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Journal of Cellular Biochemistry 112:2106–2114 (2011)

Journal of Cellular Biochemistry

Endostatin Suppresses Colorectal Tumor-Induced Lymphangiogenesis by Inhibiting Expression of Fibronectin Extra Domain A and Integrin α9

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ABSTRACT

Endostatin is a natural occurring anti-angiogenic peptide and has been shown to inhibit tumor lymphangiogenesis by suppressing the expression of tumor-stimulating growth factors. We have previously shown that fibronectin alternative extra domain A (EDA) facilitates lymphangiogenesis of colorectal tumors. Since it is known that EDA interacts with integrin α9 in the lymphatic endothelial cells (LECs), we hypothesized that endostatin may target EDA-integrin α9 pathway to inhibit colorectal tumor-induced lymphangiogenesis. To test this hypothesis, we examined the effect of endostatin on EDA secreted by SW480 colorectal cancer cells and treated human LECs with different doses of endostatin in the presence of conditional medium from SW480 cells. We found that endostatin significantly reduced EDA secretion by SW480 cells and the expression of integrin α9 in LECs. Immunofluorescence studies showed that EDA and integrin α9 colocalized on the cell membrane of LECs and these colocalizations were dramatically reduced by endostatin. Co-immunoprecipitation studies demonstrated that EDA interacted with integrin α9 in LECs, and showed that endostatin treatment inhibited the formation of EDA–integrin α9 complex in LECs. Furthermore, we found that the arrangement and polarity of LEC cytoskeletons were destroyed by endostatin substantially, leading to a reduced formation of tube-like structures of LECs and a suppressed chemotaxis of LECs toward SW480 cells. Consistently, EDA and integrin α9 expressions as well as lymphangiogenesis were significantly suppressed by endostatin in colorectal cancer xenografts. In conclusion, our results suggest that endostatin reduces colorectal tumor-induced lymphangiogenesis, at least in part, by inhibiting EDA-integrin α9 pathway. J. Cell. Biochem. 112: 2106–2114, 2011.

KEY WORDS: ENDOSTATIN; EDA-INTEGRIN α9; LYMPHANGIOGENESIS

Lymphatic vasculature is an important route for metastasis of most common cancer types, including colorectal cancer [Saharinen et al., 2004; Thiele and Sleeman, 2006]. Previous studies have suggested that some cytokines, such as VEGF-C and VEGF-D secreted by tumor cells promote tumor lymphangiogenesis and lymphatic metastasis [Skobe et al., 2001; Achen et al., 2005]. Due to the critical role of lymphangiogenesis in tumor lymphatic metastasis, many efforts have been encouraged to identify compounds and factors that can effectively block tumor lymphangiogenesis by targeting different signaling pathways [Eccles et al., 2007; Achen and Stacker, 2008; Karpanen and Alitalo, 2008].

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII produced by hemangioendothelioma, has been used in clinical cancer therapy as a tumor angiogenesis inhibitor [Abdollahi et al., 2004]. Of the endogenous angiogenesis inhibitors, endostatin has the broadest anti-cancer spectrum and the least toxicity. In addition to its well-known anti-angiogenesis properties, it has become clear in recent years that endostatin also inhibit tumor cells directly by suppressing tumor cell migration and invasion by down regulation or up regulation of several gene expressions in the tumor cells [Wilson et al., 2003]. This fact demonstrates that endostatin's efficacy may extend beyond its anti-angiogenic activity. Recent studies have demonstrated that endostatin also inhibits lymphangiogenesis in vivo and in vitro [Brideau et al., 2007; Zhou et al., 2010]. Although the mechanisms underlying the endostatin-mediated inhibition of tumor angiogenesis have been extensively studied, how endostatin inhibits tumor-induced lymphangiogenesis remains largely unknown.

Juanjuan Ou and Jianjun Li contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China and National Basic Research Program of China; Grant numbers: 81000965, 2010cb529403.

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Received 1 September 2010; Accepted 24 March 2011 • DOI 10.1002/jcb.23130 • © 2011 Wiley-Liss, Inc.
Published online 4 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

We have previously demonstrated that fibronectin (FN) alternatively spliced segment extra domain A (EDA) is an important prolymphangiogenic factor secreted by colorectal tumor cells [Ou et al., 2010]. FN is a key extracellular adhesion molecule in the tumor microenvironment, and various FN isomers arise through alternative splicing of three exons from one gene locus. FN alternatively spliced EDA is an exocrine protein whose expression is high in malignant tumors and tumor stroma, but low in benign tumors [Arti et al., 2008]. It was recently shown that interaction between integrin α9 and the FN EDA splice variant facilitates matrix assembly and lymphatic valve morphogenesis [Arti et al., 2008; Bazigou et al., 2009]. Although it has been shown that integrin α5β1 is an important mediator of endostatin's effects on blood endothelial cells, the role of integrin receptors in mediating endostatin effects on lymphatic endothelial cells (LECs) has yet to be studied. Integrin α9 is essential for the normal development of the lymphatic system [Huang et al., 2001; Mishima et al., 2007] and has been shown to directly bind VEGF-C and VEGF-D to enhance these growth factors-stimulated endothelial cell migration [Vlahakis et al., 2005]. It was unclear whether EDA-integrin α9 pathway can be inhibited by endostatin to suppress lymphangiogenesis of malignant tumors. In this study, we examined the effects of endostatin on the expressions and interactions of EDA and integrin α9 in LECs during colorectal tumor-induced lymphangiogenesis. Our data showed that endostatin significantly inhibits colorectal tumor-induced lymphangiogenesis not only in vitro but also in vivo, which is associated with a dramatic reduction in EDA secretion by colorectal tumor cells and integrin α9 expression in LECs. Given the critical role of EDA and integrin α9 in promoting lymphangiogenesis, our data suggest that endostatin may suppress lymphangiogenesis by inhibiting EDA-integrin α9 pathway.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

SW480 human colorectal carcinoma cell line was obtained from the American Type Culture Collection company (Manassas, VA), and maintained in DMEM (Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS; PAA Corp.), at 37°C in 5% CO_2. Human primary LECs were purchased from Mysha Co., Ltd., (Shanghai, China) and maintained in endothelial cell medium (ECM; ScienCell) supplemented with 20% FBS, at 37°C in 5% CO_2 on culture dishes pre-coated with 15 g/L glutin (Sigma). Human recombinant endostatin (Endostatin, also known as "Endostar") is kindly provided by Jiangsu Simcere Pharmaceutical Co, Ltd. (Nanjing, China).

IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence staining of cultured LECs and tumor xenografts was performed using standard procedures. All the cultures and frozen sections were fixed in ice-cold paraformaldehyde for 20 min, washed with PBS for three times (5 min each), and incubated for 30 min at room temperature in a protein-blocking solution. The cultures and sections were then incubated with the primary antibody directed against EDA (1:100; Santa Cruz Biotechnology, mouse anti-human), integrin α9 (1:100; Santa Cruz Biotechnology, rabbit anti-human), or F-actin (1:200; Chemicon) for 1 h at 37°C and then at 4°C overnight. After wash, the cultures and sections were incubated with appropriate secondary antibodies (FITC-conjugated goat anti-rabbit IgG, Santa Cruz, 1:50; FITC-conjugated goat anti-mouse IgG, Santa Cruz, 1:50; and TRITC-conjugated goat anti-mouse IgG, Beyotime, China, 1:50) at 37°C for 1 h. The cells were counterstained with Hoechst 33258 to reveal the nuclei. The specificity of a primary antibody was verified by omitting that antibody in the reaction.

TUBULOGENESIS ASSAY

After SW480 cells reached to sub-confluence in ECM, the conditional medium was harvested and centrifuged to remove cell debris. The supernatant was collected for treating LECs. LECs were cultured in ECM supplemented with 20% FBS for 12 h, and then digested with trypsin/EDTA to prepare for cell suspension. The cell suspension (1.0×10^6/ml) was seeded onto a 24-well plate coated with 0.5 ml of 4% rat-tail gelatin and treated with the supernatant of SW480 cells, the supernatant of SW480 + 20 ng/ml endostatin, or the supernatant of SW480 + 40 ng/ml endostatin. After 48 h incubation at 37°C, 5% CO_2, cells were photographed every 2 days and the quantities of the branches of tube-like structures were counted (one branch as one tube).

PROTEIN EXTRACTION AND WESTERN BLOTTING

Cell lysates were prepared with M-PER Mammalian Protein Extraction Reagent (PIERCE, PA). The proteins in the lysates were concentrated and used for SDS–PAGE after denature. Proteins in the SDS–PAGE gels were then transferred onto PVDF membranes, incubated with 5% non-fat milk in PBS for 1 h, and then incubated with either anti-integrin α9 or anti-β-actin antibody overnight. After wash, the membranes were incubated with an appropriate HRP-conjugated secondary antibody, and then developed with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biosciences). Densitometric analyses of Western blots were performed using the Scion Image software.

QUANTITATIVE REAL-TIME PCR

Total RNAs were isolated from cultured cells using the RNeasy System (QIAGEN) and then transcribed into cDNAs using the iScript Cdna Synthesis Kit (Bio-Rad). PCR reactions were set up using specific primer pairs for integrin α9 (forward: 5′-CCCGCGCCAAG GTGAAGG-3′ and reverse: 5′-GGGGCTCCAGATTGTGCAG-3′), EDA: (forward: 5′-TGGGAATGGTCGTGGGGAATG-3′ and reverse: 5′-GCCCCTCTTCATGACGCTTGTG-3′), or commercially available primers for GAPDH (SuperArray). Sequence-specific amplification was assessed by measuring the fluorescent signal of SYBR green using a Chromo4 Real-Time PCR Detector (Bio-Rad).

CELL MIGRATION ASSAY

Equal LEC numbers (30,000) were suspended in 0.5 ml of medium and seeded in the top compartment of a standard 8-μm pore Boyden chamber with 0.5 ml of medium added to the bottom compartment. To avoid minor growth rate differences in cell clones, we performed MTT assays and found that there were minimum differences in cell growth rates 12 h after seeding (data not shown). Thus, the migration assay was done at this time point. Non-migrated cells were scraped from the top compartment and the migrated cells to the bottom

compartment were fixed and stained using the Protocol HEMA 3 stain set (Fisher Scientific, Pittsburgh, PA). Membranes were excised and mounted on a standard microscope slide (Curtis Matheson Scientific, Houston, TX), the numbers of migrated cells were determined from five random fields visualized at ×20 magnification.

ELISA

EDA concentrations in the supernatants of SW480 were quantified using a human FN EDA-specific ELISA Kit (BPE301106H, RB, Shanghai Hushang Biotechnology Co., Ltd.), and then normalized to the total protein content of each clone grown to 85% confluence in a 100 mm^2 cell culture dish in 1% complete DMEM as measured by the Bradford assay.

IMMUNOPRECIPITATION

EDA–integrin α9 complex formation was analyzed by co-immunoprecipitation assay. Total protein lysates (500 μg) from each sample were immunoprecipitated in 400 μl lysate buffer containing 2 μl anti-integrin α9 rabbit polyclonal antibody and inhibitors of various proteases, phosphotases, and kinases at 4°C for 4 h with rotation. Protein A-conjugated agarose beads (25 μl) were then added into the immunoprecipitation reaction with an additional 5 h of rotation at 4°C. The antigen–antibody complexes were precipitated by a quick centrifugation, followed by four times of wash with cold PBS. Controls included an aliquot of rabbit serum to replace the integrin α9 antibody, in the immunoprecipitation reaction. The pellets were suspended in 20 μl of 2 × SDS reducing Western blot loading buffer and boiled for 5 min, followed by SDS–PAGE. The integrin α9-immunoprecipitates were subjected to Western blot assay to detect EDA and integrin α9 in the immunoprecipitates.

IN VIVO TUMOR MODELS

Four- to 6-week-old Balb/c nude mice (body weight: 16–20 g) were purchased from the Experimental Animal Center, Institute of Laboratory Animal Sciences, China, and maintained in a specific pathogen-free (SPF) environment in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996). The mice were subcutaneously injected with SW480 cells (5×10^6 cells in 100 μl PBS/mouse) at the left groin. Twelve days after tumor cell inoculation, animals with the tumor size reached approximately 50 mm^3 were received daily peritumoral injections of endostatin (10 or 20 mg/kg/day) for 2 weeks. PBS was injected into some animals as negative controls. The mice were sacrificed and the tumors were collected, half of which was fixed in 4% formalin and embedded in paraffin blocks and the remainder was snap-frozen in liquid nitrogen for histological studies. Three sections (8 μm) of each tumor were stained for EDA or integrin α9 following standard procedure. Podoplanin (Chemicon; 1:100) was used to monitor the numbers of lymphatic microvessels in the peritumoral regions, which were evaluated in five independent fields for each of the three tumors per group. All of our animal studies have been approved by the institutional Animal Care and Use Committee of the Third Military Medical University.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS (SPSS, Chicago, IL) using Student's *t*-test or one-way ANOVA. Differences were considered statistically significant when *P*-values were <0.05. Error bars represent standard error of the mean.

RESULTS

ENDOSTATIN REDUCES TUMOR CELL–INDUCED TUBULOGENESIS OF LECs IN VITRO

To determine the effect of endostatin on the tube formation of LECs in vitro, we used the 3D tube-like formation assay [Matsuo et al., 2007]. There were a lot of lymphatic tube-like structures formed in LECs cultured in the SW480 conditional medium after 24 h of culture. The branches of lymphatic tube-like structures became much more abundant after 7 days of culture and they were elongated to form typical hollow tubes. As expected, endostatin at a concentration of 20 ng/ml significantly inhibited the tube formation of LECs, and the inhibition was more effective when the endostatin concentration was increased to 40 ng/ml (Fig. 1).

ENDOSTATIN DISRUPTS CYTOSKELETON ARRANGEMENT OF LECs

The cytoskeleton arrangement is critical for the mobility of endothelial cells. To determine if endostatin alters cytoskeleton arrangement, we immunostained F-actin in LECs treated with or without endostatin. We found that the microfilament of LEC in the SW480 supernatant-treated group was arranged in parallel, displaying a polarized organization, consistent with the directional movement of LECs. Treatment with endostatin resulted in an obvious disruption of this polarized organization of microfilaments in LECs (Fig. 2).

ENDOSTATIN INHIBITS TUMOR CELL–INDUCED CHEMOTAXIS OF LECs

One of the mechanisms of tumor vasculogenesis is the directional movement of endothelial cells attracted by some cytokines secreted by malignant tumors, which is called chemotaxis. Therefore, we used Transwell assays to assess the inhibitory effect of endostatin on chemotaxis of LECs toward colorectal tumor cells. As shown in Figure 3, the chemotaxis of LECs toward SW480 cells was significantly suppressed by endostatin at 20 ng/ml, and this suppression became more dramatic at 40 ng/ml.

ENDOSTATIN SUPPRESSES EXPRESSION OF EDA AND INTEGRIN α9

Given the critical roles of EDA and integrin α9 in promoting lymphangiogenesis [Bazigou et al., 2009; Ou et al., 2010], we examined the effects of endostatin on the expression of EDA in SW480 cells and integrin α9 in LECs. The real-time PCR analysis showed that the mRNA for EDA was significantly reduced in SW480 cells treated with 40 ng/ml of endostatin for 72 h. A dose-dependent effect was observed for integrin α9 mRNA in endostatin-treated LECs (Fig. 4A). Consistently, ELISA measurements revealed a dose-dependent reduction in EDA secretion by the endostatin-treated SW480 cells (Fig. 4B). Immunoblotting showed a dramatic reduction in integrin α9 expression in LECs treated with endostatin (Fig. 4C).

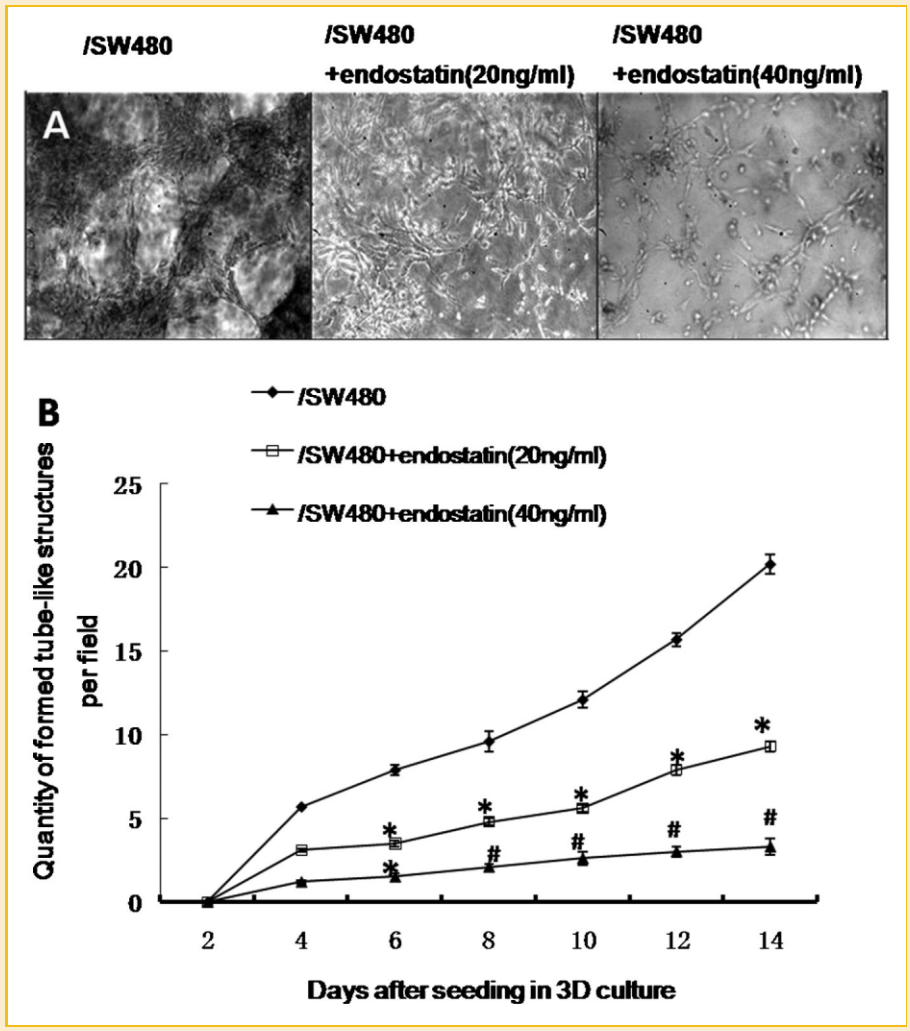

Fig. 1. Endostatin inhibits tubulogenesis in LECs. A: LECs were seeded in a 24-well plate coated with 0.5 ml of 4% rat-tail gelatin, and were divided into three treatment groups: (1) SW480 supernatant; (2) SW480 supernatant +20 ng/ml endostatin; and (3) SW480 supernatant +40 ng/ml endostatin. The tube-like structures were photographed after 48 h of the treatment (original magnification: ×100). B: Quantification of tube-like structures. Branches of tube-like structures were counted every 2 days after 48 h of the treatment. Data are presented as mean ± SEM of the values for each group. Similar results were obtained in three independent experiments. $^{*}P < 0.05$ and $\#P < 0.01$ (compared with control).

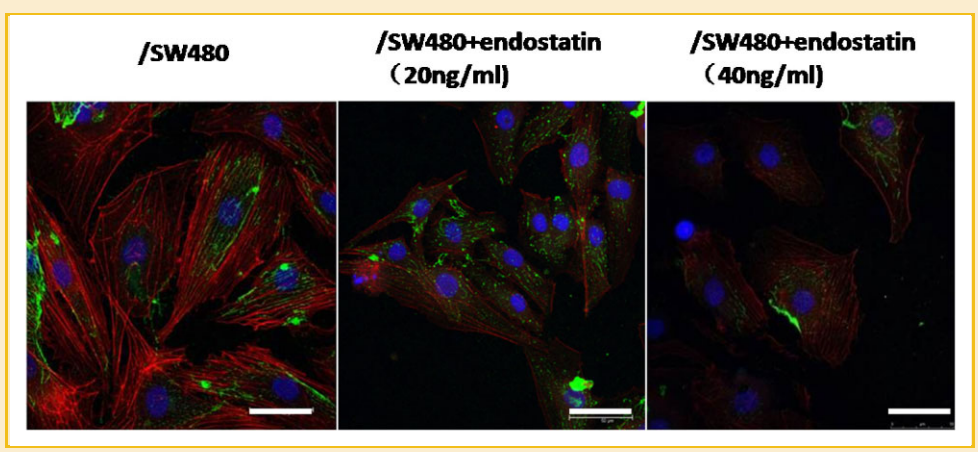

Fig. 2. Endostatin treatments disturb the arrangement and polarity of LEC cytoskeletons. LECs were grown on glutin-coated coverslips in 24-well plates, and treated with different doses (0, 20, or 40 ng/ml) of endostatin for 24 h. The immunofluorescent studies were performed as described under Materials and Methods section. Microfilaments were stained red and EDA stained green. Bar scale: 50 μM.

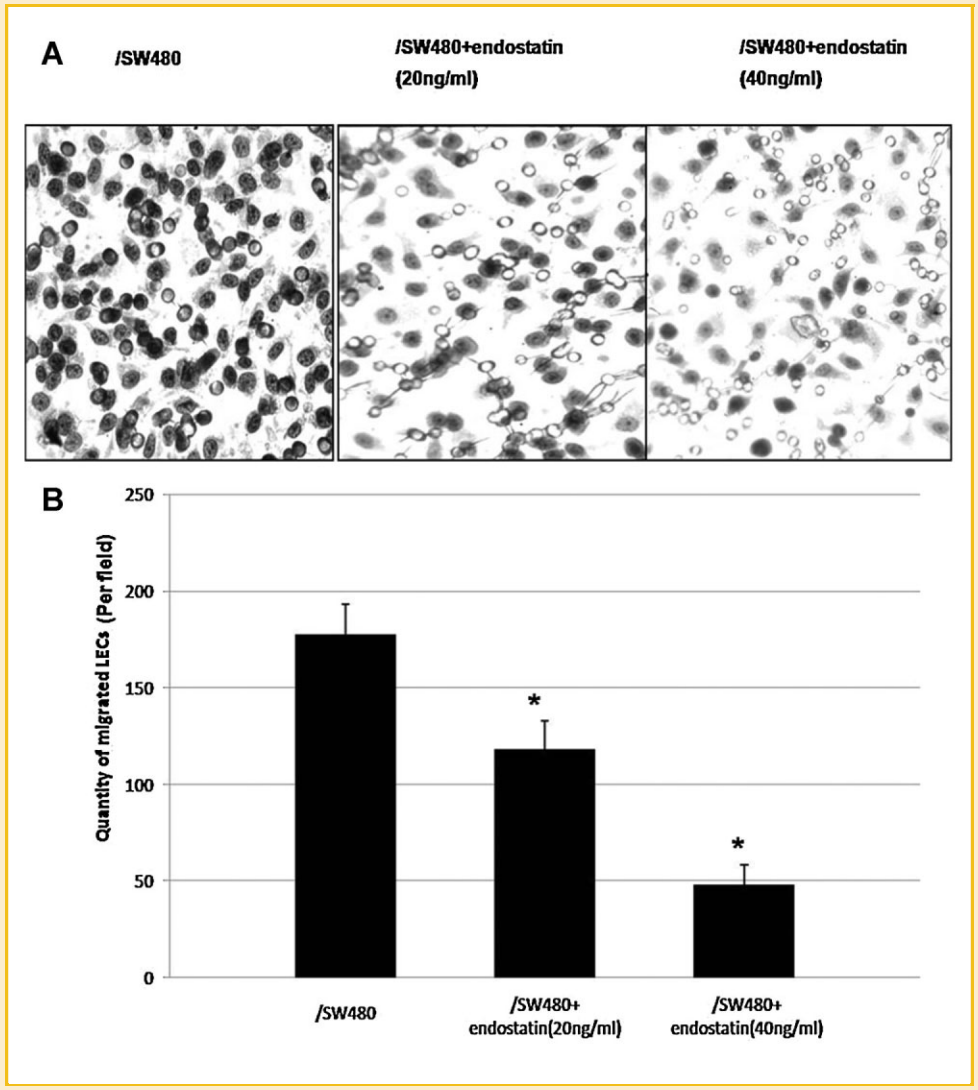

Fig. 3. Endostatin inhibits chemotaxis of LECs towards colorectal cancer cells. LECs were cultured in Transwell system and treated with different amounts (0, 20, or 40 ng/ml) of endostatin. A: LEC migration activity after 24 h of the treatment (original magnification, ×200). B: Quantification of migrated LECs after 24 h of treatment. Migrated LECs were counted as described under Materials and Methods section. Each bar represents the mean ± SEM of the values for each group. Similar results were obtained in three independent experiments. $^{*}P < 0.05$ (compared with control).

ENDOSTATIN REDUCES EDA–INTEGRIN α9 COMPLEX ON LECs

To determine if EDA interacts with integrin α9 in LECs and to define how endostatin influence this interaction, we performed co-immunoprecipitation assays. As shown in Figure 5A, endostatin treatments substantially reduced the abundance of immunoprecipitated integrin α9 and integrin α9-associated EDA in LECs. While this may be due to the decreased expression of total EDA proteins in SW480 cells and of total integrin α9 proteins in LECs as shown in Figure 4, we also found using densitometry that the relative ratio of EDA brought down by integrin α9 immunoprecipitation was substantially reduced in endostatin-treated cells (Fig. 5B). Given that EDA interacts with integrin α9 on the cell surface, this observation may suggest that the relative distribution of integrin α9 to cell membrane versus cytoplasm was reduced. To examine this possibility, we performed immunofluorescence studies. In LECs grown in SW480 conditional medium, the EDA-integrin α9 colocalization was evident on the cell surface (Fig. 5C). As expected, endostatin treatment dramatically reduced fluorescence signal (Fig. 5C), likely as a result of inhibition of EDA and integrin α9 expression (Fig. 4). Despite a much weaker immunofluorescent signal in endostatin-treated LECs, we were still able to visualize these two proteins. Interestingly, the cell surface colocalization of EDA-integrin α9 was dramatically reduced in 20 ng/ml of endostatin-treated LECs, and almost disappeared in 40 ng/ml of endostatin-treated LECs (Fig. 5C), suggesting a possible effect of endostatin on the cell surface colocalization of EDA and integrin α9.

ENDOSTATIN INHIBITS EDA–INTEGRIN α9 EXPRESSION AND LYMPHANGIOGENESIS IN VIVO

To determine if endostatin affects EDA and integrin α9 expression in vivo, we injected tumor cells into nude mice, collected tumor xenografts, and measured EDA and integrin α9 expression by

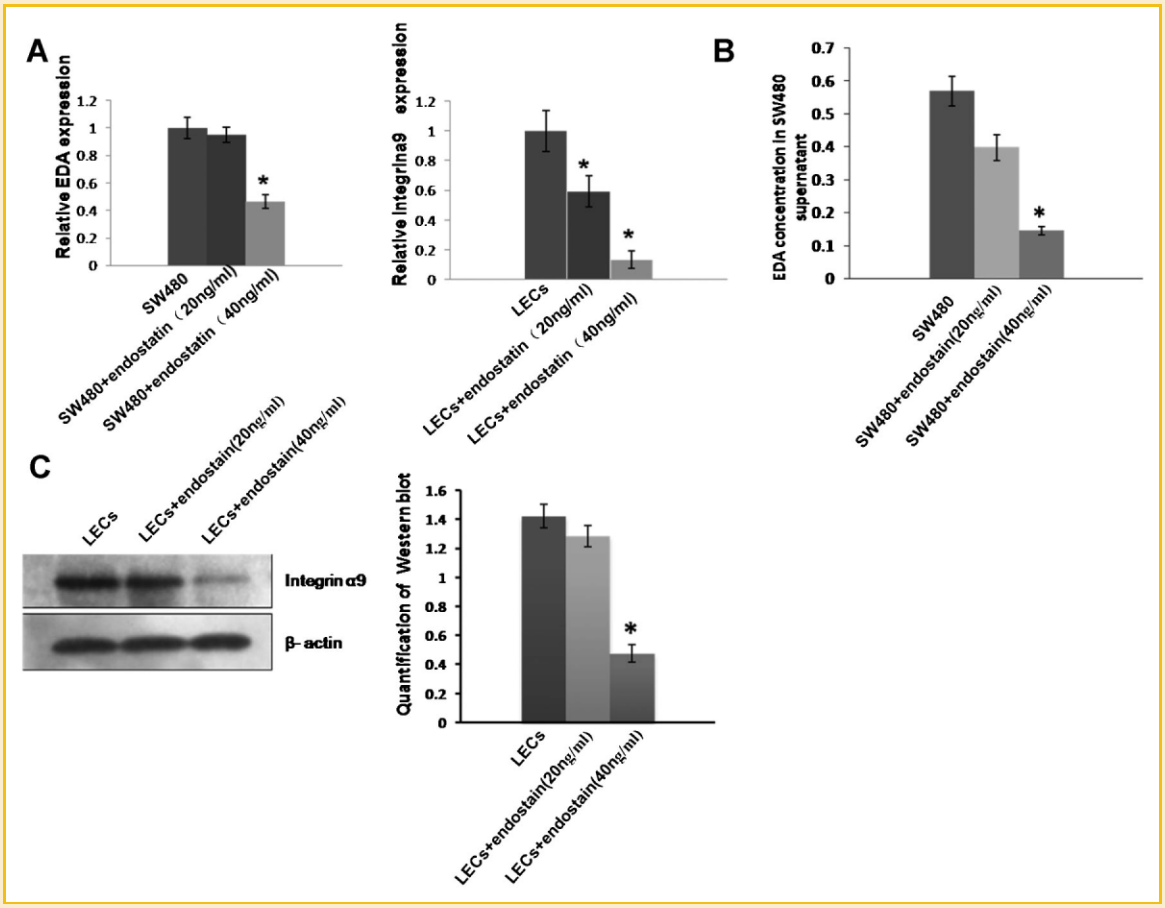

Fig. 4. Endostatin suppresses the expressions of EDA and integrin α9. SW480 cells and LECs were treated with endostatin (0, 20, or 40 ng/ml) for 24 h. A: The mRNA levels of EDA and integrin α9 determined by real time-PCR. B: Secreted EDA levels of SW480 cells determined by ELISA assays. C: Protein levels of integrin α9 detected by Western blotting. Data are presented as the mean ± SEM. $^{*}P < 0.05$ (compared with control).

immunohistocytochemistry and immunofluorescence studies. As shown in Figure 6, endostatin substantially suppressed the expression of EDA (Fig. 6A) in the xenografts and inhibited and EDA-integrin α9 colocalization in the tumor lymphatic vessels (Fig. 6B). Furthermore, we measured the numbers of lymphatic vessels of xenogfrat tumors by immunohistochemistry with a podoplanin antibody and found that endostatin treatments significantly reduced the numbers of the lymphatic microvessels in peritumoral region (Fig. 6C).

DISCUSSION

We have previously shown that FN EDA, a component of extracellular matrix of tumor microenvironment, is a potent-inducing factor of lymphangiogenesis in colorectal carcinoma cells [Ou et al., 2010]. The major finding of this study was that the recombinant human endostatin treatment significantly reduces lymphangiogenesis in cultured LECs and in tumor xenografts, which is associated with a de-arrangement of cytoskeleton, a dramatic reduction in EDA secretion by SW480 colorectal tumor cells and in the expression of EDA receptor integrin α9 in LECs. Additionally, we demonstrated that EDA interacts with integrin α9 in LECs and they colocalize at the plasma membrane of these cells. Endostatin treatments substantially reduce integrin α9 and EDA in the immunoprecipitates of LEC lysates, and the colocalization of integrin α9 and EDA on the cell surface of LECs. To the best of our knowledge, this study is the first to show that endostatin suppresses the expression and interaction of EDA and integrin α9. Given the critical role of EDA-integrin α9 pathway in mediating lymphangiogenesis [Bazigou et al., 2009; Ou et al., 2010], our findings suggest that endostatin reduces lymphangiogenesis, at least in part, through inhibiting EDA-integrin α9 pathway.

It is currently unclear how endostatin suppresses the expression of EDA in SW480 cells and integrin α9 in LECs. It has been reported that endostatin can regulate protein expression at both transcriptional [Fukumoto et al., 2005] and post-transcription levels [Schmidt et al., 2005]. Since the mRNAs for these two proteins are reduced by endostatin treatments and the protein reduction appears to match mRNA reduction (Fig. 4), it is likely endostatin inhibits transcription and/or stability of EDA and integrin α9 mRNAs under our experimental conditions. Future studies are

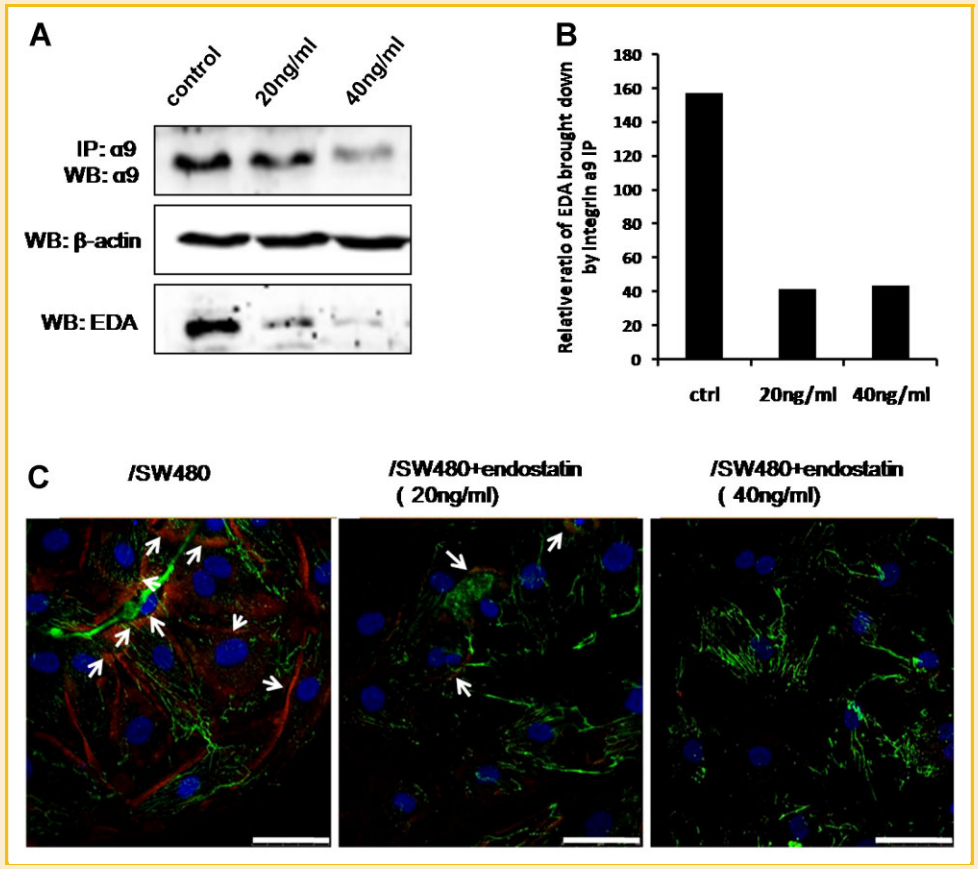

Fig. 5. Subcellular location and complex formation of EDA and integrin α9. LECs were grown on glutin-coated coverslips in 24-well plates and treated with SW480 supernatant; SW480 supernatant + 20 ng/ml endostatin; or SW480 supernatant + 40 ng/ml endostatin for 24 h. A: Co-immunoprecipitation of EDA and integrin α9. Total cell lysates were prepared from three groups of treated LECs and the same amount of total proteins in each lysate was immunoprecipitated with integrin α9 antibody. The immunoprecipitates were fractioned in SDS–polyacrilamide gel and subjected to Western blotting for detection of integrin α9 or EDA as described under Materials and Methods section. B: Densitometry was used to estimate relative ratios of densities of immunoprecipitated EDA to integrin α9 in each lysate. Immunoreactive β-actin was used as a protein input control for each total cell lysate. C: Immunofluorescence staining of EDA (green) and integrin α9 (red). Nuclei were counterstained with DAPI (blue). The colocalization of EDA and integrin α9 is shown as orange color (arrows). Bar scale: 50 μM. The experiment was repeated once and similar result obtained.

required to dissect the molecular mechanisms by which endostatin reduces mRNAs for EDA and integrin α9, and to establish whether these effects can be recapitulated in other cancer cell lines and in clinical biopsy samples from cancer patients treated with endostatin.

It has been well established that EDA can bind to its cell membrane receptor integrin α9 to promote lymphatic valve morphogenesis [Bazigou et al., 2009]. Consistently, we showed that EDA and integrin α9 co-immunoprecipitate (Fig. 5A). The treatment of LECs with endostatin reduces the immunoprecipitable complex of EDA and integrin α9, which is likely a result of reduced expression of total integrin α9 protein in these cells (Fig. 4). Because of reduced expression of integrin α9 protein, the immunofluorescence signal for integrin α9 is also weakened in endostatin-treated LECs (Fig. 5). The reduced EDA immunofluorescence signal observed in endostatin-treated LECs could also result from decreased integrin α9 expression. Alternatively, endostatin may directly inhibit expression of endogenous EDA in LECs. Intriguingly, we detected almost no integrin α9 localization to the cell membrane in the endostatin-treated LECs (Fig. 5). There are several possibility for this observation: (1) when total integrin α9 protein expression is reduced by endostatin, cell membrane-localized integrin α9 protein is also expected to be decreased, thereby reducing the enrichment of this protein on the cell surface and consequently immunofluorescence sensitivity; (2) endostatin inhibits translocation of integrin α9 from cytoplasm to the plasma membrane; (3) endostatin facilitates internalization of EDA–integrin α9 complex, resulting in reduced retention of this complex on the cell surface. It is not surprising to see reduced colocalization of EDA with integrin α9 to the cell membrane because EDA is a ligand for integrin α9. Interestingly, we found that the relative ratio of EDA brought down by integrin α9 immunoprecipitation was substantially reduced in endostatin-treated groups (Fig. 5B), suggesting that endostatin treatment may also interfere with interactions between EDA and integrin α9. Given that integrin α9 acts as a receptor for EDA on the cell membrane and intracellularly localized integrin α9 may not bind EDA, this observation may suggest that endostatin reduces integrin α9 translocation to the cell surface in addition to its expression, thereby reducing the exposure of integrin α9 to extracellular EDA. As shown in Figure 5C, integrin α9 is highly expressed on the cell membrane of un-treated LECs, and colocalizes well with EDA, but

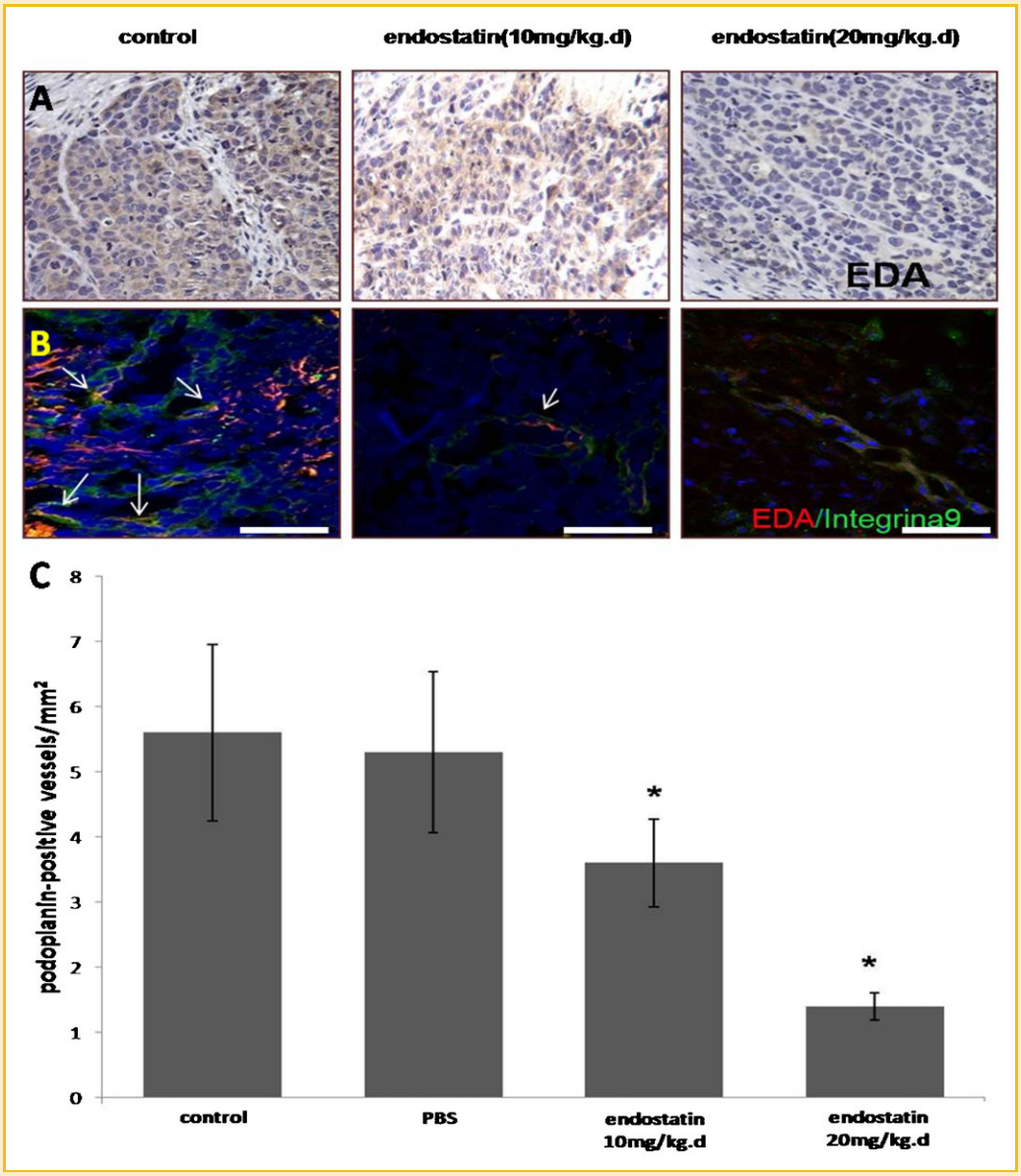

Fig. 6. Endostatin inhibits lymphangiogenesis in colorectal tumor xenografts. The nude mice were subcutaneously injected with SW480 cells (5×10^6 cells in 100 μl PBS/mouse) at the left groin. Twelve days after tumor cell inoculation, animals with the tumor size reached approximately 50 mm^3 were received daily peritumoral injections of PBS or endostatin (10 or 20 mg/kg/day) for 2 weeks and then sacrificed. A: Immunohistochemical staining of EDA in tumor xenografts (original magnification, ×100). B: Immunofluorescence staining of EDA (red) and integrin α9 (green) in tumor xenografts. Yellow colors (arrows) indicate colocalization of EDA and integrin α9. Nuclei were counterstained with DAPI (blue). Bar scale: 50 μM. C: Quantification of podoplanin-positive lymphatic vessel densities. Three sections from each SW480 cell xenografts were stained with podoplanin antibody and the peritumoral vessel density in six independent fields for each section was evaluated. Data are presented as mean ± SEM of the values for each group of treated animals. $^*P < 0.05$ (compared with control).

this co-localization is substantially reduced in the presence of endostatin. However, our immunofluorescence observation may simply reflect reduced total integrin α9 protein in endostatin-treated cells. The immunofluorescent method we used is not sensitive enough to quantify the protein translation. More sensitive methods such as biotinylation and live cell imaging are needed to determine if the relative localization of integrin α9 to cell surface versus cytoplasm is indeed reduced in endostatin-treated cells.

Activation of integrins can induce cell migration by changing the arrangement and polarity of cytoskeleton [Friedl, 2004]. Normal EDA-integrin α9 signaling may be necessary to maintain normal cytoskeleton organization. In this study, endostatin treatment causes de-arrangement of cytoskeleton in LECs, likely in part through interfering EDA-integrin α9 signaling. This cytoskeleton de-arrangement may partly explain reduced tumor-induced lymphangiogenesis observed in endostatin-treated LECs (Fig. 1) and tumor xenografts (Fig. 6).

VEGF-C and VEGF-D are well documented as pro-lymphangiogenic factors of malignant tumors. Endostatin has previously been shown to inhibit both lymphangiogenesis and lymphatic metastasis

by down-regulating VEGF-C expression in tumor cells [Fukumoto et al., 2005; Brideau et al., 2007]. Our study suggests endostatin may employ a non-classic pathway to inhibit tumor lymphangiogenesis. Target therapies to malignant tumors depend on identifications of specific proteins enriched in tumors or their microenvironments. EDA expression is much higher in malignant tumors relative to benign tissues [Rybak et al., 2007]. Inhibition of EDA by endostatin may represent a tumor-specific approach in cancer therapies.

Precise downstream signaling pathways responsible for the inhibitory effect of endostatin on EDA expression have yet to be identified. It should be noted that our results have only provided a potential link between endostatin and EDA-integrin α9 pathway, but not established a definitive role of this pathway in mediating endostatin-induced suppression of lymphangiogenesis. Loss and gain of function studies are required to address this issue. Additionally, future studies are also needed to address the crosstalk among EDA, VEGF-C, and VEGF-D. It would also be interesting to explore effects of endostatin–EDA–integrin α9 pathway on cancer stem cells because these cells are closely related to tumor vasculogenesis [Yan Leychkis et al., 2009].

In conclusion, endostatin may inhibit tumor-induced lymphangiogenesis, in part, through inhibiting EDA-integrin α9 pathway.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (81000965) and the National Basic Research Program of China (973 Program, No. 2010cb529403). We thank Dr. Liqing Yu at Wake Forest University School of Medicince for critical reading, discussion, and editing of this article.

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