Ets family members induce lymphangiogenesis through physical and functional interaction with Prox1

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Summary

Prox1 plays pivotal roles during embryonic lymphatic development and maintenance of adult lymphatic systems by modulating the expression of various lymphatic endothelial cell (LEC) markers, such as vascular endothelial growth factor receptor 3 (VEGFR3). However, the molecular mechanisms by which Prox1 transactivates its target genes remain largely unknown. Here, we identified Ets-2 as a candidate molecule that regulates the functions of Prox1. Whereas Ets-2 has been implicated in angiogenesis, its roles during lymphangiogenesis have not yet been elucidated. We found that endogenous Ets-2 interacts with Prox1 in LECs. Using an in vivo model of chronic aseptic peritonitis, we found that Ets-2 enhanced inflammatory lymphangiogenesis, whereas a dominant-negative mutant of Ets-1 suppressed it. Ets-2 also enhanced endothelial migration towards VEGF-C through induction of expression of VEGFR3 in collaboration with Prox1. Furthermore, we found that both Prox1 and Ets-2 bind to the VEGFR3 promoter in intact chromatin. These findings suggest that Ets family members function as transcriptional cofactors that enhance Prox1-induced lymphangiogenesis.

Key words: Lymphangiogenesis, Transcription, Vascular endothelial growth factor receptor 3, Ets protein family, Prox1

Introduction

The vascular system comprises blood vascular and lymphatic networks and plays important roles in the maintenance of tissue fluid homeostasis (Saharinen and Petrova, 2004). The formation and maintenance of vascular networks are regulated by the coordinated activity of signaling pathways and networks of transcription factors. Traditional in vitro and in vivo studies have revealed numerous transcription factors that determine endothelial identity during embryogenesis. Furthermore, recent attempts to elucidate transcriptional controls exercised during endothelial cell development in a genome-wide fashion have identified potential relationships between transcription factors and their direct target genes in endothelial cells (Carlsson and Mahlapuu, 2002; Hollenhorst et al., 2007; De Val and Black, 2009). These studies have identified Ets family members as transcription factors that play important roles in multiple steps in the formation of vascular networks.

Ets family transcription factors share a highly conserved DNA-binding domain and the DNA-binding consensus sequence GGA(A/T). Hollenhorst and colleagues reported that 19 Ets transcription factors are expressed in endothelial cells (Hollenhorst et al., 2007), and knockout mouse analyses have shown that several members play essential roles in vascular development. Ets-1 and Ets-2 are prototypical members of the Ets family. Cell-culture-based studies have suggested that Ets-1 induces angiogenesis through regulation of the expression of vascular endothelial growth factor receptor 2 (VEGFR2) and Tie2, a receptor tyrosine kinase for angioipoietin (Ang), both of which are required for angiogenesis (Hashiya et al., 2004) and the regulation of extracellular proteases, such as matrix metalloproteinase-9, which is involved in endothelial cell migration (Iwasaka et al., 1996). Ets-2 plays important roles in Ras–MAPK-mediated induction of the expression of aminopeptidase N (APN, also known as CD13), a potent regulator of angiogenesis (Petrovic et al., 2003). Importantly, knockdown of Ets-2, but not of Ets-1, in endothelial cells decreases the expression of APN and impairs endothelial function. These results suggest that Ets-1 and Ets-2 play important and distinct roles in angiogenesis.

In accordance with these in vitro findings, double-mutant mice for Ets-1 and Ets-2 exhibit defective blood vessel branching (Wei et al., 2009), whereas mice that were single-mutant for either Ets-1 or Ets-2 exhibited no phenotypic changes in the vascular development of the embryo proper (Bories et al., 1995; Muthusamy et al., 1995; Barton et al., 1998; Yamamoto et al., 1998). These findings suggest that Ets-1 and Ets-2 play redundant roles during embryonic vascular development. However, because these double-mutant mice die in the early stages of vascular development, the roles of Ets-1 and Ets-2 in further specification of vascular vessels, such as lymphatic development, remain to be determined.

During embryogenesis, lymphatic endothelial cells (LECs) arise by sprouting of a subset of blood vascular endothelial cells (BECs) in cardinal veins, which migrate towards the mesenchymal cells expressing VEGF-C and form the primary lymphatic plexus (Oliver, 2004; Karkkainen et al., 2004). The homeobox transcription factor
Prox1 is expressed in such lymphatic progenitor cells and induces the expression of various LEC markers, including VEGFR3, a receptor for VEGF-C (Petrova et al., 2002). Importantly, Prox1-deficient mice exhibit defects in migration of the progenitor cells towards VEGF-C, resulting in complete lack of the lymphatic system (Wigle and Oliver, 1999; Wigle et al., 2002). These reports, together with our in vitro finding that Prox1 induces LEC migration towards VEGF-C (Mishima et al., 2007), suggest that Prox1 induces lymphatic development by activating pro-lymphangiogenic signaling pathways mediated by VEGF-C–VEGFR3.

However, it remains to be determined how Prox1 induces VEGFR3 expression specifically in LECs. Although Prox1 is expressed not only in LECs but also multiple organs, including lens (Wigle et al., 1999) and liver (Sosa-Pineda et al., 2000), Prox1 induces VEGFR3 expression only in LECs. Regulation of the activities of transcription factors often crucially depends on their interaction with other transcription factors on composite DNA elements. Tissue-specific transcriptional activities of Prox1 can thus be directed by additional transcription factors.

Although Flister and colleagues recently reported that Prox1 functionally collaborates with NFκB, which is activated by inflammatory signals, to induce the expression of VEGF3 in LECs (Flister et al., 2010), the roles of inflammatory signals during embryonic lymphatic differentiation remain to be determined. To date, only the nuclear receptor COUP transcription factor 2 (COUP-TFII) has been reported as being expressed in LECs and physically and functionally interacting with Prox1 (Lee et al., 2009; Yamazaki et al., 2009). However, the effects of COUP-TFII on the transcriptional activities of Prox1 appear to be cell-type-dependent, being negative in BECs and positive in LECs (Yamazaki et al., 2009). We therefore attempted to identify transcriptional modulators of Prox1 that are capable of inducing the expression of components of pro-lymphangiogenic signals.

Here, we identified Ets-2 as a putative interactor of Prox1. Ets-2 is expressed in various types of LECs and physically interacts with Prox1. Ets-2 positively regulates Prox1-induced expression of VEGFR3. Consistent with the effects on VEGFR3 expression, Ets-2 induces LEC migration towards VEGF-C. These findings suggest that Ets-2 functions as a pro-lymphangiogenic factor in collaboration with Prox1 during lymphangiogenesis.

**Results**

**Identification of Ets-2 as a Prox1-interacting protein**

In order to identify molecules that interact with Prox1, we performed GAL4-based yeast two-hybrid screening using Prox1 as bait to screen a human bone marrow cDNA library, which has only endothelial and hemopoietic origins, to select for endothelial-specific Prox1 interactors. At total of 5 million interactions were tested with Prox1. After growth on selection medium, positive clones were obtained. One clone contained a C-terminal portion of human Ets-2 (v-ets erythroblastosis virus E26 oncogene homolog 2).

**Ets-2 is expressed in BECs and LECs**

Ets-2 has been shown to be expressed in BECs (Wei et al., 2009) and to induce the expression of APN, which is essential for capillary tube formation (Petrovic et al., 2003), suggesting that Ets-2 plays important roles in angiogenesis. However, the roles of Ets-2 in lymphangiogenesis have not been reported. To examine whether Ets-2 is expressed in LECs, we performed western blot analysis to determine Ets-2 expression in human umbilical vein endothelial cells (HUVECs) and human dermal lymphatic endothelial cells (HDLECs) in which Prox1 is expressed. As shown in Fig. 1A, Ets-2 was found in both types of cells. To confirm the in vivo significance of the finding that Ets-2 is expressed in cultured LECs, we examined...
its expression in endothelial cells in mouse embryos. LECs and BECs were obtained from E14.5 mouse embryos by FACS sorting for LYVE-1 and CD31, respectively, and subjected to RT-PCR analysis (Fig. 1B). Substantial levels of Ets2 transcripts were detected in both embryonic BECs and LECs (Fig. 1B).

Furthermore, we performed double-fluorescence staining of mouse embryonic back skin (Fig. 1C) and sections of mouse adult intestine (Fig. 1D) using antibodies for Ets-2 and LYVE-1, a LEC marker. We observed that the LYVE-1-positive cells in both tissues expressed Ets-2. Further fluorescence staining revealed that the LYVE-1-positive cells in the adult intestine also expressed Prox1 (supplementary material Fig. S1), indicating that they are LECs. These findings suggest that Ets-2 is expressed in multiple types of LECs, as well as in BECs, and that it might function in both types of cells.

**Ets-2 physically interacts with Prox1 in LECs**

Because we found that Ets-2 is expressed in LECs, we next examined whether endogenous Ets-2 interacts with Prox1 in LECs, in order to confirm the results of the two-hybrid screening. We performed co-immunoprecipitation experiments with cell lysates prepared from HDLECs (Fig. 2A) and HUVECs, in which Prox1 is not expressed (supplementary material Fig. S2A). When the lysates were subjected to immunoprecipitation with an anti-Ets-2 antibody, we detected Prox1 in the pulled-down immunoprecipitates of HDLECs, but not of HUVECs, indicating that Prox1 interacts with Ets-2 in HDLECs.

To confirm further the results of the co-immunoprecipitation assay, we examined the physical interaction of endogenous Prox1 and Ets-2 in HDLECs using an in situ proximity ligation assay (PLA). This method enables determination of the subcellular localization of endogenous protein–protein interactions at single-molecule resolution (Söderberg et al., 2006; Söderberg et al., 2008; Yamazaki et al., 2009). In native HDLECs, we detected a number of substantial fluorescence signals, indicating that endogenous Prox1 and Ets-2 interact in the nuclei of HDLECs. To determine the specificity of the signals, we knocked down Ets-2 expression by siRNA in HDLECs and carried out PLA (Fig. 2B; supplementary material Fig. S2B). The fluorescence signals observed in the HDLECs transfected with control siRNA were substantially less upon knocking down Ets-2 expression (Fig. 2B). Quantification of the in situ PLA revealed that the extent of the decrease in the fluorescence signals (Fig. 2C) was consistent with that of the decrease in the siRNA-mediated Ets-2 expression in HDLECs (supplementary material Fig. S2C,D). We also found that PROX1 expression was not altered by the decrease in Ets-2 expression (supplementary material Fig. S2C), suggesting that the decrease in the fluorescence signals upon knocking down Ets-2 expression is not due to the decreased PROX1 expression. These findings suggest that endogenous Ets-2 physically interacts with Prox1 in the nuclei of HDLECs.

**Identification of binding domains within Ets-1 and Prox1**

As noted above, Ets-1 and Ets-2 are prototypical members of the Ets family and share conserved motifs (Watson et al., 1988). We found that Ets-1 is also capable of binding to Prox1 when overexpressed in HEK-293T cells (Fig. 3A). Because extensive analyses of Ets-1 have revealed that Ets-1 has functional domains for binding its transcriptional modulators, we attempted to determine which domains of Ets-1 and Prox1 interact with one another using expression vectors encoding truncated forms of Ets-1 and Prox1. The N-terminal region of Ets-1 (Ets-1 N), containing the Pointed domain and the transcription activation domain (TAD), but not the C-terminal region containing exon VII and the Ets DNA-binding domain (Ets-1 C), were found to bind Prox1 in HEK-293T cells (Fig. 3A). These findings, together with the fact that the portion of human Ets-2 isolated during yeast two-hybrid screening extended from amino acid residues 211–469 in Ets-2, which corresponds to the TAD, exon VII and the Ets domains in Ets-1, suggest that Prox1 binds the TAD of Ets-1.

We next examined which domain of Prox1 binds Ets-1. Prox1 has a homeobox DNA-binding domain and Prospero domain in its C-terminal region (Fig. 3B). Previous studies have revealed that nuclear receptor (NR) boxes in Prox1 play important roles in its interaction with liver receptor homologue (LRH)-1 (Qin et al., 2004). Co-immunoprecipitation assays showed that the N-terminal region of Prox1, containing NR-boxes I and II, but not its C-terminal region, lacking the NR boxes (Prox1 N-Q), binds Ets-1 (Fig. 3B). The I/LXXLL motifs in the NR boxes of Prox1 have been reported to play important roles in interaction with LRH-1. In order to examine whether these motifs act similarly to mediate Prox1 binding to Ets-1, we introduced mutations (LRKLL->ARKAA in NR box I and ISQLL->ASQAA in NR box...
II), which markedly decreased the binding between Prox1 and Ets-1 (Fig. 3B). These findings suggest that Prox1 and Ets-1 interact through NR boxes I and II of Prox1 and TAD of Ets-1.

Ets-2 enhances inflammatory lymphangiogenesis

Although Ets-2 is known to be a pro-angiogenic factor (Petrovic et al., 2003), its roles in lymphangiogenesis have not yet been elucidated. To examine whether Ets-2 regulates in vivo lymphangiogenesis, we used a mouse model of chronic inflammatory lymphangiogenesis (Iwata et al., 2007; Harada et al., 2009). In this model, thioglycollate medium was intraperitoneally administered three times a week as a pro-inflammatory agent to induce chronic aseptic peritonitis in immunocompetent BALB/c mice. To investigate the function of Ets-2, adenoviruses (Ad) encoding β-galactosidase (LacZ, control), Ets-2, and TM-Ets-1, a dominant-negative Ets mutant (Fig. 3A) (Nakano et al., 2000; Pourtier-Manzanedo et al., 2003), were also intraperitoneally administered twice a week. By 16 days, inflammatory plaques consisting mainly of macrophages had formed on the peritoneal surface of the diaphragm. Diaphragms from mice were subjected to immunostaining for LYVE-1, a lymphatic marker. Compared with control diaphragms from mice injected with adenoviruses encoding β-galactosidase (Ad-LacZ), those from Ad-Ets-2-infected mice displayed increased LYVE-1-positive areas on the diaphragm (Fig. 4A), as confirmed both quantitatively and statistically (Fig. 4B). By contrast, those of Ad-TM-Ets-1-infected mice displayed significantly decreased LYVE-1-positive areas (Fig. 4A,B).

Inflammatory macrophages secrete VEGF-A, VEGF-C and VEGF-D, all of which function as pro-lymphangiogenic factors (Cursiefen et al., 2004; Schoppmann et al., 2002). Because almost all of the inflammatory plaques on the diaphragms immunostained with antibody raised against Mac-1, a macrophage marker (data not shown), we examined whether Ets-2 induced expression in macrophages of more transcripts encoding VEGF-A, VEGF-C and VEGF-D, instead of directly activating LECs in the diaphragm. Quantitative RT-PCR analyses revealed that expression of VEGFA, VEGFC and VEGFD were not positively regulated by adenovirally introduced Ets-2 in macrophages (Fig. 4C). These findings suggest that Ets-2 induces inflammatory lymphangiogenesis through direct activation of lymphatic vessels in the diaphragm.

Ets-2 and Prox1 synergistically induce VEGFR3 expression

Although Ets-2 is known to be a pro-angiogenic factor (Petrovic et al., 2003), its roles in lymphangiogenesis have not yet been elucidated. To examine whether Ets-2 regulates lymphangiogenesis, we attempted to examine whether Ets-2 directly activates LECs. VEGF-C is a lymphangiogenic growth factor that promotes migration and proliferation of LECs through activation of VEGFR3 (Makinen et al., 2001). Because Prox1 induces VEGFR3 expression (Petrova et al., 2002; Mishima et al., 2007), we examined whether Ets-2 is involved in Prox1-induced VEGFR3 expression. When Prox1 was adenovirally introduced into HUVECs, VEGFR3

Fig. 3. Analysis of interaction between domains within Ets-1 and Prox1 proteins. (A) Identification of the Prox1-interacting region in Ets-1 protein. The top panel shows a schematic illustration of Ets-1 protein containing the Pointed domain, the transcription activation domain (TAD), the exon VII domain, and the Ets DNA-binding domain. Ets-2 cDNA isolated from two-hybrid screening corresponds to the C-terminal domains of Ets-1, which includes the TAD, exon VII and the Ets domains. TM-Ets-1 contains only the Ets DNA-binding domain. Bottom panels: lysates of HEK-293T cells transfected with FLAG-tagged full-length Prox1 (F-Prox1-FL) in combination with 6Myc-tagged full-length Ets-1 (6M-Ets-1 FL) or Ets-1 deletion mutants containing the N-terminal region (6M-Ets-1 N) or the the C-terminal region (6M-Ets-1 C) were subjected to immunoprecipitation (IP) with anti-Myc antibody (Ab), followed by western blotting (WB) with anti-FLAG antibody. Expression of FLAG-tagged Prox1 and immunoprecipitation of 6Myc-tagged Ets-1 proteins was also examined. (B) Identification of the Ets-1-interacting region in the Prox1 protein. The top panel shows a schematic illustration of the Prox1 protein containing the nuclear receptor (NR) boxes, the homeodomain and the Prospero domain. Bottom panels: FLAG-tagged full-length Prox1 (F-Prox1-FL), its deletion mutant containing the N-terminal region (F-Prox1 N-Q) and that lacking amino-terminal NR boxes (F-Prox1 ΔNR) and a NR box mutant (F-Prox1 NRmt) were transfected in combination with HA-tagged full-length Ets-1 (HA-Ets-1 FL), and subjected to immunoprecipitation with anti-FLAG antibody, followed by western blotting with anti-HA antibody. Expression of FLAG-tagged Prox1 and HA-tagged Ets-1 proteins was also confirmed.
expression was induced, as previously reported (Fig. 5A). Although Ets-2 alone was also capable of inducing the VEGFR3 expression to a moderate extent, VEGFR3 expression was substantially induced when Prox1 and Ets-2 were coexpressed in HUVECs (Fig. 5A).

We next examined the effects of Ets-2 on VEGFR3 expression in HDLECs in which endogenous Prox1 was present. As shown in Fig. 5B, Ets-2 alone substantially induced VEGFR3 expression in HDLECs. This finding suggests that Ets-2 and Prox1 synergistically induce VEGFR3 expression in endothelial cells. Consistent with the finding that Ets-1 is capable of binding to Prox1, we found that Ets-1 also functionally collaborates with Prox1 to induce VEGFR3 expression in HUVECs (Fig. 5C) and HDLECs (Fig. 5D). Furthermore, we found that Ets-1 and Ets-2 are capable of inducing VEGFR3 mRNA and protein expression in HUVECs in collaboration with Prox1 (Fig. 5E), and this also occurred in HDLECs (Fig. 5F) in a similar manner. These findings suggest that Ets-1 and Ets-2 play redundant roles in the regulation of VEGFR3 expression in endothelial cells.

**Ets-2 enhances Prox1-induced endothelial migration towards VEGF-C**

Our finding that Ets-2 is involved in VEGFR3 expression prompted us to examine whether Ets-2 affects endothelial migration towards VEGF-C. To address this question, we performed Boyden chamber migration assays. Control HUVECs hardly migrated towards low concentrations of VEGF-C, whereas higher concentrations of VEGF-C induced their migration (data not shown). HUVECs, adenovirally infected with Prox1, migrated towards VEGF-C (Fig. 6A). Ets-2 also induced migration towards VEGF-C. Of note, when coexpressed with Prox1, Ets-2 enhanced migration towards VEGF-C (Fig. 6A). Additionally, Ets-2 also induced migration towards VEGF-C. Of note, when coexpressed with Prox1, Ets-2 enhanced migration towards VEGF-C (Fig. 6A). Additionally, Ets-2 also enhanced the migration of HDLECs towards VEGF-C (Fig. 6B). In the same manner as the Ets-1 collaboration with Prox1 to induce VEGFR3 expression, Ets-1 also enhanced the Prox1-induced migration of HUVECs (Fig. 6C) and HDLECs (Fig. 6D) towards VEGF-C. These findings suggest that Ets-1 and Ets-2 regulate the cellular function of BECs and LECs through upregulation of functional VEGFR3.

**Multiple Ets family members are expressed in LECs and bind Prox1**

We next examined whether Ets-2 is required for VEGFR3 mRNA and protein expression in LECs by knocking down endogenous
Ets-2 expression using siRNA. VEGFR3 expression in LECs was not decreased by the loss of Ets-2 expression (supplementary material Fig. S2C,D). Because members of the Ets family share conserved DNA-binding capacities, we hypothesized that other Prox1-interacting Ets family transcription factors regulate VEGFR3 expression.

To determine whether other Ets family members regulate the transcriptional activities of Prox1 in endothelial cells, we first examined the expression of six Ets family members [Ets-1, Net (also known as Elk-3), ERF, Fli-1, Elk-1 and TEL (also known as transcription factor ETV6)] that have been reported to be expressed in endothelial cells (Hollenhorst et al., 2007), in HUVECs and HDLECs. Semi-quantitative RT-PCR analyses revealed that all of the Ets family members examined were expressed in both types of cells (Fig. 7A). Because Ets-1 is capable of inducing VEGFR3 expression (Fig. 5) and endothelial migration towards VEGF-C (Fig. 6) in collaboration with Prox1, we further examined its expression in multiple types of endothelial cells. We observed substantial levels of Ets-1 proteins in HUVECs and HDLECs (Fig. 7B) and Ets-1 transcripts in BECs and LECs obtained from E14.5 mouse embryos (Fig. 7C).

We further examined whether these Ets family members are capable of binding to Prox1 by co-immunoprecipitation assays using HEK-293T cells. As shown in Fig. 7D, Ets-1, Net and ERF interacted with Prox1. We were able to detect the physical interaction of Prox1 and endogenous Ets-1 proteins in HDLECs when Prox1 expression was adenovirally increased (Fig. 7E), indicating that Prox1 interacts with endogenous Ets-1 in HDLECs. The physical interaction of endogenous Prox1 and Ets-1 in HDLECs was also examined by in situ PLAs. As shown in Fig. 7F, we detected a number of substantial fluorescence signals, indicating that endogenous Prox1 and Ets-1 interact in the nuclei of HDLECs, whereas the number of signals was substantially decreased when Ets-1 expression was knocked down (Fig. 7F,G; supplementary material Fig. S2C,D), suggesting that endogenous Ets-1, as well as Ets-2, physically interacts with Prox1 in HDLECs. These findings suggest that multiple Ets family members are involved in the regulation of the transcriptional activity of Prox1.

### Inhibition of Ets family transcriptional activities abolishes Prox1-induced VEGFR3 expression

Because multiple members of the Ets family appear to collaborate with Prox1 in inducing the expression of their target genes, we used TM-Ets-1 to inhibit the transcriptional activities of multiple Ets family members. When TM-Ets-1 was coexpressed with Prox1 in HUVECs, it completely abolished Prox1-mediated induction of VEGFR3 expression (Fig. 8A) and endothelial migration towards VEGF-C (Fig. 8B). These findings suggest that other members of the Ets family are involved in the regulation of Prox1-mediated VEGFR3 expression. This inhibition is not due to interference with Prox1 expression by TM-Ets-1, as shown by western blot analysis (Fig. 8C).

Prox1 induces the expression of multiple target genes including that encoding integrin α9, which is involved in endothelial cell migration towards VEGF-C (Mishima et al., 2007) and lymphatic valve formation (Bazigou et al., 2009). We found that Prox1-induced upregulation of integrin-α9 mRNA was also suppressed by TM-Ets-1 (Fig. 8D). This finding suggests that the transcriptional activities of Ets family members are required for Prox1 to regulate the expression of multiple target genes.

**Prox1 and Ets-2 bind the endogenous VEGFR3 promoter in intact chromatin**

Flister and colleagues showed that Prox1 activates the VEGFR3 promoter (Flister et al., 2010), suggesting that Prox1 regulates the transcription of VEGFR3 through direct binding to the VEGFR3 promoter. Because Ets-2 enhances Prox1-induced VEGFR3 expression and physically interacts with Prox1, we examined whether Prox1 and Ets-2 bound to the endogenous VEGFR3 promoter in intact chromatin.

Crosslinked chromatin samples prepared from HDLECs were subjected to chromatin immunoprecipitation (ChIP) assays (Fig. 9). The VEGFR3 promoter region containing putative binding consensus sequences for Prox1 and Ets-2 was pulled down with antibodies for Prox1 and Ets-2, respectively, and was amplified by semi-quantitative ChIP-PCR. These findings suggest that both Prox1 and Ets-2 bind to the VEGFR3 promoter.

**Discussion**

In the present study, we identified Ets-2 as a putative interactor of Prox1, a master regulator of lymphangiogenesis. In addition, functional studies showed that Ets-2 induces lymphangiogenesis in collaboration with Prox1 through activation of pro-lymphangiogenic signals mediated by VEGF-C–VEGFR3.

Although Prox1 induces the expression of a group of LEC markers, including VEGFR3 (Fig. 5) and integrin α9 (Fig. 8D), in collaboration with Ets family members, we and other groups have reported that Prox1 downregulates the expression of a group of BEC markers (Petrova et al., 2002; Mishima et al., 2007). Interestingly, Ets-2 increased VEGFR2 expression and counteracted the Prox1-mediated downregulation of VEGFR2 expression in HUVECs (data not shown), suggesting that Ets-2 collaborates only with the transcriptional activation by Prox1 but not with its transcriptional repression.
Ets-1 appears to synergistically activate VEGFR3 expression, together with Prox1, through protein–protein interactions in the N-terminal regions of Ets-1 and Prox1 (Fig. 3). Although Ets-1 N and Prox1 N-Q mutants are capable of binding Prox1 and Ets-1, respectively, they do not contain DNA-binding domains (Fig. 3). In order to examine whether the functional interaction between Prox1 and Ets-1 during the induction of the VEGFR3 expression depended on their DNA-binding abilities, we examined the effects of Ets-1 N and Prox1 N-Q on the expression of VEGFR3 and the migration of HDLECs. As shown in supplementary material Fig. S3, neither Ets-1 N nor Prox1 N-Q was capable of inducing VEGFR3 expression or the migration of HDLECs in collaboration with Prox1 and Ets-1, suggesting that the DNA-binding abilities of Prox1 and Ets family members are required for their functional interaction. ChIP analysis revealed that both Ets-2 and Prox1 bound the intact VEGFR3 promoter (Fig. 9). It remains to be elucidated how Ets family members activate the VEGFR3 promoter.

During embryogenesis, VEGFR3 is expressed in BECs and also plays important roles in embryonic angiogenesis (Tammela et al., 2008). Although VEGFR3 expression in the embryonic vessels is regulated by Notch signaling (Tammela et al., 2008), Ets-2 in BECs might also play a role in inducing VEGFR3 expression. When Prox1 is expressed in a subset of venous endothelial cells, VEGFR3 expression is increased in the differentiating lymphatic endothelial cells. Together with these observations, our findings that Ets-2 alone moderately induces VEGFR3 expression and substantially induces it in the presence of Prox1 suggest that the transcriptional activities of Ets-2 and/or other Ets family members are required for Prox1-induced upregulation of VEGFR3 expression. This hypothesis is strengthened by our finding that interference with the transcriptional activity of Ets-2 by expression of TM-Ets-1, a dominant-negative mutant of the Ets family, abrogates Prox1-mediated induction of VEGFR3 expression. Interestingly, Prox1-mediated induction of other target genes including that encoding integrin α9 (Mishima et al., 2007) is also inhibited by TM-Ets-1 expression in HUVECs, suggesting that Ets family members are required for regulation by Prox1 of the transcription of at least one group of its target genes.

However, we found that knockdown of Ets-2 expression in HDLECs did not alter VEGFR3 expression, although Ets-2 induced VEGFR3 expression, suggesting that other Ets family members can compensate for the decrease in expression of Ets-2. Because Ets family members share very strongly conserved DNA-binding Ets domains and core DNA-binding consensus sites [GGA(A/T)], other Ets family transcription factors might have access to the consensus sites in the VEGFR3 promoter and play a complementary role in VEGFR3 expression when Ets-2 expression is decreased.

Among the six Ets family members we examined, we found that Ets-1, Net and ERG are capable of binding to Prox1. Ets-1 and Ets-2 have been shown to play redundant roles in vascular formation (Wei et al., 2009). Gain-of-function studies showed that Ets-1 exhibited effects on VEGFR3 expression in HUVECs and HDLECs, very similar to those of Ets-2, suggesting that Ets-1 and Ets-2 can also play redundant roles in lymphangiogenesis.

Net is a ternary complex factor, and negatively regulates immediate early genes through serum-response elements. It is

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**Fig. 7. Interactions of various Ets family transcription factors with Prox1.** (A) Semi-quantitative RT-PCR analysis of the expression of Ets-1, Net, ERG, Fli-1, ELK-1 and TEL in HUVECs and HDLECs. β-actin was used as an internal control. NTC, no-template control. (B) Expression of human Ets-1 (top panel) in HUVECs and HDLECs was examined by western blot (WB) analysis. α-tubulin was used as an internal control (bottom panel). (C) Expression of Ets-1 (top panel) in BECs and LECs derived from E14.5 mouse embryos, β-actin was used as an internal control (bottom panel). NTC, no-template control. (D) Analysis of interactions between Ets family members and Prox1. HEK-293T cells were transfected with HA-tagged Ets family members and FLAG-tagged Prox1, and lysed to perform immunoprecipitation (IP) with anti-FLAG antibody, followed by western blotting with anti-HA antibody. (E) Lysates of HDLECs infected with adenoviruses encoding for FLAG-Prox1 were subjected to immunoprecipitation (IP) with anti-FLAG antibody, or normal rabbit IgG as a negative control, followed by western blotting with anti-Ets-1 antibody (top panel). Precipitation of Prox1 was confirmed (bottom panel). (F) A PLA was carried out to detect the proximal location of Ets-1 and Prox1 (observed as red dots). HDLECs transfected with negative control siRNA (siNC, top panel) and those with siRNA for Ets-1 (siEts-1, bottom panel) were subjected to PLA after being treated with antibodies to Ets-1 and Prox1. All samples were counterstained with TOTO-3 (blue) to visualize nuclei. Note that a specific interaction between Ets-1 and Prox1 was confirmed (bottom panel). (G) Results of the in situ PLA (F) were quantified by counting the number of dots per nuclei. Each value represents the mean number of dots in >10 nuclei. Error bars represent s.d.
expressed in sites of vasculogenesis during mouse development. The hypomorphic mutant of Net, in which Net mutant protein lacking the Ets DNA-binding domain is knocked-in, develops defects in the blood vascular and lymphatic systems (Ayadi et al., 2001). Net has been reported to be a modulator of lymphatic phenotype and might thus be a candidate modulator of VEGFR3 expression. ETS2 repressor factor (ERF) is a ubiquitously expressed member of the Ets family and a strong transcriptional repressor (Papadaki et al., 2007). Notably, two repressors of the Ets family members activate transcription (Mavrothalassitis and Ghysdael, 2001). Net has been reported to be a modulator of lymphatic endothelial cells.

Fig. 8. Effects of TM-Ets-1 on Prox1-induced expression of VEGFR3 in endothelial cells. (A) RT-PCR analysis of VEGFR3 expression in HUVECs adenovirally infected with Prox1, TM-Ets-1 or both. (B) Analysis of chemotaxis of HUVECs infected with Prox1, TM-Ets-1 or both towards VEGF-C. Relative migration towards VEGF-C is shown as the ratio of the number of cells migrating in the presence of VEGF-C (gray bars) to that in the absence of VEGF-C (black bars). Error bars represent s.d. (C) Western blot analysis of Prox1 and TM-Ets-1 expression in HUVECs. FLAG-tagged Prox1 and TM-Ets-1 were adenovirally transduced into HUVECs that were subjected to western blot analysis using anti-FLAG antibody. Increasing amounts of TM-Ets-1 did not alter Prox1 expression. (D) RT-PCR analysis of the levels of mRNA encoding integrin α9 in HUVECs adenovirally infected with Prox1, TM-Ets-1 or both.

Despite the difference between these two experimental settings, TM-Ets-1 appears to be a potent candidate molecule for inhibition of both angiogenesis and lymphangiogenesis. Notably, an endogenous form of a dominant-negative mutant of Ets-1 (Ets-1 p27), which has a structure essentially the same as that of TM-Ets-1, has recently been identified (Laitem et al., 2009). In some tumors, angiogenesis and lymphangiogenesis occur at the same time and in the same locations (in the peripheral portion of tumors). Local administration of TM-Ets-1 to a tumor might be a potent means of inhibiting tumor growth and metastasis by blocking angiogenesis and lymphangiogenesis simultaneously.

Materials and Methods

Yeast two-hybrid screening
To construct a bait plasmid, full-length human Prox1-encoding cDNA was inserted in-frame into the pGBKTK7 GAL4 DNA-binding vector. This construct was introduced into the yeast MATa strain AH109, which was then mated with a prey-expressing MATα Y187 strain harboring a human bone marrow cDNA library (matchmaker two-hybrid System 3, Clontech Laboratories) in the pGAD vector. The strains were co-cultured overnight and then plated on synthetic defined medium deficient in leucine, tryptophan, histidine and adenine (SD – L, – W, – A, – H) with 0.5 mM 3-amino-1,2,4-triazole. Library plasmids were rescued from the yeast and sequenced.

Plasmid construction and adenovirus production
Ets-2-encoding cDNA was kindly provided by Yasufumi Sato (Tohoku University, Sendai, Japan) (Hasegawa et al., 2004). It was amplified by PCR and subcloned into the pcDE3 vector. To map the interacting domains, expression constructs producing Myc-tagged fragments of Ets-1 and Prox1 were generated by restriction enzyme digestion and/or PCR amplification. pcDNA3 constructs carrying the V5-tagged cDNA encoding Ets-1, Net, Fli-1, ERF, TEL or ELK-1 were kindly provided by Hiroyuki Sugimoto (Dokkyo Medical University, Tochigi, Japan) (Sugimoto et al., 2005). The cDNAs were subcloned into pcDNA3 vector carrying the HA epitope. All constructs were verified by sequencing. Recombinant adenoviruses encoding Prox1, its variant (Prox1 N-Q), Ets-2, Ets-1, its variant (Ets-1 N) and TM-Ets-1 were generated and used as described previously (Shirakihara et al., 2007).

Cell culture
HUVECs and HDLECs were purchased from Sanko Junyaku and Takara Bio, and cultured in endothelial basal medium (EBM) containing 2% and 5% fetal bovine serum (FBS), respectively, supplemented with endothelial cell growth supplement (Takara Bio). HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

RNA interference
siRNAs for human Ets-1 and Ets-2 (Stealth RNAi Oligo ID VHS40620, respectively) and negative control siRNA (Stealth RNAi Negative Control Low GC for Ets-1 and Med GC for Ets-2) were purchased from Invitrogen, and were introduced into cells using HiPerFect reagent (Qiagen) according to the manufacturer’s instructions.

Isolation of RNA and RT-PCR analysis
Total RNAs were extracted from various types of cells and tissues using the RNeasy Mini Kit (QiAGEN). First-strand cDNAs were synthesized by SuperScriptII reverse transcriptase (Invitrogen) using random hexamer primers according to the manufacturer’s instructions. Expression of various Ets family members was compared

Fig. 9. Binding of Prox1 and Ets-2 to the VEGFR3 promoter. ChIP analysis of the VEGFR3 promoter using HDLECs. PCR was performed to detect VEGFR3-promoter containing putative binding sequences for Prox1 and Ets-2. The α-Prox1 and α-Ets-2 lanes show amplification of target sequences within the immunoprecipitates using antibodies for Prox1 and Ets-2, respectively. The control IgG lane shows PCR amplification of samples precipitated with corresponding control IgG antibodies. Input lanes show amplification of 0.04% of total input DNA (+) or no DNA (−).
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by semi-quantitative RT-PCR analysis. PCR products were separated by electrophoresis in agarose gels and were visualized with ethidium bromide. Quantitative RT-PCR analyses were carried out in duplicates or triplicates using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR master mix (Applied Biosystems). All values of expression were normalized to those for β-actin. Each value of the analysis is shown as the ratio of each relative expression to that of control. Error bars represent the s.d. The primer sequences are shown in supplementary material Table S1.

Co-immunoprecipitation and western blot analysis

Antibodies against Ets-1, Ets-2 and VEGFR3 were obtained from Santa Cruz Biotechnology. Antibodies against FLAG, Myc and α-tubulin were obtained from Sigma-Aldrich. HRP-conjugated anti-HA (HA-POD) antibody was obtained from Roche. Immunoprecipitation and western blot analyses were performed as previously described (Watabe et al., 2003; Mochizuki et al., 2004; Lee et al., 2009). To detect the endogenous proteins, cultured HUVECs and HDLECs were subjected to immunoprecipitation using anti-Ets-2 antibody or control IgG, followed by immunoblotting with anti-PROX1 antibody. To examine the interaction of ProX1 and endogenous Ets-1 proteins, cultured HDLECs were infected with adenoviruses encoding FLAG-Prox1, lysed and subjected to immunoprecipitation using anti-FLAG antibody or control IgG, followed by immunoblotting with anti-Prox1 antibody. For mapping of protein interaction domains and identification of Ets family members that interact with ProX1, expression vectors were transfected into HEK-293T cells using FuGENE6 (Roche), and 24 hours later the cells were lysed for immunoprecipitation using antibodies for the Myc and FLAG epitopes, followed by immunoblotting for the FLAG, Myc and HA epitopes.

Chamber migration assay

The migration assay was performed as described previously (Mishima et al., 2007). As chemotaxants, 100 ng/ml and 300 ng/ml of recombinant VEGF-C (Calbiochem) were used for HDLECs and HUVECs, respectively.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Koinuma et al., 2009). HDLECs were used for HDLECs and HUVECs, respectively. ChIP assays were performed as described previously (Koinuma et al., 2009). HDLECs and HUVECs were transfected into HEK-293T cells using FuGENE6 (Roche), and 24 hours later the cells were lysed and subjected to immunoprecipitation using anti-Ets-2 antibody or control IgG, followed by immunoblotting with anti-PROX1 antibody. To examine the interaction of ProX1 and endogenous Ets-1 proteins, cultured HDLECs were infected with adenoviruses encoding FLAG-Prox1, lysed and subjected to immunoprecipitation using anti-FLAG antibody or control IgG, followed by immunoblotting with anti-Prox1 antibody. For mapping of protein interaction domains and identification of Ets family members that interact with ProX1, expression vectors were transfected into HEK-293T cells using FuGENE6 (Roche), and 24 hours later the cells were lysed for immunoprecipitation using antibodies for the Myc and FLAG epitopes, followed by immunoblotting for the FLAG, Myc and HA epitopes.

FACS analysis

We obtained LECs and BECs from mouse embryos as described previously (Hirashima et al., 2008). E14.5 mouse embryos were, after removal of the liver and spleen, dissected and digested with 1.2 units/ml Dispase (Invitrogen), 50 µg/ml DNase I, 10% FCS collagenase S-1 (Nitta Gelatin) to obtain single-cell suspensions. After blocking Fc-receptors with an anti-mouse CD16-CD32 Fc receptor (FcR; BD Pharmingen), all cells were stained with phycoerythrin (PE)-conjugated streptavidin (BD Pharmingen) to visualize LYVE-1+ cells (LECs). The cells were also co-stained with a fluorescein isothiocyanate (FITC)-conjugated anti-PECAM-1 (BD Pharmingen) to visualize LYVE-1+ cells (EPCs). Immunohistochemistry

Immunohistochemical analysis was performed with anti-Ets-2 (Aviva Systems Biology), anti-LYVE-1 (Abcam) and anti-PROX1 (R&D Systems) antibodies, followed by counterstaining with TOTO-3 (Invitrogen-Molecular Probes) as described previously (Harada et al., 2009; Hirashima et al., 2008). Stained specimens were examined using a LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for contrast manipulation and figure assembly.

Proximity ligation assay (PLA)

The Duolink in situ PLA kits were purchased from Olink. Fixation of the cells, blocking of non-specific binding of antibody and immunostaining using anti-Prox1 (Abcam), anti-Ets-1 (Santa Cruz Biotechnology) and anti-Ets-2 antibodies (Aviva Systems Biology) were performed as described above. Subsequently, a pair of secondary antibodies conjugated with oligonucleotides (PLA probes) were used according to the manufacturer’s protocol to generate fluorescence signals only when the two PLA probes were in close proximity (40 nm). The fluorescence signal from each detected pair of PLA probes was visualized as a distinct individual dot (Söderberg et al., 2006; Söderberg et al., 2008). Nuclear counterstaining and analysis of images were performed as described above.

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