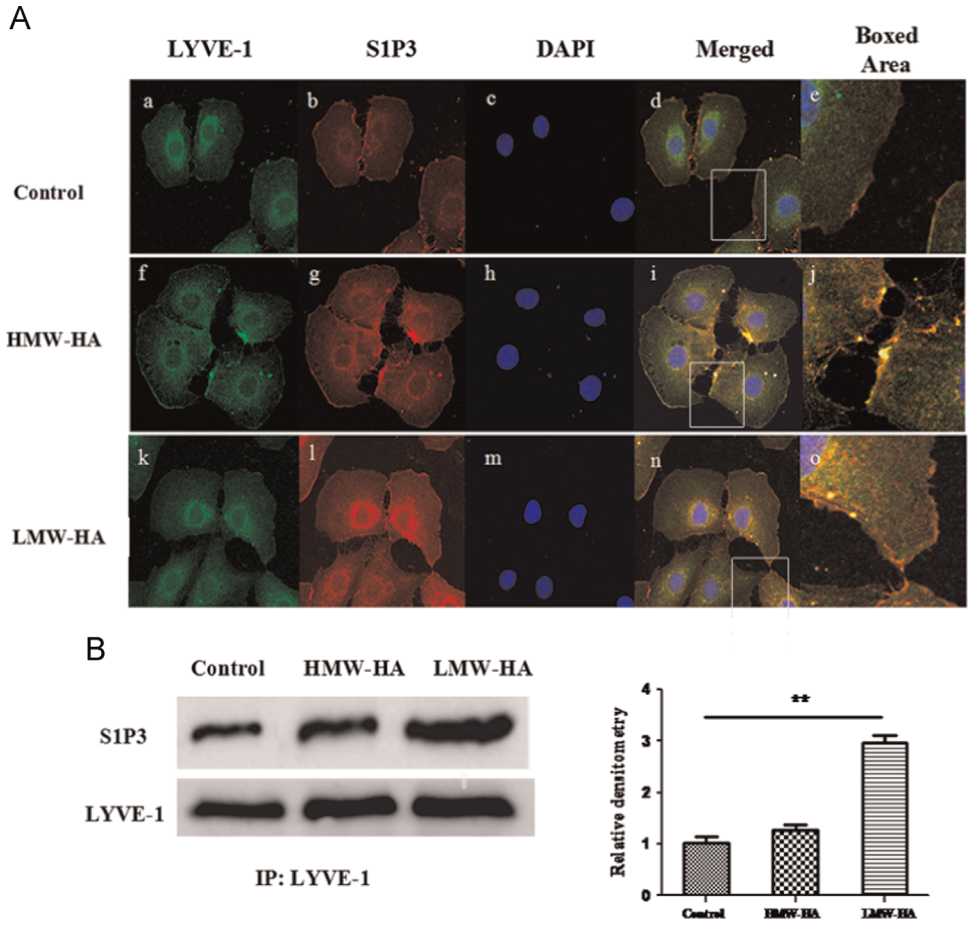

Fig. 5. S1P3 and LYVE-1 colocalize following stimulation of LECs with HMW-HA and LMW-HA. A, Immunofluorescence assays were performed as described in the materials and methods section to determine the localization of LYVE-1 and S1P3 in SVEC4-10 cells. Cells were seeded on coverslips and treated with medium only (a–e), 10 μg/ml HMW-HA (f–j) or 10 μg/ml LMW-HA (k–o) for 30 min. Cells were stained with anti-LYVE-1 antibody (a, f, and k), anti-S1P3 antibody (b, g, and l) and DAPI (e, h, and m) and merged (d, i, and n). Images were taken at × 60 magnification using confocal microscopy. The green and red labelingshow the localization of LYVE-1 and S1P3, respectively, and yellow fluorescence represents the colocalization of the two receptors in merged images. B, Co-immunoprecipitation assays were performed as described in the materials and methods section. Proteins were immunoprecipitated with anti-LYVE-1 for co-IP assays. The data shown represent one of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

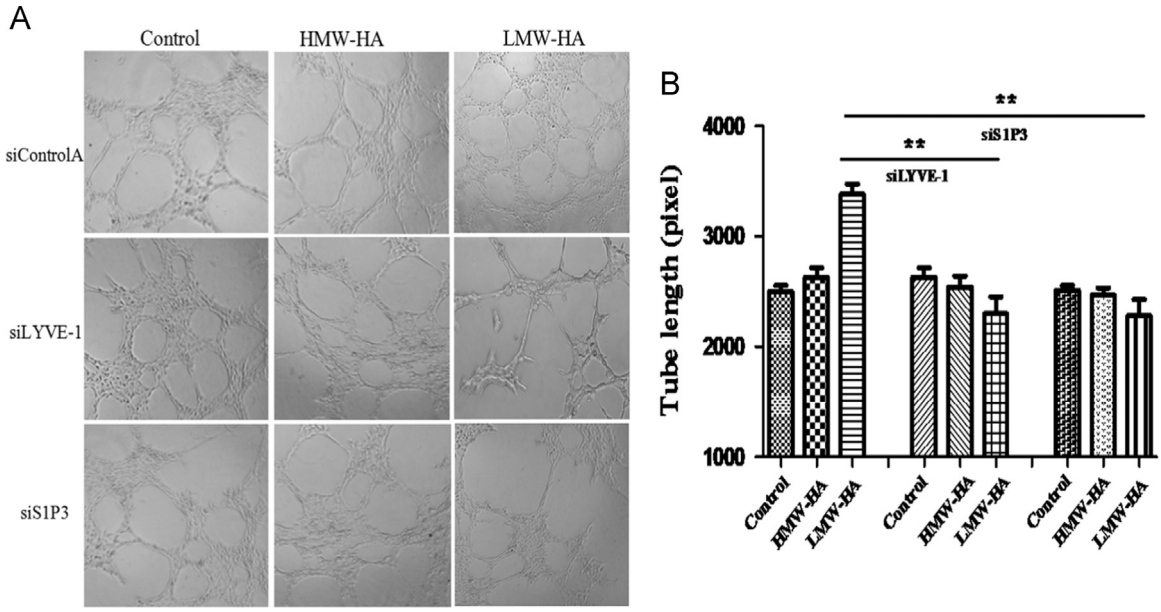

Fig. 6. Silencing of either LYVE-1 or S1P3 inhibited LMW-HA-induced tube formation. A, LYVE-1, S1P3 and scramble silenced SVEC4-10 cells were plated on Matrigel in the presence or absence of 10 μg/ml HMW-HA or LMW-HA. Images were captured after 6 h of incubation as described previously. B, The total tube length was measured and is shown as pixel length per field; the asterisks indicate a statistically significant difference compared with control ($^{**}P < 0.01$). Each treatment was performed in triplicate and experiments were repeated three times.

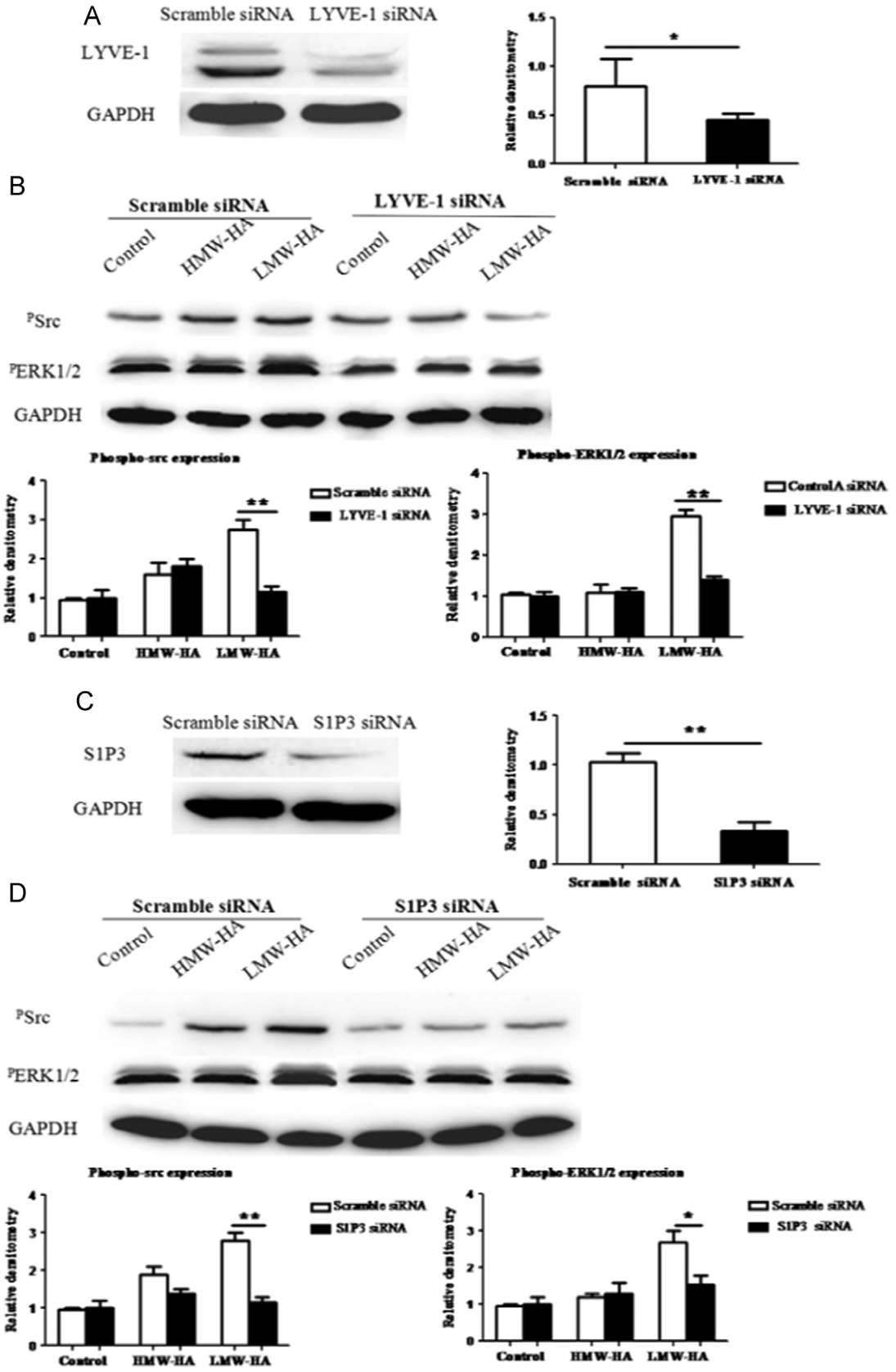

Fig. 7. Silencing of either LYVE-1 or S1P3 reduced LMW-HA-induced phosphorylation of Src kinase and ERK1/2. A and C, SVEC4-10 cells were pretreated with siRNA to silence LYVE-1 or S1P3 for 48 h, then incubated with 10 μg/ml HMW-HA or 10 μg/ml LMW-HA for 30 min. B and D, The phosphorylation of Src kinase and ERK1/2 was analyzed via Western blotting. The relative densitometry was analyzed using Image Pro Plus 6.0 software. The data shown represent one of three independent experiments. ($^{**}P < 0.01$).

S1P3 and LYVE-1 in LMW-HA-mediated lymphangiogenesis and activation of Src kinase and ERK1/2 signal transduction. The discovery of the role of S1P3 and LYVE-1 in LMW-HA-mediated lymphangiogenesis could lead to the development of improved therapeutics in the treatment of lymphangiogenesis-associated diseases, including tumor progression.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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