

# Sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells

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## Summary

Prox1 is a master regulator for the development of lymphatic vasculature and the induction of lymphangiogenesis. In this study, we identified Prox1 as a new target for small ubiquitin-like modifier 1 (SUMO-1). Lysine 556 (K556) was found to be the major sumoylation site for Prox1 in vitro and in vivo. Mutation of this site (from lysine to arginine K556R) reduced DNA binding and the transcriptional activity of Prox1. Overexpression of Prox1 in EA.hy926 endothelial cells induced expression of lymphatic endothelial cell-specific genes including vascular endothelial growth factor receptor 3 (VEGFR3), fibroblast growth factor receptor 3 (FGFR3) and p57 while expression of K556R mutant Prox1 had little effect. The induction of VEGFR3 by Prox1 in EA.hy926 endothelial cells was an indication of their response to VEGF-C-induced

lymphangiogenic signals, including the enhancement of proliferation, sprouting and tube formation and the inhibition of apoptosis. This effect is SUMO-dependent because ectopic expression of SUMO-specific protease 2 (SEN2) effectively reduced Prox1 sumoylation and Prox1-induced VEGFR3 expression. In addition, K556R mutant Prox1 could not induce lymphatic phenotypes. Taken together, our results indicate that Prox1 is a target for SUMO-1 and suggest that sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells.

Key words: Prox1, Small ubiquitin-like modifier 1, Vascular endothelial growth factor C, Vascular endothelial growth factor receptor 3, Lymphatic endothelial cells

## Introduction

Prox1 is a homeodomain transcription factor that controls the development of lymphatic vasculature (Wigle and Oliver, 1999). This gene was originally identified in mice due to its homology to the *Drosophila* homeobox protein *prospero* (Oliver et al., 1993). Prox1-expressing endothelial cells were first detected at embryonic day 10 in the jugular vein in mice. Subsequently, these cells migrated to form the lymphatic sac (Wigle et al., 2002). Biochemical and genetic analyses indicate that Prox1 is required for induction of the lymphatic endothelial cell fate and is involved in the budding of endothelial cells from the embryonic cardinal veins. Homozygous Prox1-null mice die at mid-gestation from multiple developmental defects (Wigle et al., 1999). To verify the importance of Prox1 in the control of the lymphatic vasculature phenotype, Petrova et al. overexpressed Prox1 in the blood vascular endothelial cells and found that overexpression of this gene was capable of inducing lymphatic endothelial cell-specific gene transcription (Petrova et al., 2002). In addition, the blood vascular-associated genes were simultaneously downregulated. These results suggest that Prox1 functions as a fate determination factor for the lymphatic endothelial cells.

The main function of the lymphatic vasculature is to drain the interstitial fluid from tissues and return it to the blood. Recently, the lymphatic system has been shown to be involved in the

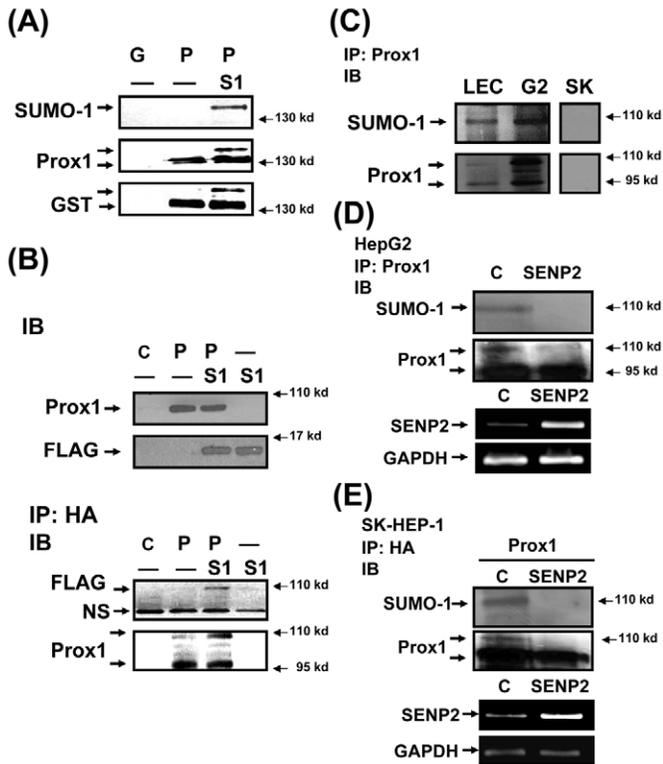
pathogenesis of several diseases, including cancer, lymphoedema and inflammation (Alitalo et al., 2005; Stacker et al., 2002). For example, lymphangiogenesis, the growth and formation of new lymphatic vessels, is crucial for the spread of malignant tumor cells to regional lymph nodes and is a prognostic indicator of the metastatic risk for human cancers (Tobler and Detmar, 2006). Although Prox1 is a master regulator of the lymphatic vasculature phenotype and lymphangiogenesis, the biological signals that control the expression of Prox1 have not yet been identified. In addition, it is also unclear whether post-translational modifications affect the transcriptional activity of Prox1.

In this study, we address whether Prox1 is a target for sumoylation in vitro and in vivo and investigate the effect of sumoylation on the biological activity of Prox1.

## Results

Prox1 is a target for SUMO-1 in vitro and in vivo

We used an expression-modification system to produce SUMO-conjugated proteins in *Escherichia coli* to test whether Prox1 is a target for SUMOs. As shown in Fig. 1A, immunoblotting detected a shift band of GST-tagged Prox1 (with a molecular mass of around 130 kDa) in *E. coli* co-transformed with SUMO-1. This band was also detected by probing the blot with anti-Prox1 and anti-SUMO-1 antibody (Fig. 1A). Co-transfection of SUMO-2 did not yield shift



**Fig. 1.** Sumoylation of Prox1 in vitro and in vivo. (A) GST (G) or GST-tagged Prox1 (P) vectors were co-transformed with pE1E2-SUMO1 (S1) vector into *E. coli*. Glutathione beads were used to purify GST-tagged Prox1. The conjugation of SUMO-1 to Prox1 was detected by anti-Prox1 or anti-SUMO-1 antibody. Anti-GST antibody was used to confirm the expression of GST-tagged Prox1. The arrows indicated the positions of target proteins. (B) 293T cells were co-transfected with control (C) or HA-Prox1 (P) vector with FLAG-tagged SUMO-1 (S1) expression vector. The expression of Prox1 and FLAG-tagged SUMO-1 in cell lysates was investigated by immunoblotting (IB). Equal amounts of cellular proteins were used for immunoprecipitation (IP) by anti-HA antibody. The immunocomplex was collected and subjected to SDS-PAGE. Western blotting was performed to detect the expression and the sumoylation status of Prox1. In addition, blots were probed with anti-FLAG to detect SUMO-1-conjugated protein. NS, non-specific band. (C) Prox1 protein was immunoprecipitated from human HepG2 hepatoma cells (G2), lymphatic endothelial cells (LEC) or SK-HEP-1 (SK) cells. Western blotting was performed by using anti-SUMO-1 antibody to examine the endogenous sumoylation of Prox1. (D) HepG2 cells were transfected with control (C) or SENP2 vector. After 24 hours, the expression of SENP2 was studied by RT-PCR and the sumoylation of endogenous Prox1 was investigated. (E) SK-HEP-1 cells were co-transfected with HA-tagged Prox1 and control (C) or SENP2 vector. After 24 hours, the sumoylation of HA-tagged Prox1 was studied by immunoprecipitation and western blotting.

bands (data not shown). We next studied whether Prox1 could be sumoylated in vivo. HA-tagged Prox1 was co-transfected with FLAG-tagged SUMO-1 into 293T cells, which do not express endogenous Prox1. Western blotting showed the expression of HA-tagged Prox1 (with a molecular mass of around 95 kDa) in these cells after vector transfection (Fig. 1B). Immunoprecipitation and western blotting demonstrated that SUMO-1 indeed conjugated with Prox1 in vivo (Fig. 1B). It should be noted that only one band at 95 kDa was seen when total cell lysates were used for western blotting analysis. These results suggested that only a minor portion of exogenous Prox1 was sumoylated in vivo and that the sumoylated Prox1 could only be detected after enrichment by immunoprecipitation. Because Prox1 expression is very low in most

cell types, we used HepG2 human hepatoma cells and primary cultured human lymphatic endothelial cells, which have been shown to express high levels of Prox1 (Wigle et al., 2002; Rodriguez-Niedenfuhr et al., 2001), to address the endogenous sumoylation of Prox1. The Prox1 protein immunoprecipitated from lymphatic endothelial cells showed a major band at 95 kDa and a minor band at about 110 kDa (Fig. 1C). These two bands were also detected in HepG2 cells. However, the abundance of 95 and 110 kDa bands was similar in these cells.

Western blotting confirmed that the higher molecular weight band is the SUMO-1-conjugated Prox1 (Fig. 1C). The Prox1 antibody used for immunoprecipitation exhibited high specificity because no signal was detected when a poorly differentiated human hepatoma cell line SK-HEP-1, which does not express Prox1, was used for the immunoprecipitation assay (Fig. 1C). In addition, non-immune immunoglobulin could not pull down Prox1 (data not shown). To further confirm that the band around 110 kDa is the SUMO-1-conjugated Prox1, we ectopically expressed SUMO-specific protease 2 (SENP2) in HepG2 cells and found that the shift band disappeared (Fig. 1D). Similarly, expression of SENP2 also caused abolishment of the 110 kDa band in 293T cells in which HA-tagged Prox1 was ectopically expressed (Fig. 1E). Collectively, these results indicate that Prox1 is a target for SUMO-1.

Lys556 (K556) is the major sumoylation site of Prox1

We next investigated the sumoylation sites on Prox1 protein. SUMOplot ([http://www.abgent.com/tools/sumoplot\\_login](http://www.abgent.com/tools/sumoplot_login)) analysis predicted two potential sumoylation sites at Lys353 (K353) and Lys556 (K556) with SUMOplot scores of 0.91 and 0.94, respectively. More importantly, these two potential sites are highly conserved in different species (from zebrafish to human) indicating a crucial role in the control of Prox1 function (Fig. 2A). We mutated these two Lys residues to Arg and tested the sumoylation of Prox1 in vitro and in vivo. As demonstrated in Fig. 2B, transfection of K556R mutant (M2), but not K353R mutant (M1), completely abolished the sumoylation of Prox1 in the *E. coli* expression-modification system. Our data also showed that sumoylation of Prox1 in vivo was abolished after mutation of K556 (Fig. 2C). Collectively, these results suggest that K556 is the major sumoylation site of Prox1.

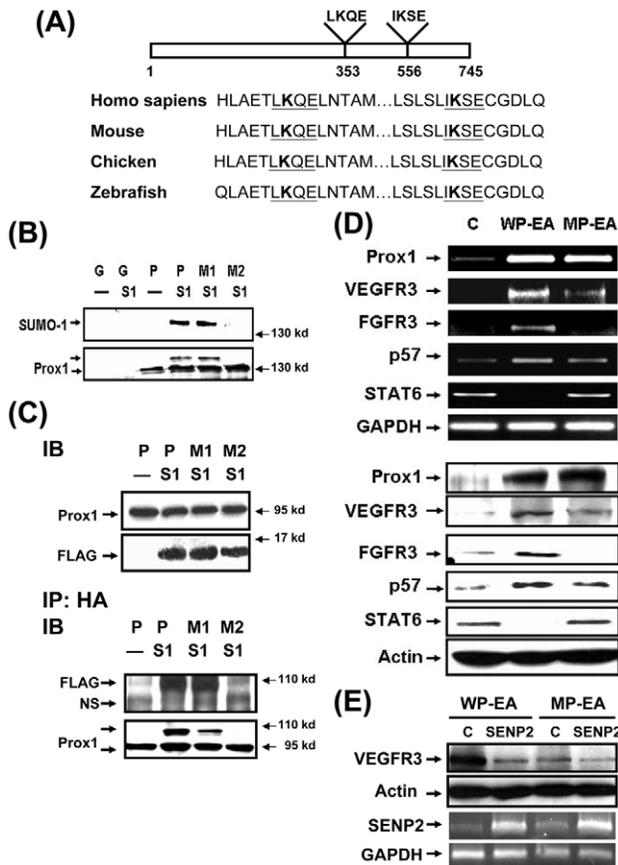
Next, we used EA.hy926 endothelial cells to study the effect of sumoylation on Prox1 function. This cell line was originally established by hybridization of A549 human lung cancer cells and human umbilical vein endothelial cells and retained some characteristics of vein endothelial cells (Rieber et al., 1993). Because lymphatic vessels originated from veins during embryonic development, we thought this cell line might be suitable for the investigation of the biological activity of Prox1. We established stable clones expressing wild-type or K556R mutant Prox1 and investigated the expression of target genes in these clones. Compared to parental cells, three lymphatic endothelial cell makers VEGFR3, FGFR3 and p57 were significantly upregulated in cells overexpressing wild-type Prox1 (WP-EA.hy926 cells) (Fig. 2D). These results were in consistent with the data of previous studies (Petrova et al., 2002; Hong et al., 2002) showing that ectopic expression of Prox1 induced these lymphatic specific genes in endothelial cells. Conversely, a blood endothelial cell marker STAT6 was downregulated.

The same experiments were repeated in cells overexpressing K556R mutant Prox1 (MP-EA.hy926 cells) and we found that modulation of the expression of these target genes was significantly attenuated in MP-EA.hy926 cells (Fig. 2D). To verify that the

upregulation of Prox1 target genes is due to sumoylation of this transcription factor, we ectopically expressed SENP2 in WP-EA.hy926 cells and found that Prox1-induced VEGFR3 transcription was significantly attenuated (Fig. 2E). By contrast, the expression of VEGFR3 was very low in MP-EA.hy926 cells and was not changed by SENP2 expression. These data suggest that sumoylation controls the transcriptional activity of Prox1.

#### Sumoylation modulates Prox1 DNA binding and transcriptional activity

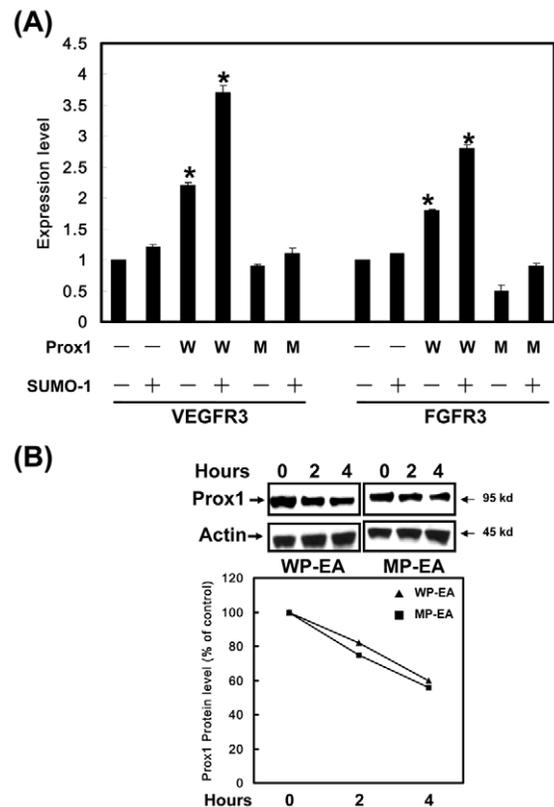
Because Prox1 is a transcription factor, we tested whether sumoylation affected Prox1 transcriptional activity. As shown in



**Fig. 2.** Lys556 (K556) is the major sumoylation site of Prox1. (A) Two potential sumoylation sites (shown in bold) were predicted in human Prox1 protein and were highly conserved in different species. (B) Expression vectors for GST (G), GST-tagged Prox1 (P), K353R (M1) or K556R (M2) mutants were co-transformed with pE1E2-SUMO1 (S1) into *E. coli* and glutathione beads used to purify GST-tagged Prox1. The conjugation of SUMO-1 to Prox1 was detected by anti-Prox1 or anti-SUMO-1 antibody. (C) 293T cells were co-transfected with HA-Prox1 (P), K353R (M1) or K556R (M2) mutants with FLAG-tagged SUMO-1 (S1) expression vector. The expression of Prox1 and FLAG-tagged SUMO-1 in cell lysates was investigated by immunoblotting (IB). Equal amounts of cellular proteins were used for immunoprecipitation (IP) by anti-HA antibody. Western blotting was performed by using anti-FLAG antibody to detect the sumoylation status of Prox1. NS, non-specific band. The blots were also probed with anti-Prox1 antibody to detect the band shift of Prox1. (D) EA.hy926 cells were transfected with control (C), wild-type Prox1 (WP-EA) or K556R mutant Prox1 (MP-EA) expression vectors to generate stable clones. Expression of Prox1-induced target genes was investigated by RT-PCR (upper panel) and western blotting (lower panel). (E) WP-EA and MP-EA cells were transfected with control (C) or SENP2 vector. After 24 hours, the expression of VEGFR3 was studied by western blotting.

Fig. 3A, quantitative RT-PCR demonstrated that overexpression of wild-type Prox1 alone induced 2.2-fold and 1.8-fold increases of mRNA levels of VEGFR3 and FGFR3, respectively. Co-transfection with SUMO-1 synergistically activated the transcription of these two target genes to 3.7-fold and 2.9-fold. However, no significant increase of VEGFR3 and FGFR3 was observed in cells transfected with K556R mutant Prox1. In addition, expression of SUMO-1 did not enhance the transcriptional activity of K556R mutant Prox1.

We also tested whether sumoylation regulated the protein stability of Prox1. Cells were treated with cycloheximide, and the level of Prox1 protein was studied at different times after treatment. Our data indicated that wild-type and K556R mutant Prox1 protein had a similar half-life in cells (Fig. 3B). We next addressed whether sumoylation affected the DNA binding activity of Prox1. Two previously reported *perspero* consensus sequences, C[A/T][C/T]NNC[T/C] and CGTCT[T/A] (Hassan et al., 1997; Cook et al., 2003) were used to blast the human VEGFR3 promoter region. Our results showed that a perfect matched sequence was identified at the -490 to -484 region and another putative binding sequence was found at the -475 to -469 region (Fig. 4A). A biotin-labeled oligonucleotide corresponding to the -507 to -460 region of human VEGFR3 promoter was used for DNA affinity precipitation



**Fig. 3.** Enhancement of Prox1 transcriptional activity by SUMO-1. (A) 293T cells were co-transfected with control (-), wild-type Prox1 (W), K556R mutant Prox1 (M) expression vectors and SUMO-1. After transfection, cells were incubated in medium containing 10% FCS for 48 hours. Quantitative RT-PCR was performed to investigate mRNA levels of VEGFR3 and FGFR3 in cells. \* $P < 0.05$  when the experimental group was compared with the control group. (B) Cells cultured in 10% FCS medium were treated with 10  $\mu\text{g/ml}$  of cycloheximide for various times. The expression of Prox1 was determined by western blotting and the Prox1 protein level of cells collected at time zero was defined as 100%. Actin was used as an internal control.



increase in extracellular signal-regulated kinase (ERK) phosphorylation, an indicator for its kinase activation, in EA.hy926 cells at 5 minutes after growth factor addition. No significant increase of AKT phosphorylation was found. By contrast, VEGF-C effectively stimulated the phosphorylation of ERK and AKT kinase in WP-EA.hy926 cells. However, this stimulation was obviously attenuated in MP-EA.hy926 cells. VEGF-C had little mitogenic effect on parental EA.hy926 and MP-EA.hy926 cells because they expressed very low levels of VEGFR3 (Fig. 5B) but VEGF-C significantly stimulated proliferation of WP-EA.hy926 cells.

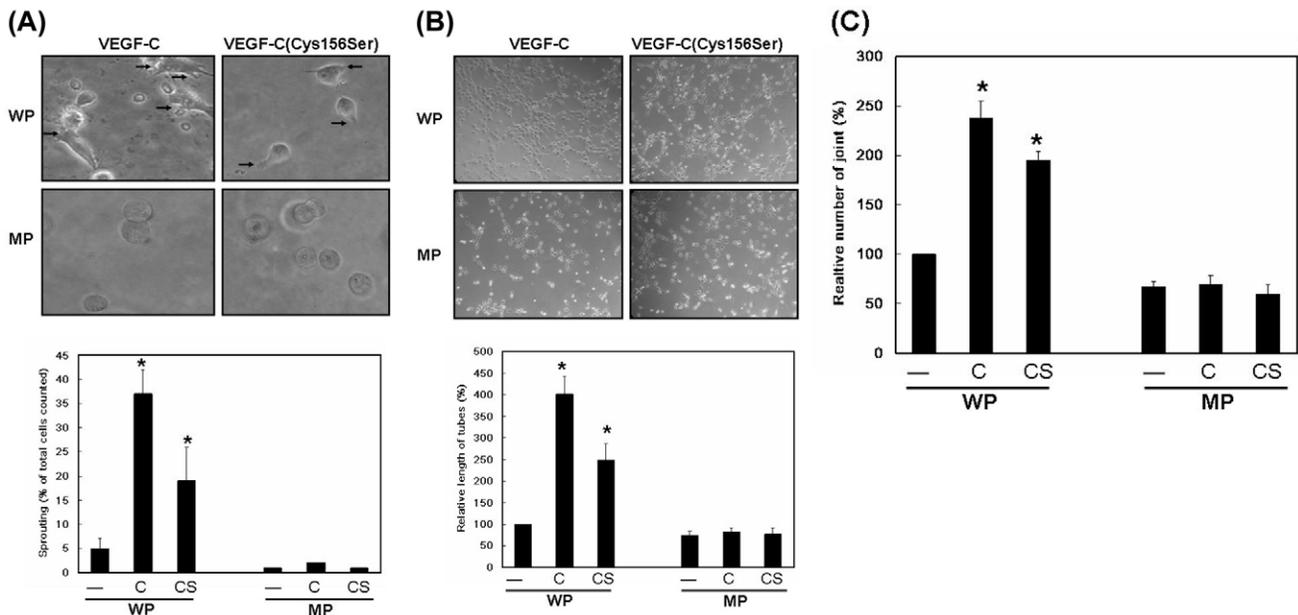
To verify that the proliferative action was mediated via VEGFR3, we also treated cells with VEGF-C (Cys156Ser), a selective agonist for VEGFR3. Our data demonstrated that VEGF-C (Cys156Ser)-stimulated growth of WP-EA.hy926 cells was comparable to the growth stimulated by VEGF-C, indicating that the mitogenic effect was mainly mediated by VEGFR3. Culture of EA.hy926, WP-EA.hy926 and MP-EA.hy926 cells under serum-free conditions for 3 days induced about 65-70% of apoptosis in these cell lines (Fig. 5C). Addition of VEGF-C attenuated the percentage of apoptotic cells to 30% in WP-EA.hy926 cells. However, VEGF-C had little protective effect on EA.hy926 and MP-EA.hy926 cells. Moreover, our results suggested that VEGFR3 was essential for the anti-apoptotic effect because VEGF-C (Cys156Ser) also effectively rescued WP-EA.hy926 cells from the apoptosis induced by serum deprivation.

We next tested the effect of VEGF-C on cell sprouting, a crucial step for the induction of migration and lymphangiogenesis. An averaged sevenfold and fourfold of increase of sprouting was found after VEGF-C and VEGF-C (Cys156Ser) stimulation in WP-

EA.hy926 cells (Fig. 6A). However, the number of sproutings of MP-EA.hy926 was very low and was not increased by VEGF-C and VEGF-C (Cys156Ser). In addition, VEGF-C and VEGF-C (Cys156Ser) increased the tube length of WP-EA.hy926 cells by fourfold and 2.5-fold, respectively (Fig. 6B). By contrast, tube formation ability was attenuated in MP-EA.hy926 cells and was not stimulated by VEGF-C and VEGF-C (Cys156Ser). Similar conclusions were obtained when the number of vessel joint was counted (Fig. 6C). These results suggest that the VEGF-C-transduced lymphangiogenic signals are significantly diminished in MP-EA.hy926 cells that are expressing K556R mutant Prox1.

## Discussion

In this study, we provide evidence that Prox1, the master regulator of lymphatic development and lymphangiogenesis, is a target for SUMO-1. In addition, we have identified the sumoylation site on this homeodomain transcription factor and suggest that sumoylation increases the transcriptional activity of Prox1. While preparing the results of our study for submission, we found that Shan et al. had also reported that sumoylation at K556 of Prox1 reduced its interaction with histone deacetylase 3 and attenuated its transcriptional corepressor activity (Shan et al., 2008). However, the biological function of mutant Prox1 was not addressed in their study. We demonstrated that mutant Prox1 could not increase VEGFR3 expression and that VEGFR3-mediated lymphangiogenic signals were significantly impaired in cells expressing K556R mutant Prox1. These data indicate that sumoylation of Prox1 is important for its role in the induction of gene transcription and lymphatic phenotypes in endothelial cells.



**Fig. 6.** VEGF-C stimulates sprouting and tube formation of Prox1-expressing EA.hy926 endothelial cells. (A) Stable clones expressing either wild-type Prox1 (WP-EA) or K556R mutant Prox1 (MP-EA) were harvested and resuspended in medium containing VEGF-C (C) or VEGF-C (Cys156Ser) (CS). Medium (100  $\mu$ l) containing 10,000 cells was mixed with 200  $\mu$ l Matrigel and the mixture was added into 24-well plates. The final concentrations for VEGF-C and VEGF-C (Cys156Ser) were 100 ng/ml and 500 ng/ml, respectively. Cell sprouting (indicated by arrows) in the three-dimensional culture of Matrigel was observed at 24 hours. The percentage of cells with sprouting in each group was counted and results from three independent assays were expressed as mean  $\pm$  s.e.m. \* $P$ <0.05, when the VEGF-C- or VEGF-C (Cys156Ser)-treated group was compared with the control group. (B) Cells were added into 24-well plates pre-coated with Matrigel and cultured in serum-free medium containing VEGF-C (C, 100 ng/ml) or VEGF-C (Cys156Ser) (CS, 500 ng/ml). Lengths of the tubes obtained from five fields were assayed using AngiogenesisImage Analyzer V.2.0.0 software. Results from three independent assays were expressed as mean  $\pm$  s.e.m. \* $P$ <0.05, when the VEGF-C- or VEGF-C (Cys156Ser)-treated group was compared with the control group. (C) The number of vessel joints was counted and expressed as mean  $\pm$  s.e.m. \* $P$ <0.05, when the VEGF-C- or VEGF-C (Cys156Ser)-treated group was compared with the control group.

The mechanism by which sumoylation regulates Prox1 activity has also been addressed in this study. Sumoylation might modulate the biological functions of target proteins by altering protein stability, subcellular localization, protein-protein interaction, DNA binding and transcriptional activity (Hay, 2005). Some proteins can be ubiquitinated and sumoylated at the same Lys residue, and sumoylation prevents their degradation via the ubiquitin-dependent pathway. For example, the inhibitor of  $\kappa$ B (I $\kappa$ B) undergoes polyubiquitylation at Lys21 and Lys22, which targets the protein for proteasome-mediated degradation (Desterro et al., 1998). Sumoylation on Lys21 blocks I $\kappa$ B ubiquitylation and stabilizes the protein. However, we find that sumoylation does not affect the half-life of Prox1 protein. Our results suggest that sumoylation modulates the DNA binding and transcriptional activity of Prox1.

Lys556 is localized near the DNA binding domain (residues 573–634) of Prox1, so it is possible that sumoylation at this site might induce conformational change and alter the DNA binding and transcriptional activity of Prox1. Indeed, mutation of the Lys556 of Prox1 significantly reduces its DNA binding activity in vitro and in vivo (Fig. 4). Sumoylation has been demonstrated to modulate the activity of various transcription factors positively or negatively. Two pioneer works clearly show that SUMO-1 conjugation activates p53 transcriptional activity (Rodriguez et al., 1999; Gostissa et al., 1999). We provide evidence that SUMO-1 modification can increase Prox1 activity. Taken together, our results suggest that sumoylation enhances Prox1-induced gene expression by increasing DNA binding and transcriptional activity.

Recent studies indicate that lymphangiogenesis plays an important role in the induction of tumor metastasis. However, the study of lymphangiogenesis is obscured by the fact that isolation of lymphatic endothelial cells is difficult. This is due to two reasons. The first reason is lack of specific markers for the identification of lymphatic endothelial cells. This difficulty has been recently resolved by the identification of several lymphatic markers, including Prox1, podoplanin and LYVE-1. Secondly, the amount of lymphatic endothelial cells isolated from primary cultures is small and these cells are not easy to use for gene transfection.

In this study, we have established a stable clone by overexpressing Prox1 in EA.hy926 endothelial cells. Characterization of the gene expression profile in the stable clone demonstrates that lymphatic endothelial cell markers including VEGFR3, FGFR3 and p57 are significantly upregulated, whereas the blood endothelial cell marker STAT6 is downregulated. These data suggest that our established clone exhibits the characteristics of lymphatic endothelial cells and that this cell line could serve as a research model for the study of lymphangiogenesis.

## Materials and Methods

### Cell lines and plasmids

EA.hy926 cells were kindly provided Ming-Hong Tai (National Sun Yat-Sen University, Kaohsiung, Taiwan). This cell line was derived by fusing human umbilical vein endothelial cells with A549 human lung cancer cells (Edgell et al., 1983). HepG2 human hepatoma cells were obtained from the cell bank of the National Health Research Institute (Maoli, Taiwan). Cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS; Hyclone) and antibiotics. Normal human lymphatic endothelial cells (No. C-12218) were purchased from PromoCell (Heidelberg, Germany) and cultured in endothelial cell growth medium MV2. pcDNA3-FLAG-Prox1 was a kind gift of You Hua Xie (Shanghai Institute for Biological Sciences, Shanghai, China). pcDNA-HA-Prox1 was generously provided by Terje Johansen (University of Tromsø, Tromsø, Norway). pcDNA-HA-SUMO-1 was kindly provided by Angela Chen (National Sun Yat-Sen University, Kaohsiung, Taiwan). SUMO-specific protease 2 (SEN2) expression vector was a generous gift of Hsiu-Ming Shih (Academia Sinica, Taipei, Taiwan). Mutations of Lys353 and

Lys556 to arginine were introduced to pcDNA-HA-Prox1 by using the QuikChange site-directed mutagenesis kit according to the procedures of the manufacturer (Stratagene, La Jolla, CA). The FGFR3 promoter-luciferase construct was kindly provided by David M. Ornitz (Department of Developmental Biology, Washington University School of Medicine, St Louis, MO).

### Reagents and antibodies

VEGF-C and VEGF-C (Cys156Ser) were purchased from R&D Systems (Minneapolis, MN). Matrigel was obtained from BD (Franklin Lakes, NJ). QuikChange site-directed mutagenesis kit was obtained from Stratagene. TransFast reagent was purchased from Promega (Madison, WI). Antibodies against GST (SC-138), HA (SC-7392), VEGFR3 (SC-28297) and p57 (SC-1040) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK (9102), anti-phospho-ERK (9101), anti-AKT (9272) and anti-phospho-AKT (9271) antibodies were obtained from New England Biolabs (Ipswich, MA). Anti-SUMO-1 antibody (33-2400) used for immunoblotting was obtained from Zymed (South San Francisco, CA). Anti-SUMO-1 antibody (ab11672) used for immunoprecipitation was purchased from Abcam (Cambridge, MA). Anti-Prox1 antibody (07-537) was purchased from UpState (Charlottesville, VA) and anti-actin antibody (MAB1501) was obtained from Millipore (Billerica, MA). Antibody against human fibroblast growth factor receptor 3 (FGFR3) (MAB7661) was purchased from R&D Systems.

### In vitro sumoylation assay

The expression-modification system used to produce SUMO-conjugated proteins in *E. coli* was established as described previously (Uchimura et al., 2004a; Uchimura et al., 2004b) and was kindly provided by Yasuhiro Uchimura and Hisato Saitoh (Kumamoto University, Kumamoto, Japan). Anti-SUMO-1 expression vector was co-transfected with pE1E2-SUMO-1 or pE1E2-SUMO-2 expression vector into *E. coli*, and glutathione beads were used to purify GST-tagged Prox1. The conjugation of SUMO to Prox1 was investigated by western blotting with anti-Prox1 or anti-SUMO antibody.

### RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and real time RT-PCR

Total RNA was isolated from cells and Prox1 expression was examined by using the OneStep RT-PCR kit as described previously (Pan et al., 2008). The primers used were: p57-forward: 5'-GAGCAGCTGCCTAGTGCC-3'; p57-reverse: 5'-GCGAAGAAATCGGAGATCAG-3'; STAT6-forward, 5'-GCATCTTCTGGGTGACTGG-3'; STAT6-reverse, 5'-AGCTGTGCAGAGACACTTGG-3'; Prox1-forward: 5'-TGGCCTATCCATTCAGAGC-3'; Prox1-reverse: 5'-ATCCTTCTGCATTGC-3'; VEGFR3-forward: 5'-GGAGAAGTCTCCCGAGCAG-3'; VEGFR3-reverse: 5'-CTTGTCGAAGATGCTGGCAGG-3'; FGFR3-forward: 5'-GACGGCA-CACCCTACGTTAC-3'; FGFR3-reverse: 5'-GGATGCCTGCATACACACTG-3'; GAPDH-forward: 5'-GAGTCAACGGATTGGTCGT-3'; and GAPDH-reverse: 5'-TGTGGTCATGAGTCTTCCA-3'. In real-time RT-PCR analysis, cDNA samples (2  $\mu$ g) were used as template for amplification reactions by using the iQ SYBR Green Supermix kit (Bio-Rad) following the manufacturer's procedures. The conditions for PCR amplification were 95°C (15 seconds), 64°C (12 seconds) and 72°C (12 seconds) for 40 cycles, and the reaction was carried out using the MJ Mini real-time PCR detection system (Bio-Rad). The results were normalized to GAPDH and the relative levels of VEGFR3 and FGFR3 from three independent experiments were expressed as mean  $\pm$  s.e.m.

### Preparation of nuclear fractions and DNA affinity precipitation assay

Preparation of nuclear extracts from WP-EA and MP-EA cells was carried out as described previously (Andrew and Faller, 1991). Biotin-labeled DNA probe 5'-CTGGGATTACAGGCGCGCACAAACCAAGCTCGGCGTATTTATTTATTTA-3' (sense strand) corresponding to the -507 to -460 region from the transcriptional start site of human VEGFR3 promoter containing two potential Prox1 binding sites (underlined) was used to interact with nuclear proteins. DAPA assays were performed as described previously (Wang et al., 2006).

### Chromatin immunoprecipitation assay

The ChIP assay was performed as described previously (Chang et al., 2006). The sequences for the primers are: sense 5'-GTCCACTGCAACCTCTTCT-3' and anti-sense 5'-GGCAAAGGGGAGCAAAT-3'. The predicted size for PCR product is 281 bp containing the -568 to -288 bp region of the human VEGFR3 promoter.

### Generation of Prox1-expressing stable clones

EA.hy926 cells were transfected with the expression vectors of pcDNA-HA-Prox1 or its K556R mutant. After 48 hours, cells were incubated with 200  $\mu$ g/ml G418 for 3 weeks. Stable clones expressing a high level of wild-type (WP-EA.hy926) or mutant (MP-EA.hy926) Prox1 were selected and used for the study of VEGF-C-induced lymphangiogenic signals.

### Immunoprecipitation and western blotting

Cells were lysed directly in a modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris (pH 7.8), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, protease inhibitor mixture and 10 mM *N*-ethylmaleimide (to preserve the SUMO-conjugated proteins). Lysates were further subjected to immunoprecipitation and western blotting as described previously (Pan et al., 2008).

### Phosphorylation of ERK and AKT kinases

EA.hy926, WP-EA.hy926 and MP-EA.hy926 cells were stimulated with 100 ng/ml of VEGF-C for different time (0-30 minutes). Cells were harvested and subjected to western blotting. The phosphorylation status of ERK and AKT kinases were studied using anti-phospho-ERK and anti-phospho-AKT antibodies.

### Analysis of protein stability

WP-EA.hy926 and MP-EA.hy926 cells cultured in 10% FCS medium were treated with 10 µg/ml of cycloheximide to block protein synthesis. Cellular proteins were harvested at various times and Prox1 protein level was determined by western blotting. Prox1 protein level in cells collected at time zero was defined as 100%.

### Cell proliferation and apoptosis assays

For cell proliferation assays, EA.hy926, WP-EA.hy926 and MP-EA.hy926 cells were seeded at a density of 8000 cells/well in 96-well plates and incubated with 100 ng/ml of VEGF-C or 500 ng/ml of VEGF-C (Cys156Ser) in serum-free medium for 24 hours. MTT assay was carried out as previously described to investigate the growth of cells (Hung et al., 1999). For apoptosis assay, cells were cultured in serum-free medium containing 100 ng/ml of VEGF-C or 500 ng/ml of VEGF-C (Cys156Ser) for 3 days. Floating cells were collected by centrifugation, and adhesive cells harvested by trypsinization. Cells were pooled and stained with propidium iodide. Apoptotic cells with condensed chromatin were counted using fluorescence microscopy.

### Cell sprouting and tube formation

For investigation of sprouting, cells were harvested by trypsinization and resuspended in medium containing VEGF-C or VEGF-C (Cys156Ser). Medium (100 µl) containing 10,000 cells was mixed with 200 µl Matrigel and the mixture added into 24-well plates. The final concentrations of VEGF-C and VEGF-C (Cys156Ser) were 100 ng/ml and 500 ng/ml, respectively. Cell sprouting in a three dimensional culture of Matrigel was observed at 24 hours after incubation. Cells ( $n=100$ ) were counted and the percentage of cells with sprouting was expressed as mean  $\pm$  s.e.m. For tube formation assay, cells (40,000/well) suspended in serum-free medium containing VEGF-C (100 ng/ml) or VEGF-C (Cys156Ser) (500 ng/ml) were added into 24-well plates pre-coated with Matrigel. The formation of tube-like structure was observed at 4 hours after cell addition. As a quantitative estimation for angiogenesis of the tubes, the vessel length and the number of vessel joints obtained from five fields were assayed using the AngiogenesisImage Analyzer V2.0.0 software (Kurabo Industries, Osaka, Japan). Results from three independent experiments were expressed as mean  $\pm$  s.e.m.

### Statistical analysis

Student's *t*-test was used to evaluate the difference between various experimental groups. Differences were considered to be significant at  $P<0.05$ .

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### References

Alitalo, K., Tammela, T. and Petrova, T. (2005). Lymphangiogenesis in development and human disease. *Nature* **438**, 946-953.  
 Andrew, N. C. and Faller, D. V. (1991). A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499.  
 Chang, H. C., Cho, C. Y. and Hung, W. C. (2006). Silencing of the metastasis suppressor RECK by RAS oncogene is mediated by DNA methyltransferase 3b-induced promoter methylation. *Cancer Res.* **66**, 8413-8420.

Cook, T., Pichaud, F., Sonnevile, R., Papatsenko, D. and Desplan, C. (2003). Distinction between color photoreceptor cell fates is controlled by Prospero in *Drosophila*. *Dev. Cell* **4**, 853-864.  
 Cross, M. J., Dixelius, J., Matsumoto, T. and Claesson-Welsh, L. (2003). VEGF- receptor signal transduction. *Trends Biochem. Sci.* **28**, 488-494.  
 Desterro, J. M., Rodriguez, M. S. and Hay, R. T. (1998). SUMO-1 modification of IkkappaBalpha inhibits NF-kappaB activation. *Mol. Cell* **2**, 233-239.  
 Edgell, C. J., McDonald, C. C. and Graham, J. B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA* **80**, 3734-3737.  
 Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M. and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. *EMBO J.* **18**, 6462-6471.  
 Hassan, B., Li, L., Bremer, K. A., Chang, W., Pinsonneault, J. and Vaessin, H. (1997). Prospero is a panneuronal transcription factor that modulates homeodomain protein activity. *Proc. Natl. Acad. Sci. USA* **94**, 10991-10996.  
 Hay, R. T. (2005). SUMO: a history of modification. *Mol. Cell* **18**, 1-12.  
 Hong, Y. K., Harvey, N., Noh, Y. H., Schacht, V., Hirakawa, S., Detmar, M. and Oliver, G. (2002). Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev. Dyn.* **225**, 351-357.  
 Hung, W. C., Chang, H. C. and Chuang, L. Y. (1999). Activation of caspase-3-like proteases in apoptosis induced by sphingosine and other long-chain bases in Hep3B hepatoma cells. *Biochem. J.* **338**, 161-166.  
 Makinen, T., Veikkola, T., Mustjoki, M., Karpainen, T., Catimel, B., Nice, E. C., Wise, L., Mercer, A., Kowalski, H. and Kerjaszki, D. (2001). Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J.* **20**, 4762-4773.  
 Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E. P., Doe, C. Q. and Gruss, P. (2003). Prox 1, a prospero-related homeobox gene expressed during mouse development. *Mech. Dev.* **44**, 3-16.  
 Olsson, A., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006). VEGF receptor signaling in control of vascular function. *Nat. Rev. Mol. Cell Biol.* **7**, 359-371.  
 Pan, M. R., Hou, M. F., Chang, H. C. and Hung, W. C. (2008). Cyclooxygenase-2 up-regulates CCR7 via EP2/EP4 receptor signaling pathways to enhance lymphatic invasion of breast cancer cells. *J. Biol. Chem.* **283**, 11155-11163.  
 Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaszki, D., Yla-Herttuala, S. and Alitalo, K. (2002). Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* **21**, 4593-4599.  
 Rieber, A. J., Marr, H. S., Comer, M. B. and Edgell, C. J. (1993). Extent of differentiated gene expression in the human endothelium-derived EA.hy926 cell line. *Thromb. Haemost.* **69**, 476-480.  
 Rodriguez, M. S., Desterro, J. M. P., Lain, S., Midgley, C. A., Lane, D. P. and Hay, R. T. (1999). SUMO-1 modification activates the transcriptional response of p53. *EMBO J.* **18**, 6455-6461.  
 Rodriguez-Niedenfuhr, M., Papoutsis, M., Christ, B., Nicolaidis, K. H., von Kaisenberg, C. S., Tomarev, S. I. and Wilting, J. (2001). Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. *Anat. Embryol.* **204**, 399-406.  
 Shan, S. F., Wang, L. F., Zhai, J. W., Qin, Y., Ouyang, H. F., Kong, Y. Y., Liu, J., Wang, Y. and Xie, Y. H. (2008). Modulation of transcriptional corepressor activity of prospero-related homeobox protein (Prox1) by SUMO modification. *FEBS Lett.* **582**, 3723-3828.  
 Stacker, S. A., Achen, M. G., Jussila, L., Baldwin, M. E. and Alitalo, K. (2002). Lymphangiogenesis and cancer metastasis. *Nat. Rev. Cancer* **2**, 573-583.  
 Tobler, N. E. and Detmar, M. (2006). Tumor and lymph node lymphangiogenesis -impact on cancer metastasis. *J. Leukoc. Biol.* **80**, 691-696.  
 Uchimura, Y., Nakao, M. and Saitoh, H. (2004a). Generation of SUMO-1 modified proteins in *E. coli*: towards understanding the biochemistry/structural biology of the SUMO-1 pathway. *FEBS Lett.* **564**, 85-90.  
 Uchimura, Y., Nakamura, M., Sugawara, K., Nakao, M. and Saitoh, H. (2004b). Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in *Escherichia coli*. *Anal. Biochem.* **331**, 204-206.  
 Wang, C. H., Chang, H. C. and Hung, W. C. (2006). p16 inhibits matrix metalloproteinase-2 expression via suppression of Sp1-mediated gene transcription. *J. Cell. Physiol.* **208**, 246-252.  
 Wigle, J. T. and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769-778.  
 Wigle, J. T., Chowdhury, K., Gruss, P. and Oliver, G. (1999). Prox1 function is crucial for mouse lens-fibre elongation. *Nat. Genet.* **21**, 318-322.  
 Wigle, J. T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M. D., Jackson, D. G. and Oliver, G. (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* **21**, 1505-1513.