FIGURE LEGENDS

Figure 1. Transgenic overexpression of apelin induces larger blood vessels. (**A**) Schematic representation of the K14-apelin expression cassette. (**B**) Quantitative real-time PCR analysis of apelin mRNA in the ear skin from 3-day-old wt mice and animals from three different founder lines of K14-apelin Tg mice. Threshold values for target genes normalized against the level of GAPDH (dCt) are shown under the graph. (**C**) Immunohistochemical staining of apelin expression in the dorsal skin sections from 6-week-old wt or K14-apelin Tg mice. Positive reactions are revealed by purple coloration. (**D**) Comparison of blood vessels in lectin-stained whole mounts of ear skin from 8-week-old wt or K14-apelin Tg mice. ECs were stained by perfusion of FITC-conjugated isolectin (green) and mural cells were stained with anti-αSMA antibody (red). (**E,F**) Quantitative evaluation of the vascular diameter (**E**) and vascular density (**F**) of capillaries in the dermis from wt and K14-apelin Tg mice. Data were obtained by counting 5 random fields from 5 different mice (total 25 fields). $*P<0.01$. (**G**) Immunohistochemical staining of APJ expression in the dorsal skin serial sections from 2 day -old (neonate) or 8 week -old (adult) mice. Blood vessels were detected by immunohistochemical staining with anti-CD31 Ab. Positive reactions are visualized as a purple coloration.

Figure 2. Requirement for endogenous apelin for recovery of hindlimb perfusion after induction of ischemia. (**A**) Immunohistochemical staining of muscle sections from sham operated (sham) or ischemic (ischemia) hindlimb with anti-CD31 (green) and anti-APJ (red) antibodies. (**B,C**) Quantitative real-time PCR analysis of APJ (**B**) or apelin (**C**) mRNA expression in $CD31^{+}CD45^{-}$ ECs or $CD31^{-}$non-ECs derived from muscle of sham operated (sham) or ischemic (ischemia) hindlimb. Levels were normalized against the level of expression of CD31 as an EC marker. Threshold values for target genes normalized against the level of CD31 (dCt) are shown under the graph. (**D**) Gross appearance of foot pad from

sham-operated (sham) or ischemic wt and apelin$^{-/-}$ mice 14 days after resection of the femoral artery. (**E**) Representative laser Doppler-images of mouse hind limbs from wt or apelin$^{-/-}$ mice 14 days after surgery. Red indicates greater flow; blue indicates less flow. (**F**) Quantification of laser Doppler-monitored blood flow measurements. Data are from ratio of ischemic right leg versus non-ischemic left leg in mice. *P < 0.01 (n>10).

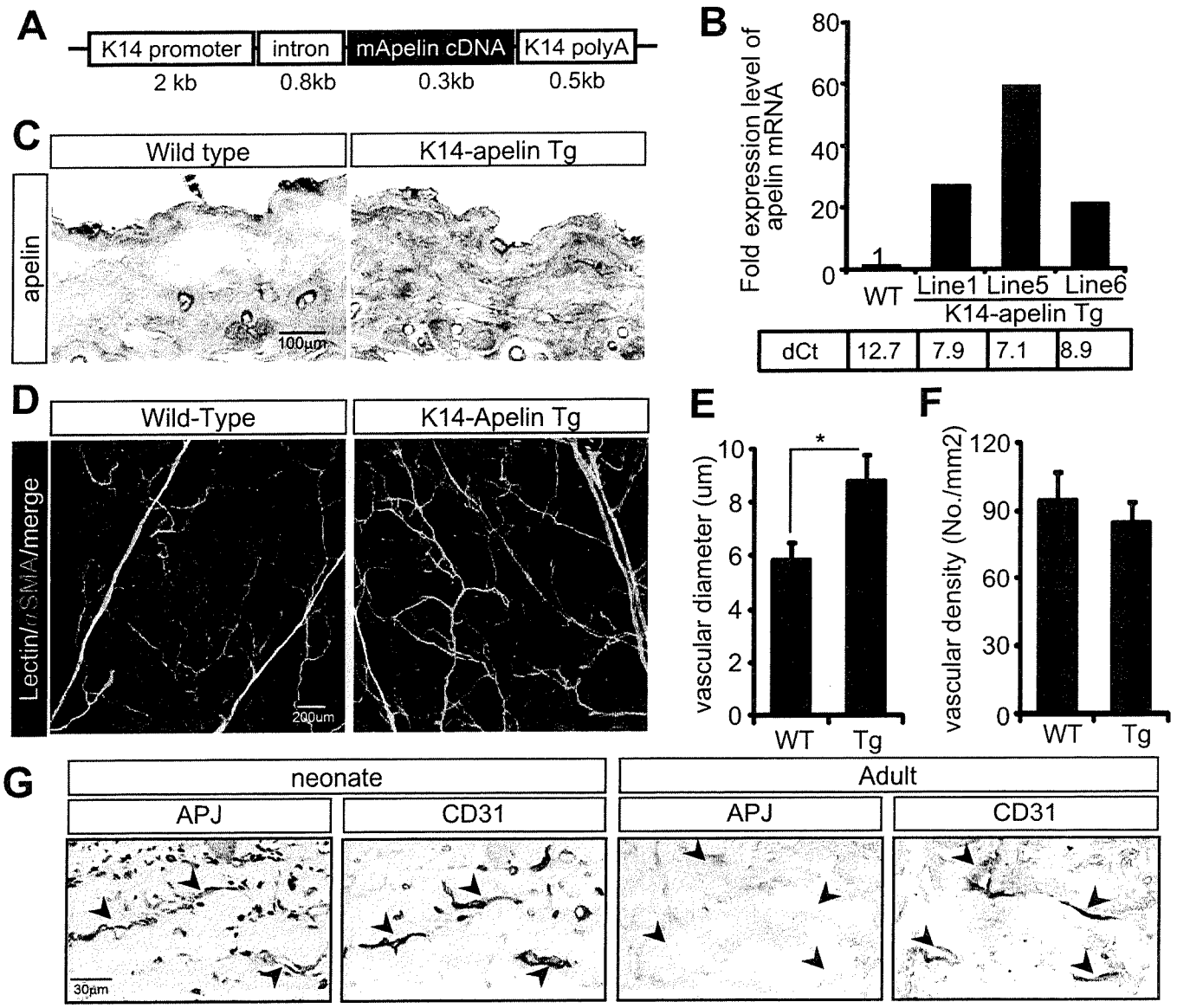
Figure 3. Apelin gene transfer promotes development of larger vessels in the hindlimb ischemia model. (**A**) Gross appearance of foot pad from sham-operated mice (sham) or ischemic mice injected with genes as indicated 14 days after resection of the femoral artery. (**B**) Representative laser Doppler-images of a mouse hindlimb 14 days after surgery and gene transfer as indicated. Red indicates greater flow; blue indicates less flow. (**C**) Quantification of laser Doppler-monitored blood flow measurements. Data are from ratio of ischemic right leg versus non-ischemic left leg mice injected with genes as indicated. *P < 0.01 (n>10). (**D**) CD31 staining of quadriceps muscle from mice 14 days after surgery and gene transfer as indicated. Muscle from sham-operated (sham) mice was used for comparison. (**E,F**) Quantitative evaluation of the vessel number (**E**) and the percentage of enlarged blood vessels >30μm relative to the total number of blood vessels (**F**) in mice 14 days after surgery and gene transfer as indicated. *P < 0.01.

Figure 4. Apelin suppresses VEGF-induced vascular edema. (**A**) Histological analysis of hindlimb muscles 14 days after resection of the femoral artery and gene therapy as indicated. (**B**) Miles assay using VEGF and apelin. Left-hand panels show representative images of the vascular leakage induced by VEGF in the presence or absence of apelin (1 or 10 ng/ml). PBS was used as a negative control. The right-hand panel shows the Evans blue dye content eluted from dissected skin (mean±SEM, n=10). *P<0.01.

Figure 5. Requirement for endogenous apelin for inhibition of VEGF- or histamine-induced vascular edema. (**A,B**) Vessel permeability was assessed by the Miles assay. Apelin-deficient (KO) and wt (WT) mice (**A**) or K14-apelin Tg (Tg) and wt (WT) mice (**B**) were injected with 15 μL of PBS in the presence or absence of rhVEGF165 (100ng/ml) or histamine (100μM) intradermally. Thirty min after injection, the inner side of the dorsal skin was inspected. Representative images of the vascular leakage are presented in the upper panels. Bottom panels show the quantification of extravasated Evans blue dye content eluted from dissected skin (mean ±SEM, n=10). *P<0.05.

Figure 6. Apelin inhibits VEGF mediated hyperpemeability (**A**) Effect of apelin on VE-cadherin-mediated endothelial junctions. VE-cadherin expression was studied in HUVEC monolayers. HUVECs cultured with or without pretreatment with apelin for 10 minutes were stimulated with VEGF (20ng/ml) for 10 minutes. F-actin (red) and VE–cadherin (VE-cad.; green) staining is shown. Arrowheads indicate the gaps between adjacent ECs and lack of VE-cadherin. Nuclei were labeled with TOPRO-3 (TOP.; blue). (**B**) Suppression of VEGF-mediated VE-cadherin internalization by apelin. HUVEC cell surface VE-cadherin labeled by anti-VE-cadherin antibody and stimulated with VEGF (20ng/ml) in the presence (apelin) or absence (control) of apelin (100ng/ml). VE-cadherin internalization was visualized in fixed cells using secondary fluorescent antibodies (green). To confirm the internalization of VE-cadherin, cell surface bound VE-cadherin antibodies were removed by a mild acid wash before fixation (right two lines of images). Nuclei were labeled with TOPRO-3 (blue). (**C**) The internalization of endogenous VE-cadherin [in the right two lines of (**B**)] was quantified by the fluorescence intensity of secondary antibodies per cell. *P<0.01. (**D**) Western blot analysis of cell surface and cytoplasmic VE-cadherin protein on HUVECs that had been stimulated with VEGF (20 ng/ml) and/or apelin (100 ng/ml) for 20 min. The purity of cytoplasmic protein was confirmed by the lack of expression of the cell surface protein

N-cadherin. GAPDH expression was analyzed as a control for an unrelated intracellular protein.



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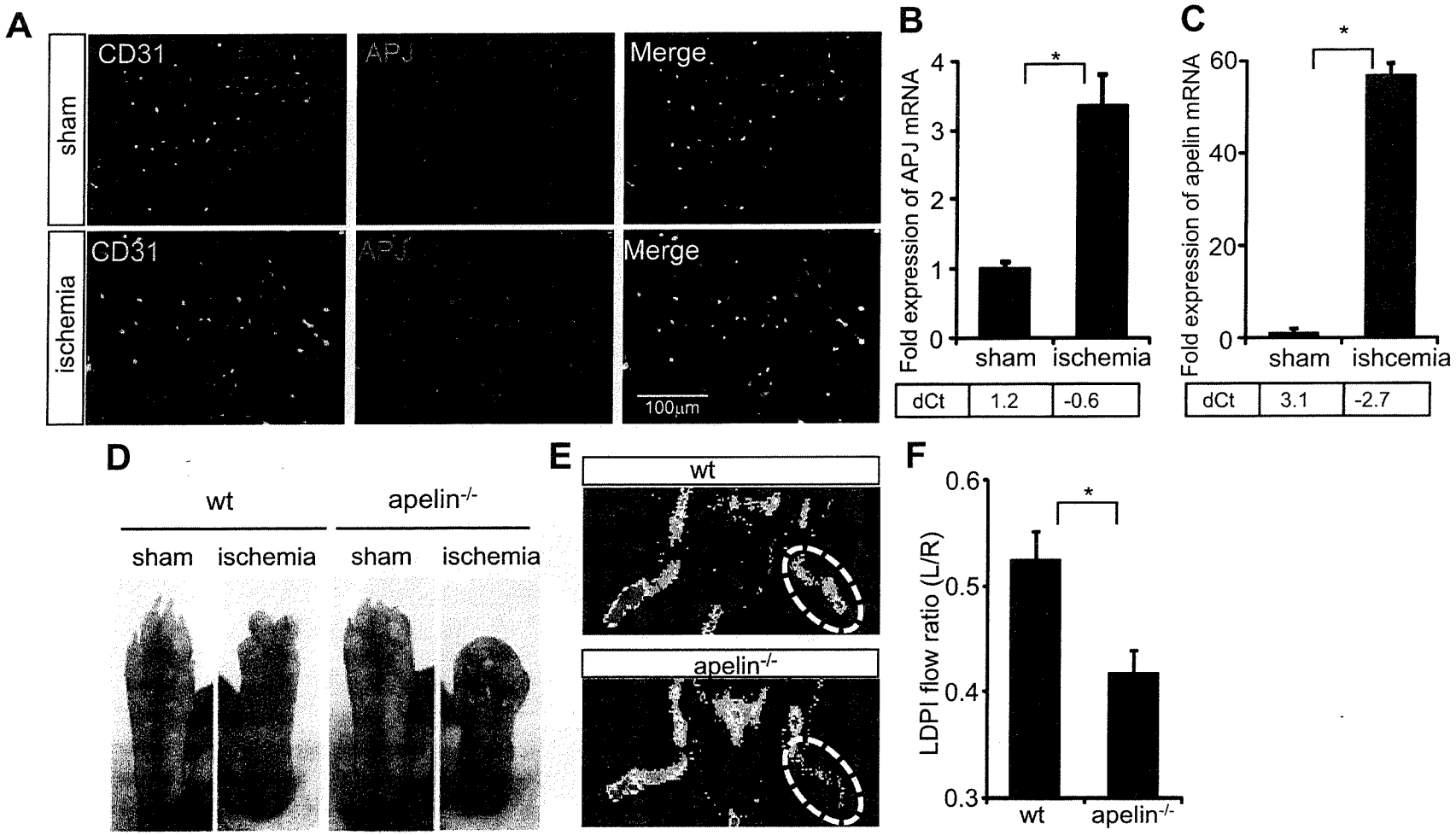

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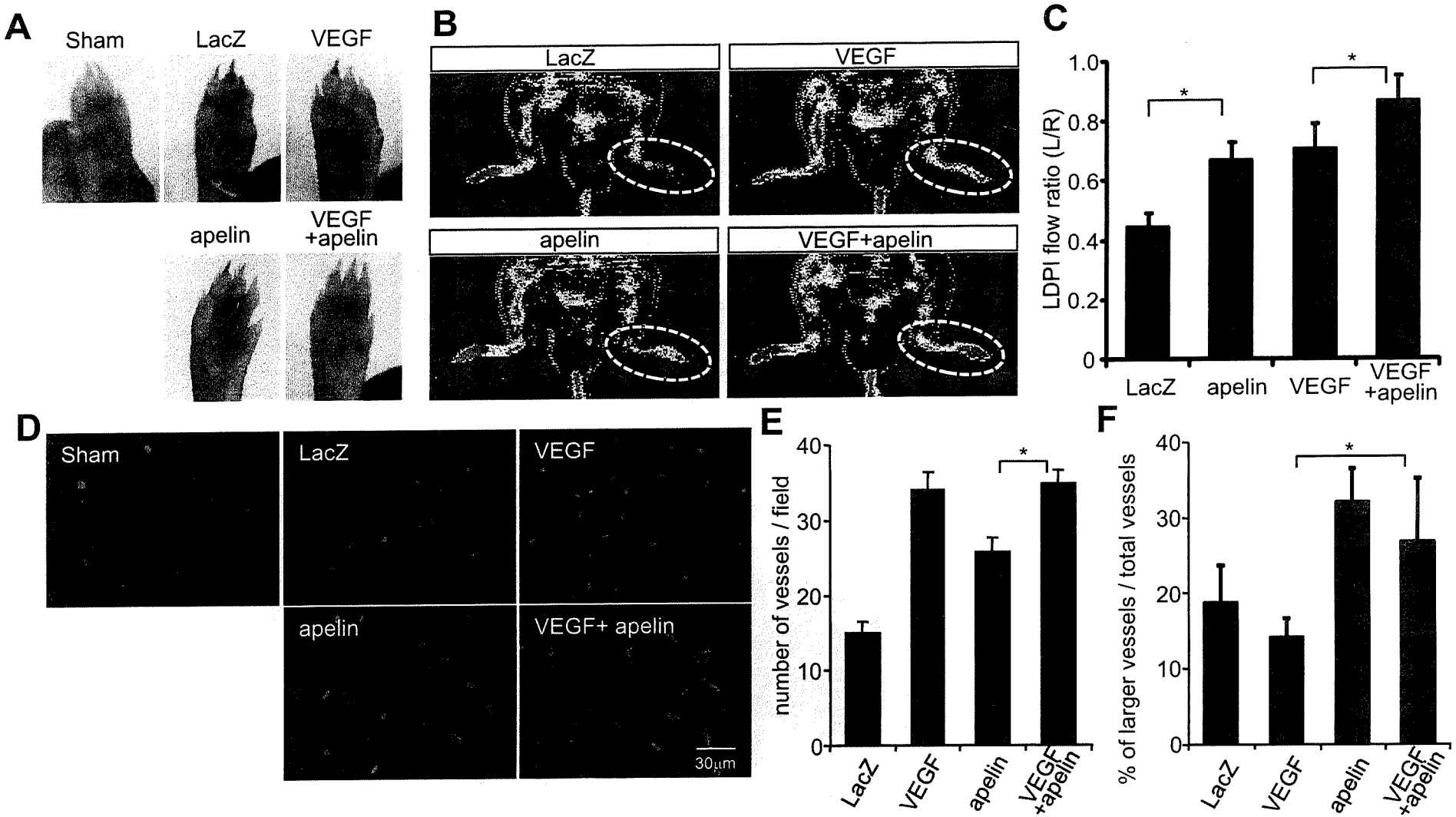

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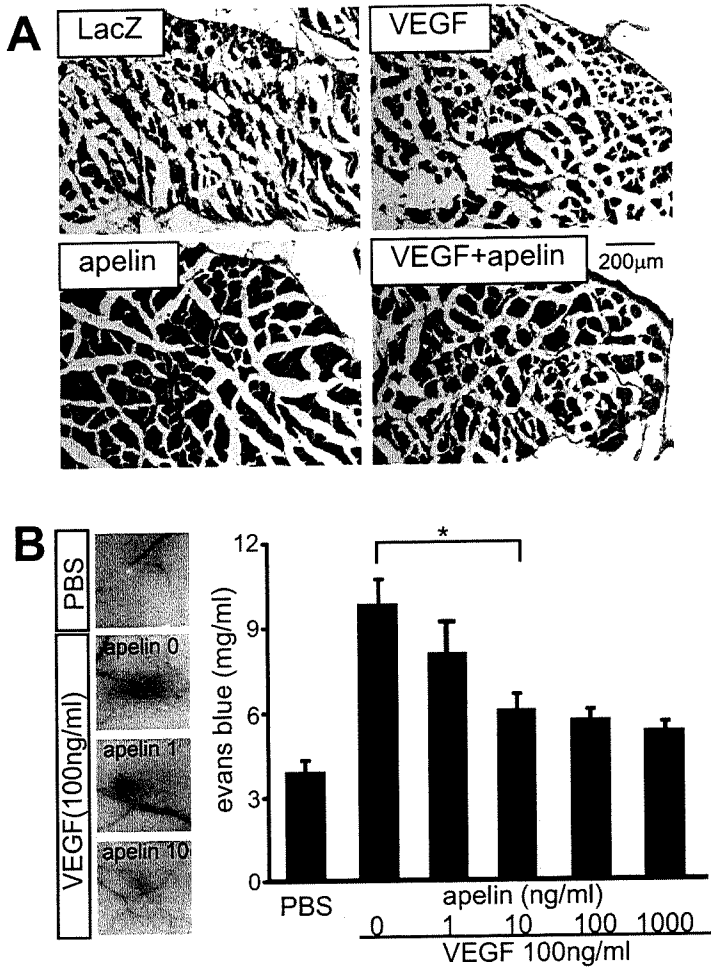

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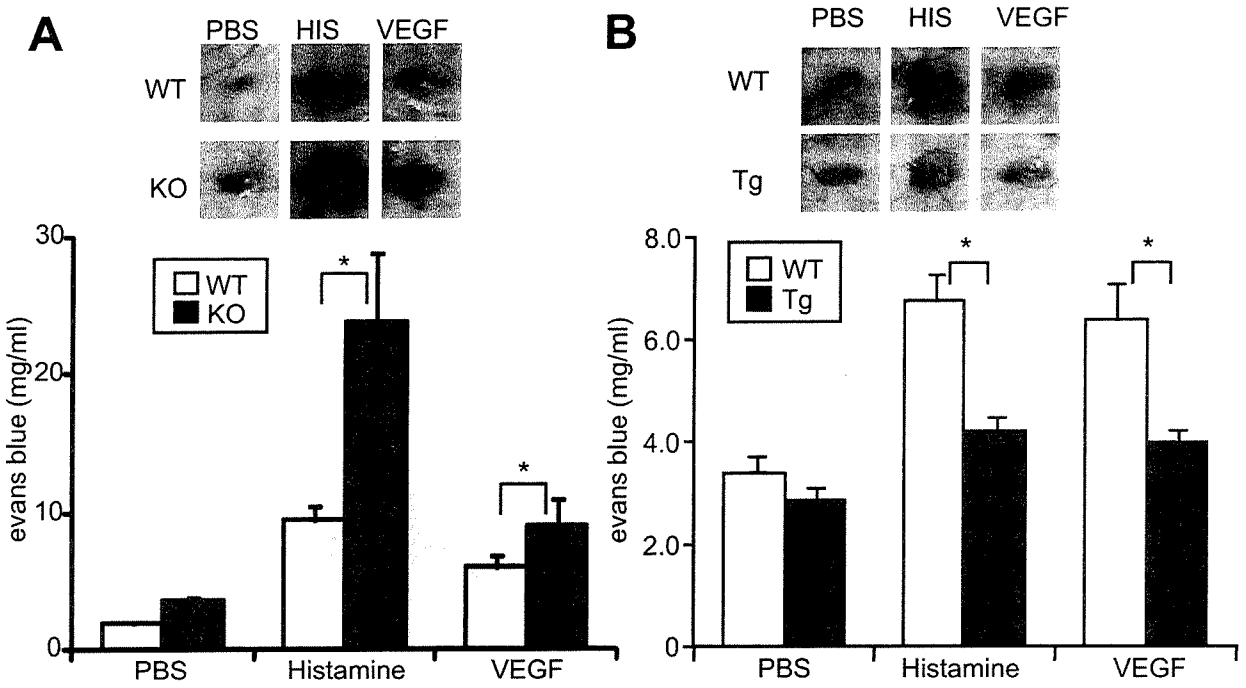

Figure 5 Kidoya H. et al.

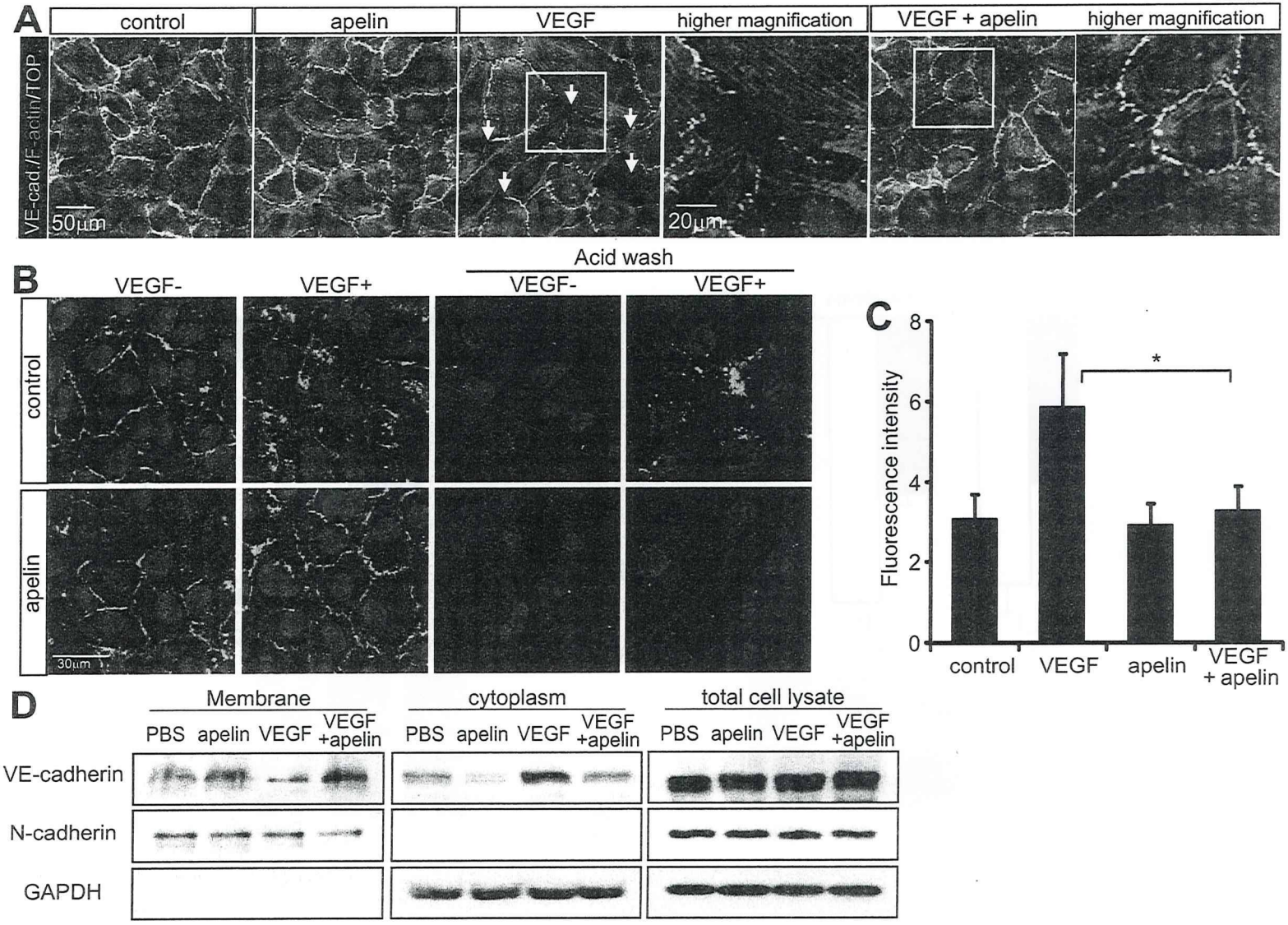

Figure 6 Kidoya H. et al.

Identification of targets of Prox1 during in vitro vascular differentiation from embryonic stem cells: functional roles of HoxD8 in lymphangiogenesis

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Summary

During lymphatic development, Prox1 plays central roles in the differentiation of blood vascular endothelial cells (BECs) into lymphatic endothelial cells (LECs), and subsequently in the maturation and maintenance of lymphatic vessels. However, the molecular mechanisms by which Prox1 elicits these functions remain to be elucidated. Here, we identified FoxC2 and angiopoietin-2 (Ang2), which play important roles in the maturation of lymphatic vessels, as novel targets of Prox1 in mouse embryonic-stem-cell-derived endothelial cells (MESECs). Furthermore, we found that expression of HoxD8 was significantly induced by Prox1 in MESECs, a finding confirmed in human umbilical vein endothelial cells (HUVECs) and human dermal LECs (HDLECs). In mouse embryos, HoxD8 expression was significantly higher in LECs than in BECs. In a model of inflammatory lymphangiogenesis, diameters of lymphatic vessels of the diaphragm were increased by adenovirally transduced HoxD8. We also found that HoxD8 induces Ang2 expression in HDLECs and HUVECs. Moreover, we found that HoxD8 induces Prox1 expression in HUVECs and that knockdown of HoxD8 reduces this expression in HDLECs, suggesting that Prox1 expression in LECs is maintained by HoxD8. These findings indicate that transcriptional networks of Prox1 and HoxD8 play important roles in the maturation and maintenance of lymphatic vessels.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/21/3923/DC1

Key words: HoxD8, Prox1, Lymphangiogenesis, Angiopoietin-2

Introduction

The lymphatic system plays very important roles in the maintenance of tissue-fluid homeostasis and mediation of the afferent immune response (Karpanen and Alitalo, 2008). Insufficiency or obstruction of function of this system results in lymphedema, characterized by leaking of tissue fluid and swelling of affected tissue. Furthermore, in many types of cancer, the lymphatic vessels provide a major pathway for tumor metastasis to lymph nodes. Understanding of the molecular mechanisms that govern the formation of the lymphatic system is thus of crucial importance.

Embryonic lymphatic endothelial cells (LECs) arise by sprouting from the jugular veins and migrate towards mesenchymal cells expressing vascular endothelial growth factor (VEGF)-C, leading to the formation of the primary lymphatic plexus (Oliver, 2004). During these processes, the prospero-related homeobox-1 (Prox1) transcription factor marks the first LECs within embryonic cardinal veins. Importantly, in Prox1-deficient mice, the migration of endothelial cells expected to express Prox1 towards VEGF-C is arrested, resulting in complete lack of the lymphatic vasculature and embryonic lethality (Wigle and Oliver, 1999; Wigle et al., 2002). Transcription-profiling analyses have shown that Prox1 induces the expression of various LEC markers – including VEGFR3, a receptor for VEGF-C – in human dermal microvascular endothelial cells (HDMECs) (Hong et al., 2002; Petrova et al., 2002). Recently, we also showed that Prox1 inhibits sheet formation of mouse embryonic stem (ES)-cell-derived endothelial cells (MESECs) and induced various LEC markers, such as integrin α9 (Mishima et al., 2007). Furthermore, we confirmed that Prox1 induces integrin-α9 expression in human umbilical vein endothelial cells (HUVECs) and human dermal LECs (HDLECs), and found that integrin α9 is required for Prox1-induced inhibition of sheet formation and promotion of migration towards VEGF-C. Interestingly, Prox1 also downregulates the expression of blood vascular endothelial cell (BEC) markers such as VEGFR2 (Petrova et al., 2002; Mishima et al., 2007), suggesting that Prox1 regulates the differentiation of embryonic BECs to LECs by reprogramming gene expression profiles.

The primary lymphatic plexus, which is composed of thin lymphatic vessels, undergoes enlargement and maturation to form collecting lymphatic vessels that have characteristics different from those of primary lymphatic vessels, such as valve formation and attachment of smooth-muscle cells. FoxC2 transcription factor and angiopoietin-2 (Ang2), a ligand for Tie2 receptor tyrosine kinase, have been implicated in these maturation processes (Gale et al.,

2002; Dellinger et al., 2008; Petrova et al., 2004). Prox1 expression is maintained in mature LECs, and is required for maintenance of the expression of LEC markers and characteristics of LECs (Mishima et al., 2007; Srinivasan et al., 2007; Johnson et al., 2008). However, the molecular mechanisms by which Prox1 regulates the maturation of lymphatic vessels remain to be elucidated.

In the present study, we identified novel target genes of Prox1 in MESECs. Expression of FoxC2 and Ang2 was induced by Prox1. Furthermore, we found that Prox1 induces the expression of HoxD8, which is capable of increasing the caliber of lymphatic vessels in an in vivo model of inflammatory lymphangiogenesis. These findings suggest that Prox1 induces the expression of a distinct group of genes to promote the maturation of lymphatic vessels.

Results

Identification of Prox1 targets in mouse ES-cell-derived endothelial cells

We previously showed that *Prox1* transgene expression in MESECs induces the expression of a group of genes that induce the differentiation of BECs to LECs (Mishima et al., 2007). To further identify the Prox1 target genes that are involved in lymphatic development, we performed cDNA microarray analysis using endothelial cells derived from mouse ES cells carrying a tetracycline (Tc)-inducible *Prox1* transgene. Among approximately 400 genes whose levels of expression were regulated by Prox1 (supplementary material Tables S1 and S2), we found that expression of integrin α9, cyclin E1 and fibroblast growth factor receptor 3 (FGFR3), all of which have been reported to be Prox1 targets (Mishima et al., 2007; Petrova et al., 2002; Shin et al., 2006), was induced by Prox1 (Table 1). These findings suggest that cDNA microarray analysis identified an appropriate set of Prox1 targets.

Prox1 induces the expression of Ang2 and FoxC2

Among the candidate molecules of Prox1 targets identified by the cDNA microarray analysis, we next searched for factors that have been implicated in lymphatic formation. Although Ang2 and FoxC2 have been implicated in the maturation of lymphatic vessels (Gale et al., 2002; Dellinger et al., 2008; Petrova et al., 2004), the molecular mechanisms by which their expression is regulated in LECs have not yet been elucidated. Because we found that their expression was induced by Prox1, we first confirmed the effects of Prox1 on the expression of Ang2 and FoxC2 in MESECs by quantitative reverse transcriptase (RT)-PCR analysis. As previously reported (Mishima et al., 2007), the removal of Tc from culture of VEGFR2-expressing (VEGFR2+) cells derived from Tc-Prox1 ES cells induced *Prox1* transgene expression, whereas that from Tc-Empty ES cells did not (Fig. 1A,E). As shown in Fig. 1B,C, *Prox1* transgene expression significantly promoted the expression of Ang2 and FoxC2.

We next examined whether these effects of Prox1 are observed in other types of endothelial cells. HUVECs were infected with adenovirus encoding Prox1 (Ad-Prox1) or non-coding adenoviruses (Ad-Null) (Fig. 2Aa,E). When *Prox1* was transduced into HUVECs, Ang2 expression was significantly induced (Fig. 2Ba,E). By contrast, FoxC2 expression was only mildly induced (Fig. 2Ca,E).

Because LECs, but not BECs, express Prox1 endogenously (Fig. 2Ab,E), we expected that the levels of expression of endogenous Ang2 and FoxC2 might be higher in HDLECs than in HUVECs. As shown in Fig. 2Bb and E, Ang2 expression was higher in HDLECs than in HUVECs. However, the levels of expression of *FoxC2* mRNA were comparable in HDLECs and HUVECs, and the expression of FoxC2 protein was only slightly higher in HDLECs than in HUVECs (Fig. 2Cb,E).

We previously showed that endogenous Prox1 expression in HDLECs is required for maintenance of the expression of LEC markers, including VEGFR3 and integrin α9 (Mishima et al., 2007).

Table 1. Examples of genes that are upregulated by Prox1

Category	Gene symbol	Gene name
Transcription factors	*Foxc1*	Forkhead box C1
	Foxc2	Forkhead box C2
	Foxp1	Forkhead box P1
	Hoxd8	Homeobox D8
	Hoxd9	Homeobox D9
	Id1	Inhibitor of DNA binding 1
	Id2	Inhibitor of DNA binding 2
	Id3	Inhibitor of DNA binding 3
	Id4	Inhibitor of DNA binding 4
	Irx3	Iroquois related homeobox 3
	Irx5	Iroquois related homeobox 5
	Klf4	Kruppel-like factor 4
	Snai1	Snail homolog 1
Adhesion molecules	*Itga8*	Integrin alpha 8
	Itga9	Integrin alpha 9
Growth factors	*Angpt2*	Angiopoietin 2
	Fgf13	Fibroblast growth factor 13
	Igf1	Insulin-like growth factor 1
Cytokines and chemokines	*Cxcl12*	Chemokine (C-X-C motif) ligand 12
Cell-cycle control	*Aurka*	Aurora kinase A
	Aurkb	Aurora kinase B
	Ccna2	Cyclin A2
	Ccnb1	Cyclin B1
	Ccne1	Cyclin E1
	Ccnf	Cyclin F
	Plk4	Polo-like kinase 4
	Sgol1	Shugoshin-like 1
	Skp2	S-phase kinase-associated protein 2 (p45)
Receptors	*Acvr2b*	Activin receptor IIB
	Fgfr3	Fibroblast growth factor receptor 3
	Pdgfra	Platelet derived growth factor, alpha polypeptide
	Lifr	Leukemia inhibitory factor receptor
	Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19
Other	*Fst*	Follistatin
	Hmgb3	High mobility group box 3
	Jag1	Jagged 1
	Plce1	Phospholipase C, epsilon 1
	Prkcm	Protein kinase C, mu
	Prom1	Prominin 1, CD133
	Racgap1	Rac GTPase-activating protein 1
	Thbs4	Thrombospondin 4

To identify genes whose expression was increased by Prox1 in MESECs, three criteria were applied to 45102 genes in the GeneChip Mouse Genome 430 2.0 Array. (1) Signal intensities in Tc-Prox1/Tc– (MESECs expressing the *Prox1* transgene) were >36, and given 'present' calls. (2) Signals were increased by Prox1 more than twofold compared with control Tc-Prox1/Tc+ (MESECs not expressing the *Prox1* transgene). (3) Signals were not increased in Tc-Empty/Tc– compared with Tc-Empty/Tc+ (in order to remove genes whose expression is altered by Tc); 310 genes met these restrictions, some of which are listed here. Complete lists of genes regulated by Prox1 are included in supplementary material Tables S1 and S2.

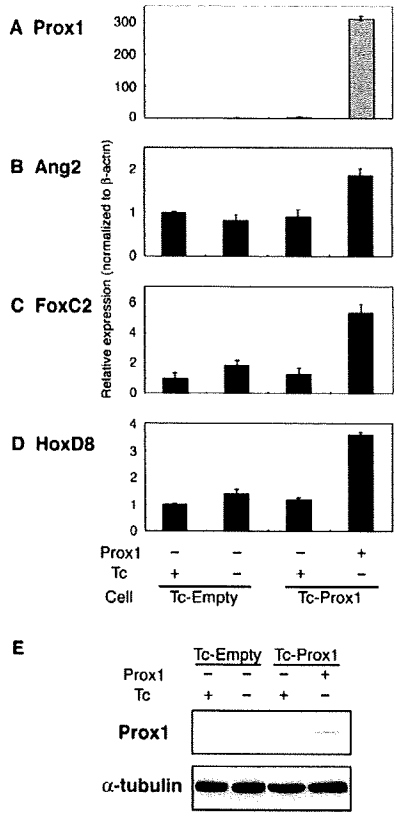

Fig. 1. Identification of Prox1 targets in MESECs. (A-D) Effects of the *Prox1* transgene (A; light grey bar) on expression of Ang2 (B), FoxC2 (C) and HoxD8 (D) were examined in MESECs by quantitative RT-PCR analyses. VEGFR2-expressing endothelial progenitor cells were sorted from differentiated ES cells carrying a tetracycline (Tc)-regulated transgene encoding mouse Prox1 (Tc-Prox1) or no transgene (Tc-Empty), and re-differentiated in the presence (+) or absence (−) of Tc. Expression of *Prox1* transgene was induced in the absence of Tc in Tc-Prox1 cells (A). Bars, s.d. (E) Levels of Prox1 (upper panel) and α-tubulin (lower panel; internal control) proteins were examined by immunoblotting.

To examine the roles of endogenous Prox1 in HDLECs, HDLECs were transfected with siRNAs for *Prox1* (siProx1) or negative control siRNA (siNC). When Prox1 expression was knocked-down (Fig. 2Ac,E), the expression levels of *Ang2* and *FoxC2* mRNAs were not significantly altered, and those of Ang2 and FoxC2 proteins were only weakly repressed (Fig. 2Bc,Cc,E). These results suggest that Prox1 is able to induce the expression of Ang2 in embryonic and mature endothelial cells, and that Prox1 differentially induces FoxC2 expression in embryonic and mature endothelial cells.

HoxD8 is a novel target of Prox1 in LECs

To identify novel targets of Prox1 involved in lymphangiogenesis, we focused on transcription factors whose expression was upregulated by Prox1 in MESECs. In addition to FoxC2, we identified homeobox transcription factors including HoxD8 and HoxD9 (Table 1). Homeobox (Hox) genes encode transcription factors, which play crucial roles in cell proliferation, migration and differentiation (Pearson et al., 2005). Hox proteins bind DNA weakly, but gain specificity and affinity by interaction with other proteins, termed modulators or cofactors. We therefore examined whether HoxD8 and/or HoxD9 form transcription complexes and function in a lymphatic-vessel-specific fashion. Some Hox-family members, such as HoxA9 and HoxD3, have been implicated in development of the cardiovascular system and endothelial-cell activation during neovascularization (Bruhl et al., 2004; Boudreau et al., 2004). However, the roles of HoxD8 in the vascular system have not yet been reported. Furthermore, no Hox-family members have yet been implicated in lymphangiogenesis.

Induction of HoxD8 expression by Prox1 in MESECs was confirmed by quantitative RT-PCR analysis (Fig. 1D). We also found that Prox1 induces HoxD8 expression in HUVECs (Fig. 2Da), suggesting that HoxD8 is induced by Prox1 in various types of endothelial cell.

We next examined the expression of HoxD8 in LECs. As shown in Fig. 2Db, the level of expression of HoxD8 in HDLECs was higher than that in HUVECs. Furthermore, knockdown of Prox1 expression in HDLECs led to a decrease in HoxD8 expression (Fig. 2Dc). These results suggest that the amount of *HoxD8* transcripts is increased during Prox1-induced differentiation of BECs into LECs and that this increased expression is maintained by Prox1 in LECs, although these results were not confirmed at protein level because of the lack of antibodies that are able to detect endogenous HoxD8 proteins.

Among paralogous group-8 Hox genes, *HoxD8* alone is expressed in LECs

HoxD8 is one of the paralogous group-8 Hox genes: this group also includes *HoxB8* and *HoxC8*. HoxD8-mutant mice exhibited two anterior homeotic transformations of thoracic vertebrae, although both of these phenotypes exhibited very low penetrance (van den Akker et al., 2001). Phenotypes of double and triple mutants revealed that HoxB8, HoxC8 and HoxD8 have redundant functions at the upper thoracic and sacral levels, including positioning of the hindlimbs. To examine whether other group-8 Hox genes are involved in lymphangiogenesis, we determined the expression of *HoxB8*, *HoxC8* and *HoxD8* in HUVECs and HDLECs by conventional RT-PCR analysis. Whereas all paralogous group-8 Hox genes were expressed in HUVECs, only *HoxD8* was expressed in HDLECs (Fig. 3A). Furthermore, Prox1 failed to induce the expression of HoxB8 and HoxC8 in HUVECs (Fig. 3B), whereas it induced HoxD8 expression (Fig. 2Da), suggesting important functions for HoxD8 among the paralogous group-8 Hox genes in LECs.

HoxD8 is expressed in LECs of mouse embryos

Previous studies showed that HoxD8 is expressed along the anteroposterior axis in the nervous system, vertebral column, gut and kidney at the posterior end of the embryo, and that expression of it extends anteriorly (Izpisùa-Belmonte et al., 1990; van den Akker et al., 2001). However, expression of it in the developing lymphatic vessels has not been reported. To examine the in vivo significance of our finding that Prox1 induces HoxD8 expression in various cultured endothelial cells, we determined the levels of expression of HoxD8 in BECs and LECs derived from mouse embryos. BECs and LECs were isolated from embryonic day 14.5 (E14.5) mouse embryos by fluorescence-activated cell sorting (FACS) using antibodies for CD31 and LYVE-1, respectively, as described in Materials and Methods (Hirashima et al., 2008). The level of expression of HoxD8 in LECs was significantly higher than that in BECs (Fig. 4). This result suggests that the in vitro induction

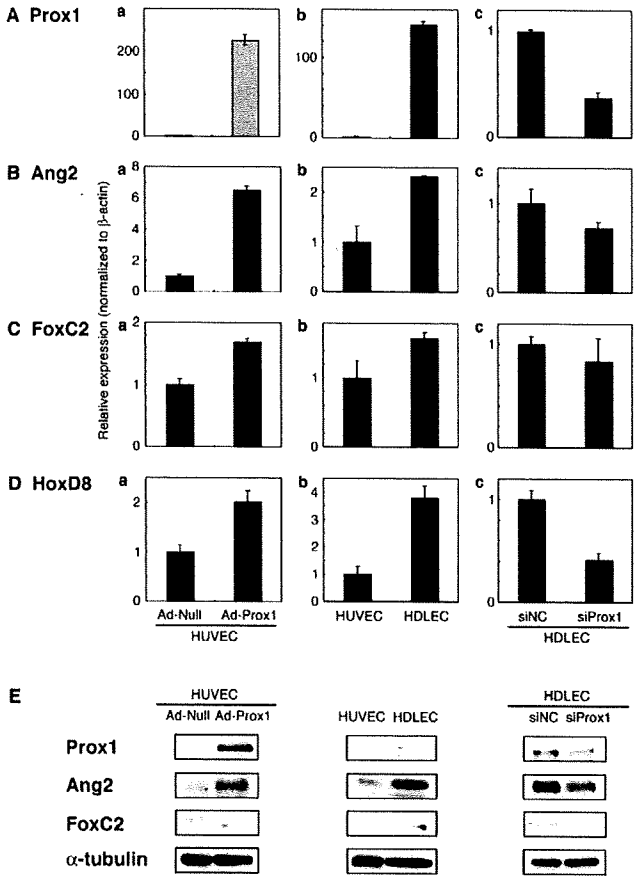

Fig. 2. Roles of Prox1 in the expression of its targets in BECs and LECs. (Aa-Da) Effect of gain-of-function of Prox1 via the *Prox1* transgene (A; light grey bar) on the endogenous expression of Ang2 (B), FoxC2 (C) and HoxD8 (D). HUVECs were infected with adenovirus encoding Prox1 (Ad-Prox1) or non-coding adenovirus (Ad-Null), followed by quantitative RT-PCR analyses. (Ab-Db) Expression of endogenous Prox1 (A; dark grey bar), Ang2 (B), FoxC2 (C) and HoxD8 (D) was examined in native HUVECs and HDLECs. (Ac-Dc) Effect of loss-of-function of Prox1 (A) on the expression of Ang2 (B), FoxC2 (C) and HoxD8 (D). HDLECs were transfected with siRNA for *Prox1* (siProx1) or negative control siRNA (siNC), followed by quantitative RT-PCR analyses. Bars, s.d. (E) Levels of Prox1, Ang2, FoxC2 and α-tubulin (internal control) proteins were examined by immunoblotting.

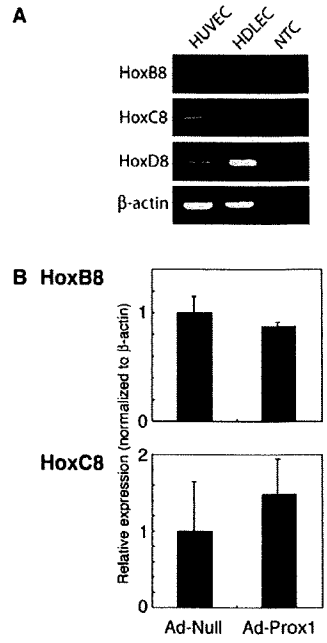

Fig. 3. Expression of paralogous group-8 Hox genes in BECs and LECs. (A) Expression of HoxB8, HoxC8 and HoxD8 in HUVECs and HDLECs was examined by conventional RT-PCR analysis. β-actin was used as an internal control. NTC, no template control. (B) Effect of the *Prox1* transgene on the expression of HoxB8 (top) and HoxC8 (bottom). HUVECs were infected with adenovirus encoding Prox1 (Ad-Prox1) or non-coding adenovirus (Ad-Null), followed by quantitative RT-PCR analyses. Bars, s.d.

of HoxD8 expression by Prox1 demonstrated in the present study might mimic the process of embryonic lymphatic development.

HoxD8 increases the diameters of lymphatic vessels in an in vivo mouse model of inflammatory lymphangiogenesis

To examine the roles of HoxD8 in lymphangiogenesis, we used a mouse model of chronic inflammation (Iwata et al., 2007). In this model, chronic aseptic peritonitis was induced by repeated intraperitoneal injection of thioglycollate medium, a proinflammatory agent, into immunocompetent BALB/c mice. As shown in Fig. 5A, outgrowths of lymphatic vessels were detected by anti-LYVE-1 staining in the inflammatory plaques formed on the peritoneal surface of the diaphragm. When adenoviruses encoding β-galactosidase (*lacZ*) were injected intraperitoneally in combination with thioglycollate medium, these lymphatic vessels were stained for anti-β-galactosidase (Fig. 5A), suggesting that newly generated lymphatic vessels are transduced by adenoviruses.

When adenoviruses encoding HoxD8 were injected, the diameters of lymphatic vessels increased significantly compared with those injected with adenoviruses encoding β-galactosidase (Fig. 5B,C). These findings suggest that HoxD8 regulates the caliber of lymphatic vessels.

The inflammatory plaques formed on the surface of the diaphragm consist of macrophages that express VEGF-A, VEGF-C and VEGF-D (Iwata et al., 2007). To examine whether HoxD8 that was transduced into macrophages alters the expression of angiogenic and/or lymphangiogenic factors, we determined the levels of expression of VEGF-A, VEGF-C and VEGF-D in the plaques that mainly consisted of macrophages. As shown in Fig. 5D, levels of expression were not statistically changed by adenovirally transduced *HoxD8*. These results suggest that the effects of HoxD8 on the lymphatic vessels are not indirectly mediated through VEGF-A, VEGF-C or VEGF-D secreted from macrophages.

HoxD8 induces the expression of Ang2

Tie2 receptor tyrosine kinase is activated by its ligand, Ang1, and transduces signals to induce maturation of blood and lymphatic vessels. Ang2 is considered as an antagonist or agonist for Tie2, depending on endothelial-cell status. Furthermore, lymphatic phenotypes in knockout mice deficient for Ang2 were rescued by knocking-in the *Ang1* gene in the *Ang2* locus (Gale et al., 2002), suggesting that Ang2 might function as an agonist for Tie2 to induce

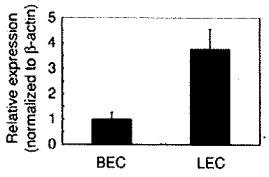

Fig. 4. Expression of HoxD8 in BECs and LECs derived from mouse embryos. E14.5 mouse embryos were dissociated, followed by FACS sorting with anti-CD45, -LYVE-1 and -CD31 antibodies, as described in Materials and Methods (Hirashima et al., 2008). Equivalent amount of total RNAs prepared from CD45– CD31+ LYVE-1– BEC fractions (1.5% in CD45– cells) and CD45– CD31– LYVE-1+ LEC fractions (0.2% in CD45– cells) were subjected to quantitative RT-PCR analysis of transcripts for HoxD8. Bars, s.d.

maturation of lymphatic vessels. To examine the relationship between HoxD8 and Ang2, both of which are targets of Prox1, we examined the level of expression of Ang2 in HDLECs transfected with siRNAs for *HoxD8* (siHoxD8) or siNC (Fig. 6A). A decrease in HoxD8 expression (Fig. 6A, top) resulted in partial decrease in the amounts of transcripts (Fig. 6A, bottom) and proteins (Fig. 6B) of Ang2 in HDLECs.

We next examined the effects of gain-of-function of HoxD8 on Ang2 expression in HUVECs. When *HoxD8* as well as *Prox1* was transduced into HUVECs, the level of *Ang2* transcripts was increased (Fig. 6C), which was confirmed at protein level (Fig. 6D). These results suggest that both Prox1 and HoxD8, one of the targets of Prox1, induce Ang2 expression.

HoxD8 maintains Prox1 expression in LECs

Although Prox1 expression is maintained in mature LECs, the molecular mechanisms by which it is maintained have not yet been elucidated. We therefore examined whether HoxD8, the expression of which is induced by Prox1, plays any roles in the regulation of Prox1 expression. When HoxD8 expression in HDLECs was knocked-down by siRNA (Fig. 6A, top), endogenous expression of Prox1 decreased (Fig. 7A), suggesting that HoxD8 is required for the maintenance of endogenous Prox1 expression in LECs. Moreover, we found that the expression of a *HoxD8* transgene (Fig. 7B, top) induced endogenous Prox1 expression in HUVECs (Fig. 7B, bottom). These results were confirmed at the protein level by western blot analysis (Fig. 7C). Taken together, these findings suggest that HoxD8 plays an important role in positive feedback of Prox1 expression in LECs.

Discussion

In the present study, we identified a novel group of Prox1 targets using cDNA microarray analysis of MESECs. We found that Prox1 induces the expression of Ang2 and FoxC2, both of which are involved in the maturation of lymphatic vessels (Gale et al., 2002; Dellinger et al., 2008; Petrova et al., 2004). We also found that Prox1 induces the expression of HoxD8, which increased the diameter of lymphatic vessels in an in vivo model of inflammatory lymphangiogenesis. Furthermore, HoxD8 maintains endogenous Prox1 expression in LECs.

Although previous studies (Petrova et al., 2002; Hong et al., 2002; Shin et al., 2006) identified various LEC markers as Prox1 targets using microarray analyses of mature endothelial cells (HDMECs), they did not find that Ang2 or FoxC2 expression was upregulated

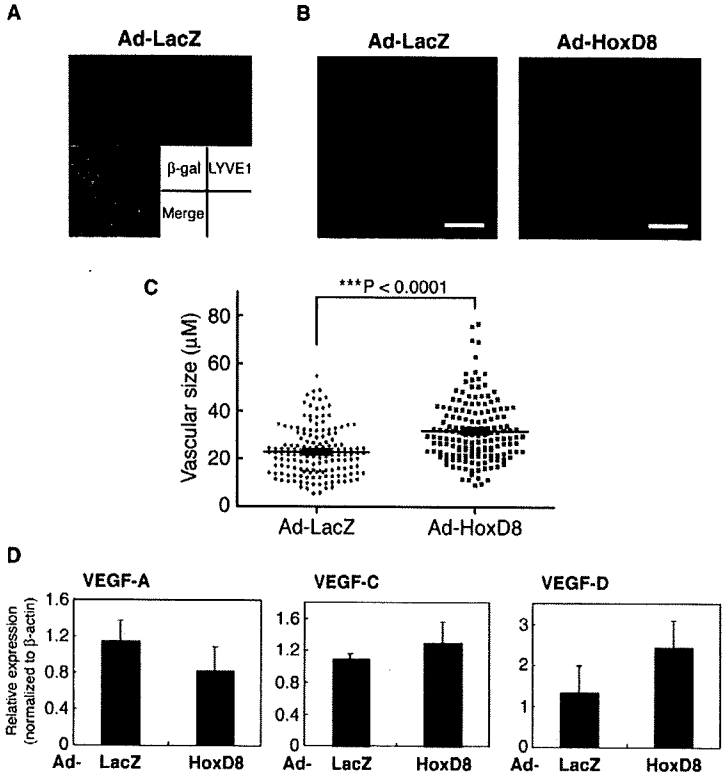

Fig. 5. Effects of HoxD8 on lymphangiogenesis in a mouse model of chronic aseptic peritonitis. (A) Repeated intraperitoneal injection of thioglycollate medium in combination with adenoviruses encoding β-galactosidase resulted in the formation of inflammatory plaques on the peritoneal surface of the diaphragm. Whole-mount staining with anti-LYVE-1 (red) and anti-β-galactosidase (green) is shown. (B) Adenoviruses encoding β-galactosidase or HoxD8 were injected in combination with thioglycollate medium, followed by immunostaining for LYVE-1. Scale bars: 200 μm. (C) Diameters of lymphatic vessels in plaques were quantified. Points represent individual values and long bars represent mean of the diameters of LYVE-1-positive vessels. Short bars, s.e. (150 vessels from three mice were examined); ***$P<0.0001$. (D) Effects of HoxD8 on expression of angiogenic and/or lymphangiogenic factors in plaques. Plaques that mainly consist of macrophages were obtained from the peritoneal surface of the diaphragm, followed by quantitative RT-PCR analyses for VEGF-A, VEGF-C and VEGF-D. Bars, s.e.; $P>0.05$.

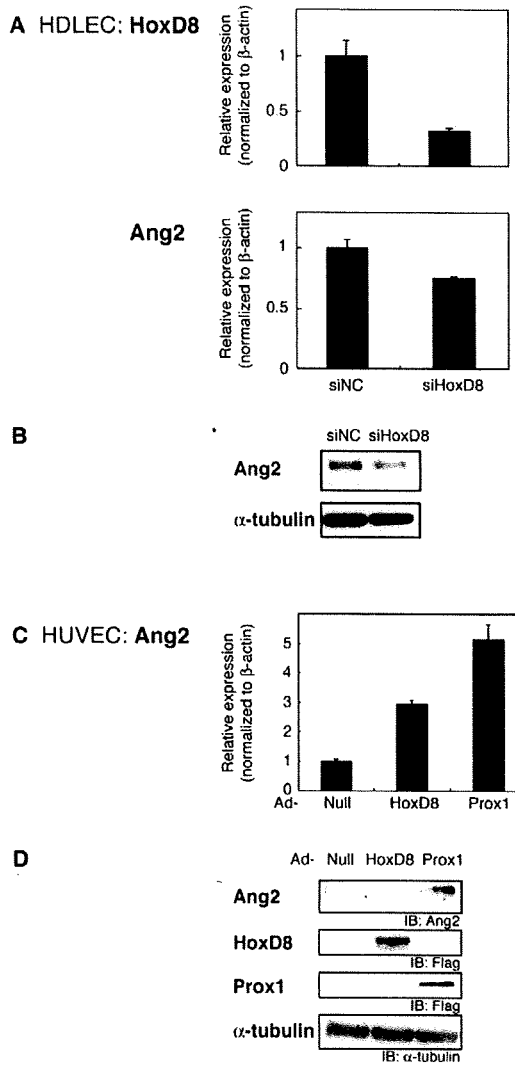

Fig. 6. Effects of HoxD8 on Ang2 expression in LECs and BECs. (A) Effect of loss-of-function of HoxD8 on expression of Ang2 in LECs. HDLECs were transfected with siRNA for *HoxD8* (siHoxD8) or negative control siRNA (siNC), followed by quantitative RT-PCR analyses for HoxD8 (top) and Ang2 (bottom). (B) Levels of Ang2 (top) and α-tubulin (bottom; internal control) proteins were examined by immunoblotting. (C) Effect of gain-of-function of HoxD8 and Prox1 on expression of Ang2. HUVECs were infected with adenovirus encoding HoxD8 (Ad-HoxD8) or Prox1 (Ad-Prox1), or non-coding adenovirus (Ad-Null), followed by quantitative RT-PCR analyses for Ang2. Bars, s.d. (D) Protein levels of endogenous Ang2 and α-tubulin (internal control) were examined by immunoblotting (IB). Expression of adenovirally introduced FLAG-tagged HoxD8 and Prox1 proteins was confirmed by immunoblotting (IB) using an anti-FLAG antibody.

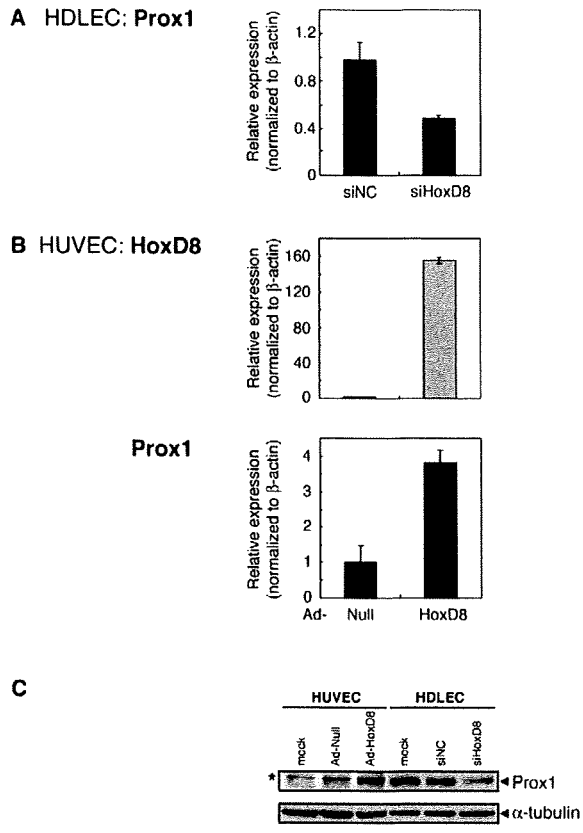

Fig. 7. Effects of HoxD8 on Prox1 expression in LECs and BECs. (A) Effect of loss-of-function of HoxD8 on the expression of Prox1 in LECs. HDLECs were transfected with siRNA for HoxD8 (siHoxD8) or negative control siRNA (siNC), followed by quantitative RT-PCR analyses for Prox1. (B) Effect of gain-of-function of HoxD8 on expression of Prox1. HUVECs were infected with adenovirus encoding HoxD8 (Ad-HoxD8) or non-coding adenovirus (Ad-Null), followed by quantitative RT-PCR analyses for HoxD8 transgene (top; light grey) and endogenous Prox1 (bottom). Bars, s.d. (C) Expression of endogenous Prox1 protein in HUVECs and HDLECs is shown by western blotting. Asterisk represents non-specific bands.

by Prox1. As shown in Fig. 2Ca, induction of FoxC2 by Prox1 in HUVECs was not as potent as that in MESECs (Fig. 1C), which might explain why previous studies did not identify these genes as targets of Prox1. By contrast, VEGFR3 expression was not upregulated by Prox1 in our analysis. VEGFR3 expression has been reported to be high in embryonic endothelial cells (Tammela et al., 2008), which might have led to the failure of VEGFR3 induction by Prox1 in MESECs. These results suggest that MESECs might represent a different status of endothelial cells from HDMECs and HUVECs, and confirm the usefulness of the cDNA microarray analysis performed in the present study.

Ablation of Ang2 or FoxC2 does not result in defects in the formation of the primary lymphatic plexus, which is severely affected in Prox1-knockout mice (Wigle and Oliver, 1999). However, remodeling and maturation of lymphatic vessels is defective in mice deficient for Ang2 or FoxC2 (Gale et al., 2002; Dellinger et al., 2008; Petrova et al., 2004). In Ang2-deficient mice, lymphatic vessels do not mature or they exhibit a collecting vessel phenotype, without proper recruitment of smooth-muscle cells or postnatal remodeling (Dellinger et al., 2008). FoxC2-knockout mice exhibit abnormal lymphatic vascular patterning, increased pericyte recruitment of lymphatic vessels and defective valve formation (Petrova et al., 2004), which lead to the phenotype of lymphedema-distichiasis syndrome (LD). The present finding that Prox1 induces the expression of Ang2 and FoxC2 suggests

that Prox1 indirectly induces the maturation of embryonic lymphatic vessels through induction of a group of maturation-inducing factors.

We identified HoxD8 as a novel target of Prox1. Hox genes play very important roles during embryonic development. We found that HoxD8 increases the caliber of lymphatic vessels in a model of inflammatory lymphangiogenesis. However, this effect of HoxD8 on the caliber size did not mimic the functions of Prox1. We found that adenoviruses encoding Prox1 significantly decreased the diameters of lymphatic vessels (supplementary material Fig. S1A,B) and that Prox1 seemed to increase the number of lymphatic vessels as compared with the control (supplementary material Fig. S1A). Both Prox1 and HoxD8 induced the expression of Ang2, which is involved in the remodeling and maturation of lymphatic vessels. Similar to Prox1, however, adenoviruses encoding Ang2 significantly decreased the diameters of lymphatic vessels (supplementary material Fig. S1C,D), suggesting that Ang2 is not involved in the HoxD8-mediated increase of the caliber of lymphatic vessels. Several lines of evidence have suggested that Ang2 that is present in smooth-muscle cells plays important roles in the formation of lymphatic systems (Gale et al., 2002; Dellinger et al., 2008). The effects of adenovirally introduced Ang2 on the lymphatic formation (supplementary material Fig. S1C,D) might mimic the effects caused by Ang2 secreted by smooth-muscle cells.

Furthermore, studies using in-vitro-cultured HDLECs revealed that the decrease in HoxD8 expression failed to affect the number of HDLECs (supplementary material Fig. S2A). We also found that the decreased HoxD8 expression did not alter the migration of HDLECs towards VEGF-C (supplementary material Fig. S2B). Nonetheless, adenovirally introduced HoxD8 might have caused inflammatory cells to secrete a different profile of lymphangiogenic factors. Although we showed that HoxD8 does not significantly alter the expression of VEGF-A, VEGF-C or VEGF-D in macrophages (Fig. 5D), it might have induced the expression of other cytokines that regulate the caliber size of lymphatic vessels. The molecular mechanisms by which HoxD8 regulates the diameter of lymphatic vessels remain to be elucidated in the future.

We found that both Prox1 and HoxD8 induce Ang2 expression. During angiogenesis, FoxC2 controls Ang2 expression by direct activation of the Ang2 promoter and promotes maturation of blood vessels (Xue et al., 2008). Taken together with the present finding that Prox1 induces the expression of FoxC2 and HoxD8, these results suggest that the transcriptional networks that include Prox1, HoxD8 and FoxC2 play important roles in Ang2 expression during lymphatic development.

Recently, Sox18 was reported to play important roles in the initial induction of Prox1 expression in venous endothelial cells by direct binding to the *Prox1* promoter (François et al., 2008). Sox18 is expressed in a subset of venous endothelial cells prior to Prox1 expression and is coexpressed with Prox1 during the formation of the primary lymphatic plexus. However, Sox18 expression in LECs ceases during the maturation of lymphatic vessels. Endogenous Prox1 expression in LECs thus requires transcriptional inducers that are expressed in LECs. In the present study, we found that HoxD8 maintains endogenous Prox1 expression in LECs. These results suggest a novel positive-feedback-loop mechanism in which Prox1 expression is maintained by HoxD8, the expression of which is induced by Prox1. Our findings also indicate that HoxD8 plays crucial roles in the maturation and maintenance of lymphatic vessels, and suggest the possibility that HoxD8 can serve as a new therapeutic target in the treatment of inflammatory diseases, lymphedema, and lymphatic metastasis of tumors.

Materials and Methods

Cell culture and adenovirus infection

Maintenance, differentiation, culture and cell sorting of Tc-inducible MGZ5TcH2 ES cell lines were performed as described (Yamashita et al., 2000; Masui et al., 2005; Mishima et al., 2007). Briefly, VEGFR2-expressing endothelial progenitor cells derived from Tc-Empty or Tc-Prox1 ES cells were cultured in the absence or presence of Tc for 3 days. HUVECs and HDLECs were purchased from Sanko Junyaku and TaKaRa, and cultured in endothelial basal medium (EBM) containing 2% and 5% fetal bovine serum (FBS), respectively, supplemented with endothelial-cell growth supplement (TaKaRa). Recombinant adenoviruses encoding mouse Prox1 and HoxD8 were generated and used as described (Yamazaki et al., 2009). The *HoxD8* construct in the pSG5 vector was kindly provided by Vincenzo Zappavigna (University of Modena and Reggio Emilia, Italy). Purification of adenoviruses was performed using Virakit for adenovirus 5 (Virapur).

FACS analysis

We obtained LECs and BECs from mouse embryos as described previously (Hirashima et al., 2008). E14.5 mouse embryos, after removing their liver and spleen, were dissected and digested with 1.2 U/ml Dispase (Gibco), 50 μg/ml DNaseI (Roche) and 0.05% collagenase S-1 (Nitta Gelatin) to obtain single-cell suspension. After blocking Fc-receptors with an anti-mouse CD16/CD32 Fc receptor (FcR; PharMingen), all cells were stained with phycoerythrin (PE)-conjugated CD45 antibody (PharMingen) to sort CD45– non-hematopoietic cells using AutoMACS (Miltenyi Biotec). The cells were also stained with biotinylated anti-LYVE-1 antibody (ALY7; eBioscience) followed by allophycocyanin-conjugated streptoavidin (PharMingen) to visualize LYVE-1+ cells (LECs). The cells were also co-stained with a fluorescein isothiocyanate (FITC)-conjugated anti-PECAM-1/CD31 antibody (PharMingen) to visualize CD31+ cells (BECs). We sorted CD31+ LYVE-1– cells as BECs and CD31+ LYVE-1+ cells as LECs using FACS Vantage (Beckton Dickinson).

RNA isolation, and microarray and RT-PCR analyses

Total RNAs were extracted using the RNeasy Mini Kit (QIAGEN) to perform microarray and RT-PCR analyses. Oligonucleotide microarray analysis was performed using GeneChip Mouse Genome 430 2.0 Array (Affymetrix) according to the manufacturer's instructions. FileMaker Pro software (Filemaker) was used for statistical analysis. RNAs were reverse transcribed by random priming using Superscript III Reverse Transcriptase (Invitrogen). Expression of various Hox-family members was compared by RT-PCR analysis. PCR products were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide. Quantitative RT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green (Applied Biosystems). The primer sequences are shown in supplementary material Table S3.

RNA interference and oligonucleotides

siRNAs were introduced into cells using HiPerFect reagent (QIAGEN) according to the manufacturer's instructions. The target sequence for human *HoxD8* was 5′-UUUGUCUUCCUCUUCGUCUACCAGG-3′ (Stealth RNAi; Invitrogen), whereas that for human *Prox1* was 5′-CACCUUAUUCGGGAAGUGCAA-3′. Negative control was obtained from Invitrogen (Stealth RNAi Negative Control Med GC).

Immunohistochemistry, immunofluorescence microscopy and western blotting

Whole mounts of diaphragms were fixed with 10% neutral-buffered formalin for 1 hour at 4°C and washed overnight in phosphate-buffered saline (PBS) containing 10%, 15% and 20% sucrose at 4°C, followed by immunostaining. Immunostaining was carried out with anti-β-galactosidase (1:1000 dilution; Cappel) and anti-LYVE-1 (1:200 dilution; Abcam) antibodies. Stained specimens were examined using an LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for contrast manipulation and figure assembly. Antibodies to FLAG epitope and α-tubulin for western blot analysis were obtained from Sigma. Anti-Prox1 and -Ang-2 antibodies were from Chemicon and Abcam, respectively. Anti-human FoxC2 was described previously (Mani et al., 2007). Western blot analyses were performed as described (Watabe et al., 2003). The bound antibody was detected using a chemiluminescent substrate (ECL; Amersham) and a LAS-4000 Luminescent image analyzer (Fuji Photo Film).

Model of chronic aseptic peritonitis

BALB/c mice at 5 weeks of age, obtained from Charles River Laboratories, were used. The model of chronic aseptic peritonitis was described previously (Iwata et al., 2007). We intraperitoneally injected 2 ml of 3% thioglycollate medium (BBL thioglycollate medium, BD Biosciences) into BALB/c mice every 2 days for 2 weeks to induce peritonitis. Adenovirus encoding β-galactosidase or HoxD8 was also intraperitoneally injected twice per week during the same period. The mice were then

sacrificed, and their diaphragms excised and prepared for immunostaining as described above. Statistical analysis was performed using GraphPad Prism5 (GraphPad Software). Results were expressed as individual values and mean values ± s.e. Differences were evaluated by Mann-Whitney test and considered statistically significant at $P<0.05$. Plaques consisting of macrophages were obtained from the peritoneal surface of the diaphragm and treated with RNAlater (Ambion), followed by RNA isolation and quantitative RT-PCR analyses for VEGF-A, VEGF-C and VEGF-D.

Production of adenovirus

Recombinant adenoviruses encoding mouse Prox1 and HoxD8 were generated and used as described (Yamazaki et al., 2009). *Ang2* construct was kindly provided by Gou Young Koh (Korea Advanced Institute of Science and Technology, Republic of Korea).

Cell-proliferation assay

Cells were seeded at a density of 5×10^4 cells/well in 12-well plates and transfected with siRNAs using HiPerfect (QIAGEN). Cells were trypsinized and counted by a Coulter counter on day 2 after siRNA transfection. The experiments were performed in triplicate.

Chamber migration assay

Migration assay was performed as described previously (Mishima et al., 2007). As a chemoattractant, 100 ng/ml of recombinant VEGF-C (Calbiochem) were used.

We thank Vincenzo Zappavigna and Gou Young Koh for providing the *HoxD8* and *Ang2* plasmids, respectively, and all the members of the Department of Molecular Pathology of the University of Tokyo for discussion. This research was supported by KAKENHI (Grants-in-Aid for Scientific Research) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Enhancement of vascular progenitor potential by protein kinase A through dual induction of Flk-1 and Neuropilin-1

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Fine tuning of vascular endothelial growth factor (VEGF) signaling is critical in endothelial cell (EC) differentiation and vascular development. Nevertheless, the system for regulating the sensitivity of VEGF signaling has remained unclear. Previously, we established an embryonic stem cell culture reproducing early vascular development using Flk1 (VEGF receptor-2)$^+$ cells as common progenitors, and demonstrated that cyclic adenosine monophosphate (cAMP) enhanced VEGF-induced EC differentiation. Here we show that protein kinase A (PKA) regulates sensitivity of Flk1$^+$ vascular progenitors to VEGF signaling for efficient EC differentiation. Blockade of PKA perturbed EC differentiation and vascular formation in vitro and ex vivo. Overexpression of constitutive active form of PKA (CA-PKA) potently induced EC differentiation and vascular formation. Expression of Flk1 and Neuropilin-1 (NRP1), which form a selective and sensitive receptor for $VEGF_{165}$, was increased only in CA-PKA–expressing progenitors, enhancing the sensitivity of the progenitors to $VEGF_{165}$ by more than 10 times. PKA activation induced the formation of a $VEGF_{165}$, Flk1, and NRP1 protein complex in vascular progenitors. These data indicate that PKA regulates differentiation potential of vascular progenitors to be endothelial competent via the dual induction of Flk1 and NRP1. This new-mode mechanism regulating "progenitor sensitivity" would provide a novel understanding in vascular development and regeneration. (Blood. 2009;114:3707-3716)

Introduction

Vascular endothelial growth factor (VEGF) signaling is a key regulator of vascular development during embryogenesis as well as neovascularization in the adult.[1-3] Intensity of VEGF signaling is strictly controlled during vascular development through ligand-receptor interaction.[4,5] Flk1 (also designated as VEGF receptor-2) is tyrosine-phosphorylated much more efficiently than Flt1 (VEGF receptor-1) upon VEGF binding and is thought to be the major receptor in endothelial cells (ECs) for VEGF-induced responses.[6-8] Whereas Flk1-null mice die at embryonic day 8.5 (E8.5) to E9.5 with no organized blood vessels,[9] Flt1-null mice die at midgestation with vascular overgrowth and disorganization.[10,11] Flt1 tyrosine kinase–deficient homozygous mice, in which VEGF can bind to the cell-surface domain of Flt1 but cannot conduct kinase signaling, developed normal vessels and survived,[12] indicating that VEGF signal intensity on Flk1 is regulated by absorption of VEGF to the higher affinity receptor, Flt1. VEGF-A heterozygotes die early in gestation due to failure in vascular system formation.[13] On the other hand, 2- to 3-fold overexpression of VEGF-A from its endogenous locus results in aberrant heart development and lethality at E12.5 to E14,[14] indicating that strictly balanced VEGF function is important in normal embryogenesis.

Neuropilin-1 (NRP1) is a type 1 membrane protein, which is expressed in particular classes of developing neurons[15,16] and functions as a receptor for the class 3 semaphorins mediating semaphorin-elicited inhibitory axon guidance signals to neurons.[17,18] NRP1 is also expressed in ECs of blood vessels and endocardial cells of the heart.[15,16,19] NRP1, together with Flk1, forms a specific receptor for $VEGF_{165}$, an isoform of VEGF, and the Flk1-$VEGF_{165}$-NRP1 complex potently enhances Flk1 signaling.[20] Coexpression of NRP1 with Flk1 in cultured ECs enhanced $VEGF_{165}$ binding to Flk1 and VEGF-elicited mitogenic and chemotactic activities.[20] Overexpression of NRP1 in mouse embryos resulted in an excess production of blood vessels and malformed hearts.[15] NRP1-null mice die midway through gestation at E10.5 to E12.5 and exhibit defects in the heart, vasculature, and nervous system.[16] These findings indicate that NRP1 plays an important role in regulating vascular development, and Flk1/NRP1 system would be important for controlling VEGF signal intensity. However, the regulatory mechanisms of Flk1/NRP1 expression in vascular development are not fully elucidated.

In the early embryo and in differentiating embryonic stem (ES) cells, Flk1 expression marks a common progenitor for both blood and endothelium.[21-24] To elucidate the mechanisms underlying vascular development, we have developed a novel ES cell differentiation system that exhibits early vascular development using Flk1$^+$ cells as common progenitors for vascular cells.[25] ES cell–derived Flk1$^+$ cells can differentiate into both ECs and mural cells (MCs: vascular smooth muscle cells and pericytes) and form mature vascular-like structures in vitro. We recently reported that adrenomedullin/cyclic adenosine monophosphate (cAMP) pathway enhanced EC differentiation and induced arterial EC appearance from Flk1$^+$ progenitors.[26] In the present study, to further elucidate

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the mechanisms of EC differentiation from vascular progenitor cells, we examined roles of cAMP pathways in EC differentiation. Here we report that protein kinase A (PKA) activation remarkably enhanced EC differentiation and vascular formation from Flk1$^+$ vascular progenitors. PKA markedly increased the sensitivity of vascular progenitors to VEGF through dual up-regulation of Flk1 and NRP1 and played a pivotal role in EC differentiation. This new-mode molecular system regulating "progenitor sensitivity" would offer novel insights for vascular development as well as molecular targets for vascular regeneration strategies.

Methods

Generation of ES cells carrying an inducible expression unit in ROSA locus

Murine ES cell line (EStTA5-4), expressing tetracycline-transactivator protein and containing the puromycin resistance gene,[27] was a kind gift from Dr T. Era (Kumamoto University, Kumamoto, Japan). We generated an ES cell line (EStTA-ROSA) by inserting a knockin vector carrying loxP and mutant loxP, loxP511, recombination sites flanking neomycin-resistant and herpes simplex virus thymidine kinase (HSV-TK) genes (a kind gift from Dr K. Tanimoto [University of Tsukuba, Tsukuba, Japan] and Dr P. Soriano [Mt Sinai School of Medicine, New York, NY]) into ROSA locus[28] of EStTA5-4 (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Neomycin (200 μg/mL)–resistant colonies were selected and homogenous insertion of the loxP sites into ROSA locus was confirmed by Southern blotting using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics; supplemental Figure 1B-C). The probes were generated by polymerase chain reaction (PCR) amplification using the primer pair, 5′ probe: 5′-TTCAACAGGGATATCGCAAGG and 5′-AGCCTGGTAG-CAGGAAGATC, and Neo probe: 5′-CTCGACGTTGTCACTGAA and 5′-AAGAACTCGTCAAGAAGGCG.

Generation of ES cells for CA-PKA expression

cDNA for constitutive active form (CA)–PKA (a kind gift from Dr G. S. McKnight, University of Washington, Seattle, WA)[29] was introduced into the downstream region of tetracycline responsive element–regulatable cytomegalovirus promoter of Exchange vector (supplemental Figure 1A).

Stable ES cells that express the CA-PKA under the control of the tetracycline responsive element–regulatable cytomegalovirus promoter were generated by introduction of Exchange vectors and pBS185 (Cre expression vector) to EStTA-ROSA cells using mouse ES cells Nucleofector Kit (Amaxa Biosystems). Cells were then plated on 10-cm dishes containing 1 μg/mL doxycycline (Dox$^+$). After 1 day, the medium was changed to Dox$^+$ medium with 200 μg/mL hygromycin. After 10 days, the medium was changed to Dox$^+$ medium with 200 μg/mL hygromycin and 1 μg/mL ganciclovir. Total hygromycin- and ganciclovir-resistant colonies were collected and subjected to further studies.

Antibodies

Monoclonal antibodies for murine Flk1 (AVAS12) and murine vascular endothelial (VE)–cadherin (VECD1, for fluorescence-activated cell sorting [FACS]) were described previously.[24] Monoclonal antibodies for murine CD31 (1:500), VE-cadherin (for immunostaining, 1:200), and endothelial nitric oxide synthase (eNOS; 1:200) were purchased from BD Pharmingen. Monoclonal antibodies for murine α-smooth muscle actin (SMA; 1:1000) were from Sigma-Aldrich. Antibodies for SM22α (1:400) and calponin (1:500) were from Abcam. Polyclonal antibodies for murine Claudin-5 (1:100) were from Invitrogen. Polyclonal antibodies for murine VEGF and rat Neuropilin1 were from R&D Systems.

Cell culture

ES cell lines, D3, EStTA-ROSA, and CA-PKA–introduced EStTA-ROSA were maintained as described.[26] Induction of differentiation of these ES cell lines was performed using differentiation medium (DM; alpha minimal essential medium [MEM; Gibco] supplemented with 10% fetal calf serum [Japan Bioserum Co Ltd] and 5×10^{-5} M 2-mercaptoethanol [Gibco]) as previously described.[25,26] In brief, undifferentiated ES cells were cultured in the absence of leukemia inhibitory factor on collagen type IV–coated dishes (Becton Dickinson) at cell density 0.75 to 1×10^3 cells/cm^2 for 96 to 108 hours. Cultured cells were harvested and subjected to magnetic cell sorting (MACS) purification. Purified Flk1$^+$ cells were then plated onto type IV–coated dishes at cell density 0.75 to 1×10^4 cells/cm^2 in DM. After 3 days of Flk1$^+$ cell differentiation (Flk-d3), induced ECs were then examined by immunohistochemistry and flow cytometric analysis. Various reagents, human $VEGF_{165}$, $VEGF_{121}$ (R&D Systems), 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP; Nacalai Tesque), γ-secretase inhibitor, DAPT, PI3K inhibitor, LY294002, GSK3β inhibitor, Bio, Akt inhibitor, TAT-Akt-in, PKA inhibitor, PKI, H89, p38 inhibitor, SB202190, MEK inhibitor, PD98059, PKCαβ inhibitor, PKCη inhibitor, PKCζ inhibitor, H-Ras inhibitor, FTI-277 (Calbiochem), and phospholipase C (PLC) inhibitor, U73122 (Tocris Cookson Inc) were occasionally added to the Flk1$^+$ cell culture. (DAPT, LY294002, Bio, TAT-Akt-in, SB202190, PD98059, PKCαβ inhibitor, PKCη inhibitor, and PKCζ inhibitor did not inhibit cAMP effect.) Human $VEGF_{165}$ was used as the representative of VEGF isoforms unless stated otherwise. In serum-free culture, a defined medium, SFO3 (Sanko Junyaku; including insulin, transferrin, sodium selenite, and ethanolamine), was used instead of DM.[25]

Three-dimensional culture

Three-dimensional culture was performed as described previously.[25] Briefly, Flk1$^+$ cells (4×10^5 cells/mL) were incubated in DM with VEGF on uncoated Petri dishes for 16 hours to induce aggregation. Aggregates were resuspended in 2 × DM and mixed with an isovolume of collagen I-A gel (3 mg/mL; Nitta Gelatin). We plated 250 to 300 μL of this mixture onto a lucent insert disk, Cell disk (Sumitomo Bakelite), in 24-well dishes. After 30 minutes at 37°C to allow polymerization, we added 500 μL DM. To monitor vascular formation, collagen-embedded Flk1$^+$ cell aggregates were cultured in a temperature- and gas-controlled chamber (37°C, 5% CO_2), and phase-contrast images were acquired every 10 minutes with Metamorph software (Molecular Devices) for up to 5 days.

Cell sorting and flow cytometric analysis

After induction of Flk1$^+$ cells, cultured cells were harvested and stained with allophycocyanin (APC)–conjugated anti-Flk1 antibody (AVAS12).[24] Flk1$^+$ cells were sorted by auto MACS (Miltenyi Biotec) using anti-APC MicroBeads (Miltenyi Biotec). At Flk-d3, cultured cells were harvested and stained with monoclonal antibodies for phycoerythrin-conjugated CD31 (Mec13.3; BD Pharmingen) together with APC-conjugated VECD1 or biotin-conjugated CXCR4 (BD Pharmingen) followed by streptavidin-conjugated APC (BD Pharmingen) or AVAS12, then subjected to analysis by FACSVantage or FACSAria (Becton Dickinson).

Immunocytochemistry

Immunostaining for cultured cells was carried out as described.[25,26] Briefly, 4% paraformaldehyde-fixed cells were blocked by 1% skim milk (BD Biosciences) and incubated overnight with primary antibodies at 4°C. For immunohistochemistry, anti–rat immunoglobulin G (IgG) conjugated with alkaline phosphatase and anti–mouse IgG horseradish peroxidase (Invitrogen) were used as secondary antibodies. For immunofluorescent staining, anti–mouse, –rat, –rabbit, or –goat IgG antibodies conjugated with Alexa488 or Alexa546 (Invitrogen) were used for secondary antibodies. Nuclei were visualized with DAPI (4.6 diamidino-2-phenylindole; Invitrogen). Double staining for NRP1 and CD31 was performed using anti-NRP1 antibody (1:100; R&D Systems) as first antibody, followed by secondary antibody, Alexa Fluor 488–conjugated donkey anti–goat IgG (1:500; Molecular Probes). CD31$^+$ cells were visualized using phycoerythrin-