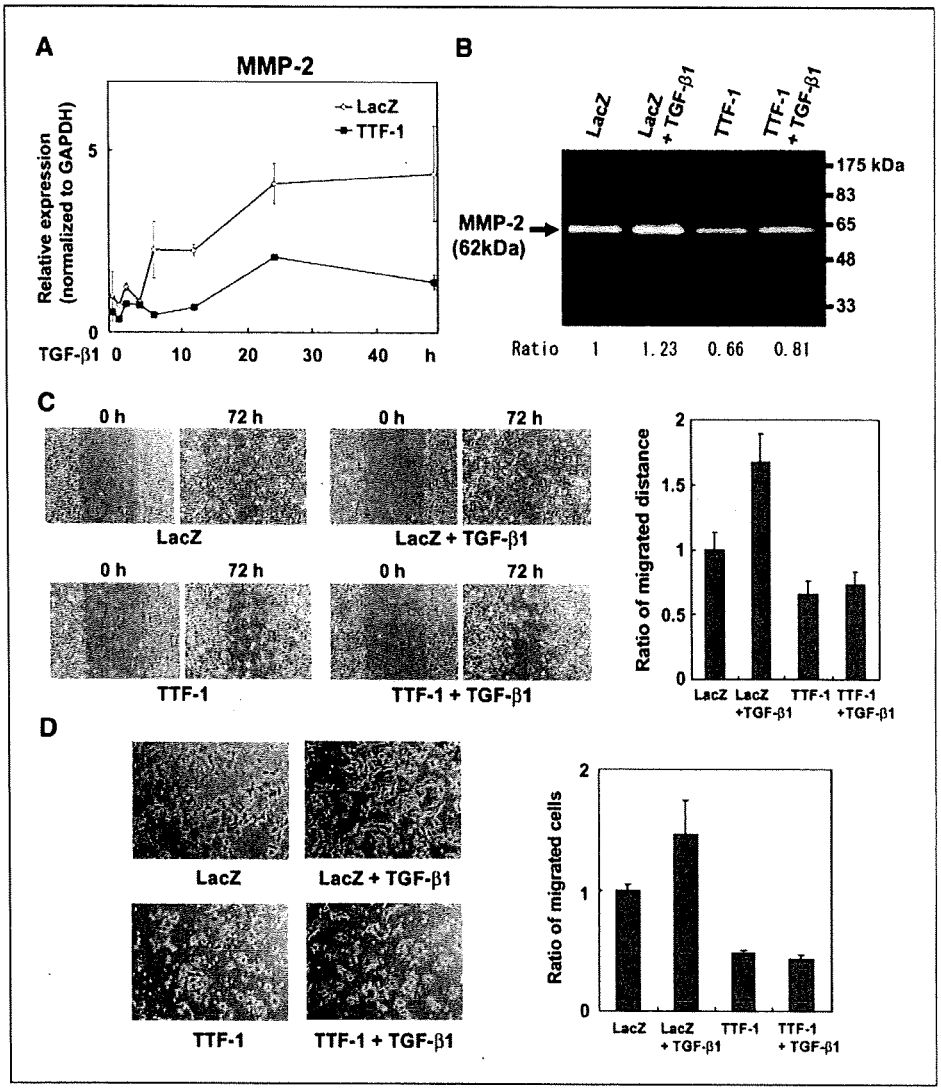

Figure 2. TTF-1 attenuates MMP-2 activity, cell migration, and invasive capacity. *A*, quantitative RT-PCR. Kinetic expression of MMP-2 after TGF-β1 treatment in LacZ-transduced or TTF-1–transduced cells. *Bars*, SD. The values indicate the fold difference compared with 0 h control of LacZ-expressing cells. *B*, gelatin zymography. Gelatin digestion by activated MMP-2 was quantified and relative intensity to control is indicated. Molecular mass markers are in kDa. *C*, *left*, cells infected with Ad-LacZ or Ad-TTF-1 were scratched and incubated with or without TGF-β1 for 72 h; *right*, quantitation of wound healing assay. The distance of cell migration was measured after 24 h by time-lapse video microscopy at eight fields for each group. *Bars*, SD. *D*, *left*, cell invasion assay. The migrated cells were stained with crystal violet. *Right*, quantitation of invasion assay. The migrated cells were counted, and the sum of five random fields was obtained for each well. Each experiment was performed in triplicate. *Bars*, SD.

cancer cells, we used chambers coated with collagen IC. TGF-β treatment resulted in increased number of migrated cells on the lower face of the chambers. TTF-1–expressing cells showed impaired migration through the filters, and the action of TGF-β was completely antagonized by TTF-1 (Fig. 2*D*, *left*). Quantitation of these results revealed that TTF-1 inhibited the invasive capacity of lung adenocarcinoma cells and TGF-β failed to restore it (Fig. 2*D*, *right*).

TTF-1 negatively regulates the expression of molecules involved in EMT. In A549 cells, ectopic TTF-1 inhibited the induction of TGF-β target genes, Smad7 and PAI-1, which are regulated by Smad pathway (Fig. 3*A*). Despite of these striking differences, phosphorylation of Smad2 or Smad3 after TGF-β treatment displayed no significant difference between the control and TTF-1–expressing cells (Supplementary Figs. S5*A* and S5*B*). Next, we knocked down the expression of endogenous TTF-1 in H441 cells. Transfection of TTF-1 siRNA effectively suppressed the expression of TTF-1 (Fig. 3*B*, *left*). TTF-1 knockdown resulted in enhanced induction of Smad7 and PAI-1 after TGF-β stimulation in H441 cells (Fig. 3*B*, *right*), consistent with the results in A549 cells. Recent data have shown that Smad3 physically interacts with TTF-1 and regulates the transcription of the TTF-1 target gene SPB (19, 20). Taken together, it is suggested that TTF-1 suppresses Smad-mediated transcription of a subset of TGF-β target genes in the nucleus and, thereby, inhibits TGF-β–mediated EMT in lung adenocarcinoma cells.

We further explored the antagonistic effects of TTF-1 against TGF-β–induced EMT. Expression of E-cadherin is regulated by multiple transcription factors, including zinc finger transcriptional repressors Snail and Slug (8). TTF-1 suppressed the basal expression level of Snail and Slug, and their rapid induction after TGF-β treatment was also inhibited by TTF-1 (Fig. 3*C*, *left*). Suppressed expression of Snail was also shown by immunoblotting (Fig. 3*C*, *right*). Although LY364947 treatment suppressed the expression of Snail and Slug after 24 h in LacZ-transduced cells, it did not induce further decrease in TTF-1–transduced cells (Supplementary Figs. S4*B* and *C*).

Luciferase assay showed that Snail or Slug suppresses the human E-cadherin promoter activity, antagonizing the action of TTF-1 to enhance it (Supplementary Fig. S6*A*). Furthermore, adenoviral

transduction of human Snail resulted in down-regulation of E-cadherin and up-regulation of N-cadherin and fibronectin, mimicking the effect of TGF-β (Supplementary Fig. S6*B*). These results support the idea that Snail and Slug are involved in the regulation of EMT in A549 cells, as previously described in other cell types.

Recently, platelet-derived growth factor (PDGF) signaling (21) and collagen I (22) have been reported to be involved in TGF-β–induced EMT. In A549 cells, TGF-β stimulation resulted in the induction of PDGF-B and α1(I) collagen, whereas this effect was blocked by ectopic TTF-1 (Supplementary Figs. S7*A* and *B*). These results suggest that TTF-1 blocks EMT and induces epithelial differentiation by suppression of an array of events leading to EMT. In addition, induction of CTGF after TGF-β treatment was also inhibited by TTF-1 (Supplementary Fig. S7*C*). Thus, it is also suggested that TTF-1 can act as an antifibrotic factor in cancer, as well as in fibrotic disorders, through down-regulation of fibrotic factors.

Silencing of TTF-1 modulates epithelial phenotypes and enhances TGF-β–mediated EMT. To further address the effect of TTF-1 on TGF-β–induced EMT, we knocked down endogenous TTF-1 in H441 cells. Control or TTF-1 siRNA was transfected at 0 and 72 hours in the presence or absence of continuous TGF-β stimulation, and the cell morphology was examined at 144 hours (Fig. 4*A*), because it was previously reported that alveolar epithelial cells undergo EMT when chronically treated with TGF-β for >144 hours (23). Silencing of TTF-1 in H441 cells resulted in morphologic changes to a flattened or elongated shape with decreased cell-cell attachment (Fig. 4*A*). TGF-β treatment led to the reorganization of actin stress fibers, whereas cell-cell adhesions were sustained (Fig. 4*A* and Supplementary Fig. S8*A*). The cells with combined treatment of TTF-1 knockdown and TGF-β showed impaired cell-cell attachment, and fibroblast-like cells were frequently found when cultured at low cell density (Fig. 4*A, bottom*).

H441 cells were also immunostained for E-cadherin, ZO-1, and pan-cytokeratin (Supplementary Fig. S8*B*). E-cadherin staining on

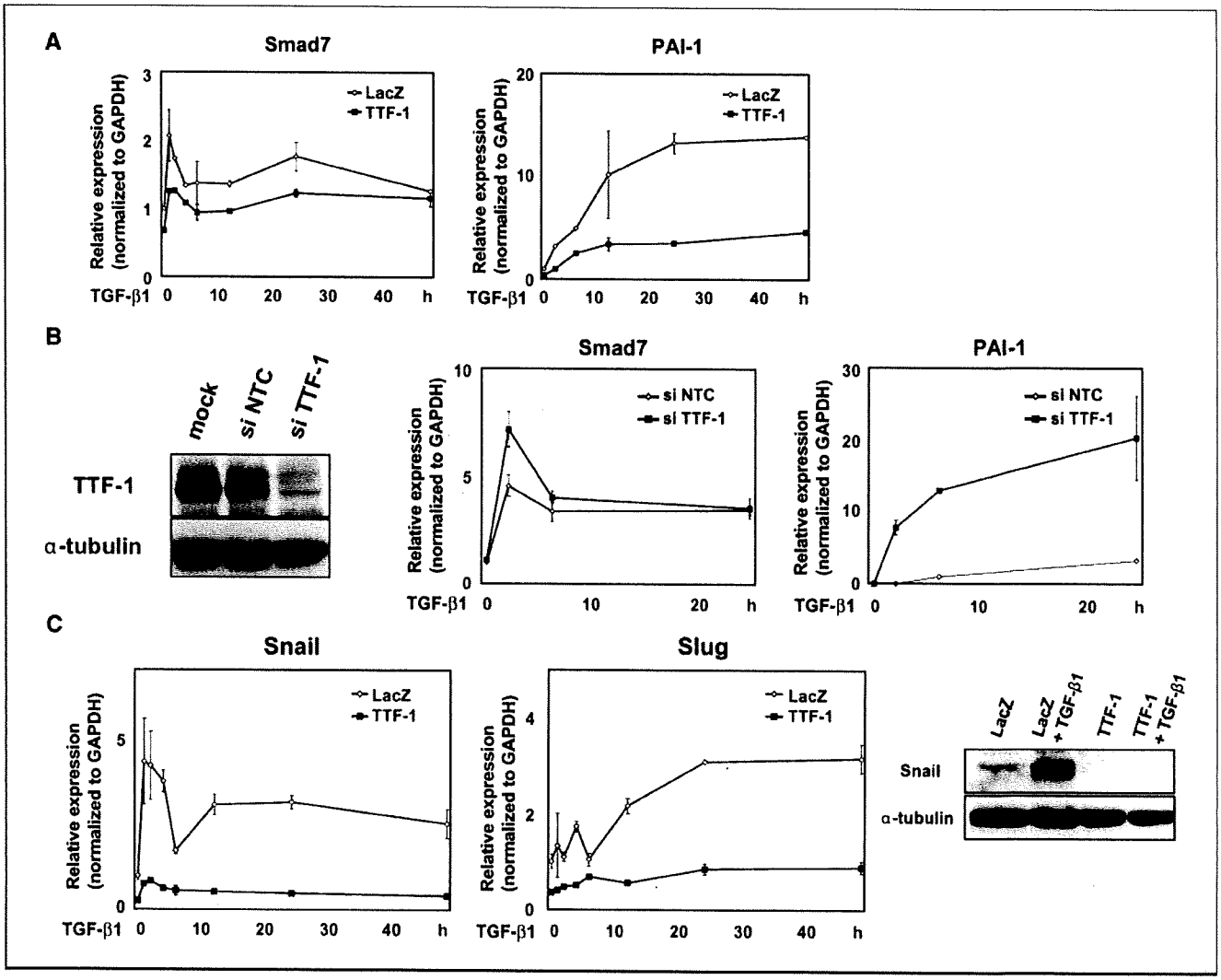

Figure 3. TTF-1 down-regulates the molecules involved in EMT. *A*, quantitative RT-PCR. Kinetic expression of Smad7 and PAI-1 indicated as in Fig. 2*A*. *B, left,* immunoblotting of TTF-1 in H441 cells transfected with mock, control siRNA (*si NTC*), and siRNA for TTF-1 (*si TTF-1*). α-Tubulin was used as a loading control. *Right,* quantitative RT-PCR. Kinetic expression of Smad7 and PAI-1. H441 cells were transfected with si NTC or si TTF-1 and treated with TGF-β1 for the indicated time periods. *C, left,* quantitative RT-PCR. Kinetic expression of Snail and Slug. *Right,* immunoblotting of Snail in A549 cells infected with Ad-LacZ or Ad-TTF-1 and treated with or without TGF-β1 for 24 h.

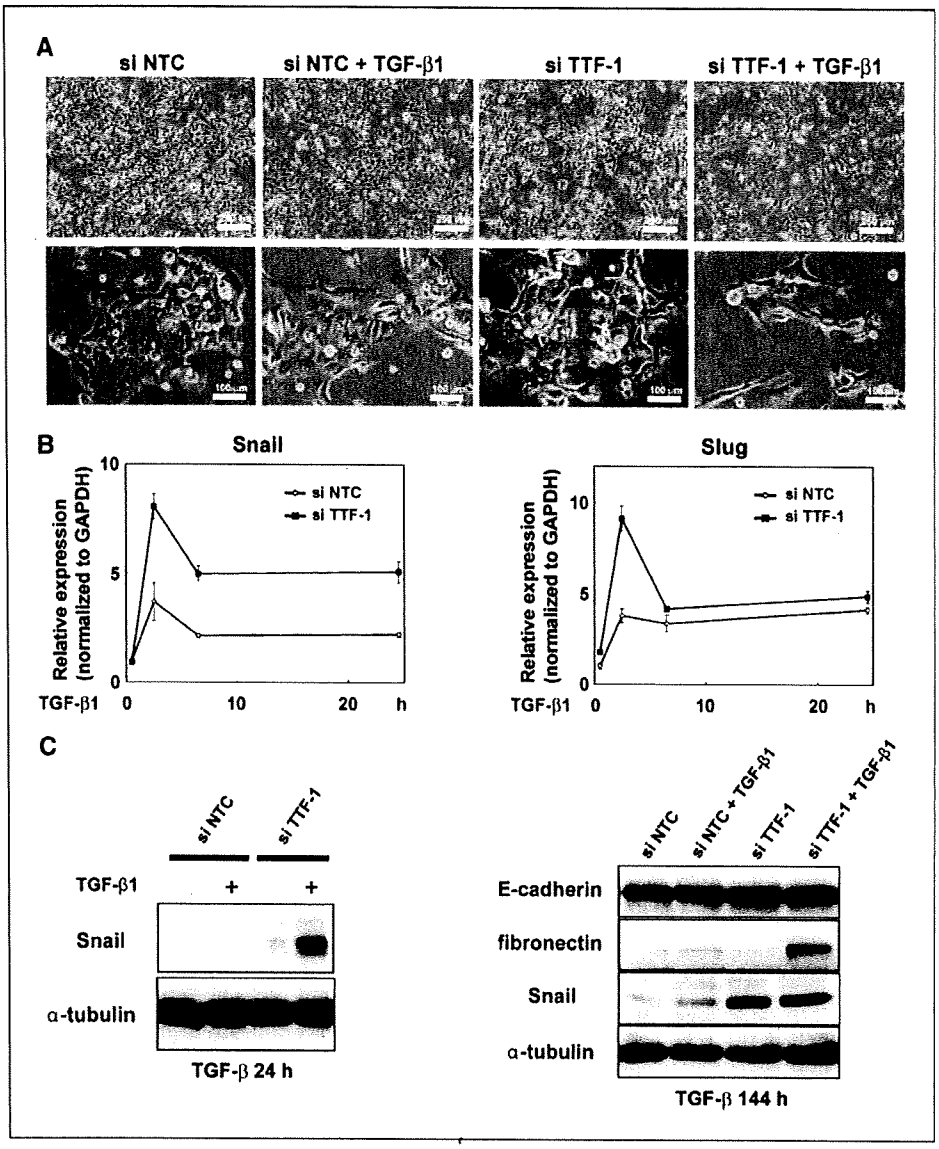

Figure 4. Silencing of TTF-1 enhances TGF-β–mediated EMT. *A*, phase contrast microscopy of H441 cells transfected with si NTC or si TTF-1 and incubated with or without TGF-β1 for 144 h. *B*, quantitative RT-PCR. Kinetic expression of Snail and Slug indicated as in Fig. 3*B*. *C*, *left*, immunoblotting of Snail in H441 cells transfected with si NTC or si TTF-1 and treated with or without TGF-β1 for 24 h; *right*, immunoblotting of E-cadherin and mesenchymal markers (fibronectin and Snail). H441 cells were transfected with si NTC or si TTF-1 at 0 and 72 h and incubated with or without TGF-β1 for 144 h.

the cell membrane was clearly observed in H441 cells. In contrast to A549 cells, E-cadherin expression was persistent even after TGF-β treatment. TTF-1 knockdown alone failed to significantly suppress its expression, but simultaneous treatment with TGF-β resulted in loss of cell-cell adhesions and substantially decreased E-cadherin staining. Irregular staining of ZO-1 was noted in H441 cells, and TTF-1 knockdown or TGF-β treatment led to its reduced expression. Pan-cytokeratin expression was decreased but sustained even after TGF-β treatment or TTF-1 knockdown. Together with the results in A549 cells, cytokeratins might be persistently expressed in lung cancer cells with mesenchymal phenotypes, consistent with the clinical findings that most lung cancer cells keep expressing cytokeratins.

We next examined the effect of TTF-1 knockdown on both TGF-β–mediated rapid induction of Snail or Slug and expression of EMT markers. Consistent with the observations in A549 cells (Fig. 3*C*), silencing of TTF-1 resulted in enhanced induction of Snail and Slug (Fig. 4*B*). Enhanced induction of Snail was also shown by immunoblotting (Fig. 4*C*, *left*). We also studied the effect of chronic exposure (144 hours) to TGF-β. TTF-1 knockdown resulted in enhanced expression of Snail, and the induction of fibronectin mediated by TGF-β was enhanced under the condition that TTF-1 was knocked down (Fig. 4*C*, *right*). These observations support the action of TTF-1, which inhibits EMT mediated by TGF-β. Contrary to the immunocytochemical observations (Supplementary Fig. S8*B*), E-cadherin expression was not significantly affected by either TGF-β treatment or TTF-1 knockdown in a bulk population of the cells cultured at high cell density (Fig. 4*C*, *right*). This result suggested that E-cadherin expression is retained by other mechanisms that might overcome the effect of TGF-β or TTF-1 in H441 cells cultured at high cell density.

Reciprocal regulation of TTF-1 expression and TGF-β signaling. To address the effect of TGF-β on the expression of TTF-1, we used two different lung adenocarcinoma cell lines, H441 and LC-2/ad, which endogenously express TTF-1. TGF-β treatment for 72 hours suppressed the expression of TTF-1 mRNA and protein

in both cell lines (Fig. 5*A* and *B*), and blockade of TGF-β signaling with LY364947 resulted in restoration of TTF-1 expression suppressed by TGF-β (Fig. 5*B*). These findings were consistent with the previous report, showing reduced expression of TTF-1 in alveolar epithelial cells undergoing EMT (23).

To examine the effect of TTF-1 on the expression of TGF-β ligands, semiquantitative RT-PCR was performed for the three isoforms of TGF-β in A549 cells. Transcription of TGF-β2 was down-regulated by TTF-1, whereas expression levels of TGF-β1 transcripts were not significantly different between the control and the TTF-1–transduced cells (Fig. 5*C*). Transcripts of TGF-β3 were not detected in A549 cells (data not shown). Down-regulation of TGF-β2 expression was further confirmed by quantitation of TGF-β2 protein in the conditioned media (Fig. 5*D*). Taken together, reciprocal regulation between TTF-1 and TGF-β signaling has been observed, suggesting that enhancement of autocrine TGF-β signaling accelerates the decrease of TTF-1 expression and vice versa.

TTF-1 inhibits tumor progression *in vivo*. To address the effect of TTF-1 *in vivo*, we used a mouse syngenic model. Mouse LLC cells stably expressing green fluorescent protein (GFP) or TTF-1 were generated by retroviral gene transfer and were inoculated into syngenic C57/BL6 mice. Retroviral transduction was confirmed by GFP fluorescence (Supplementary Fig. S9*A*). LLCs cells lacked TTF-1 expression, and ectopic TTF-1 was located in the nucleus (Supplementary Fig. S9*B*). Expression of TTF-1 resulted in retardation of tumor growth (Fig. 6*A*), and survival rate was prolonged (Fig. 6*B*). Blood vessel density was lower in the TTF-1–expressing tumor, suggesting that TTF-1 expression might affect tumor-stromal interactions (Fig. 6*C* and *D*).

Discussion

In the present study, we showed that TTF-1 inhibits EMT in response to TGF-β and restores epithelial phenotypes in lung adenocarcinoma cells, leading to suppression of cell migration and invasion. TTF-1 abrogated TGF-β–mediated induction of Snail and Slug, which regulate the changes in gene expression patterns that underlie EMT (9). On the other hand, expression profiles of other factors that have been implicated in EMT, such as δEF-1 (ZEB1) and SIP1 (15), HMGA2 (24), and Twist1 (25), suggested that they are not involved in either TGF-β–mediated EMT or the effect of TTF-1 in A549 cells (data not shown). The mechanism of how TTF-1 inhibits TGF-β–mediated EMT could be explained by multiple mechanisms. One is the suppression of Smad-mediated transcription of EMT-inducing molecules, such as Snail and Slug (Fig. 3), as suggested by the recent findings that Smad3 physically interacts with TTF-1 and regulates its transcriptional activity (19, 20). We have also shown the importance of another pathway; i.e., attenuation of autocrine TGF-β signaling by TGF-β2 down-regulation (Fig. 5).

Accumulating evidence of genomic analyses revealed that TTF-1 gene is amplified in 10% to 15% of lung adenocarcinomas, and *in vitro* studies further support the concept that TTF-1 acts as a lineage-specific oncogene (26–28). On the other hand, the functional significance of TTF-1 in other subsets of lung adenocarcinomas, wherein TTF-1 expression is reduced or lost, still remains to be elucidated.

It is reported that TTF-1 expression is high in well-differentiated carcinomas and relatively low in poorly differentiated carcinomas (13). According to the classification of lung adenocarcinomas into terminal respiratory unit (TRU) type and

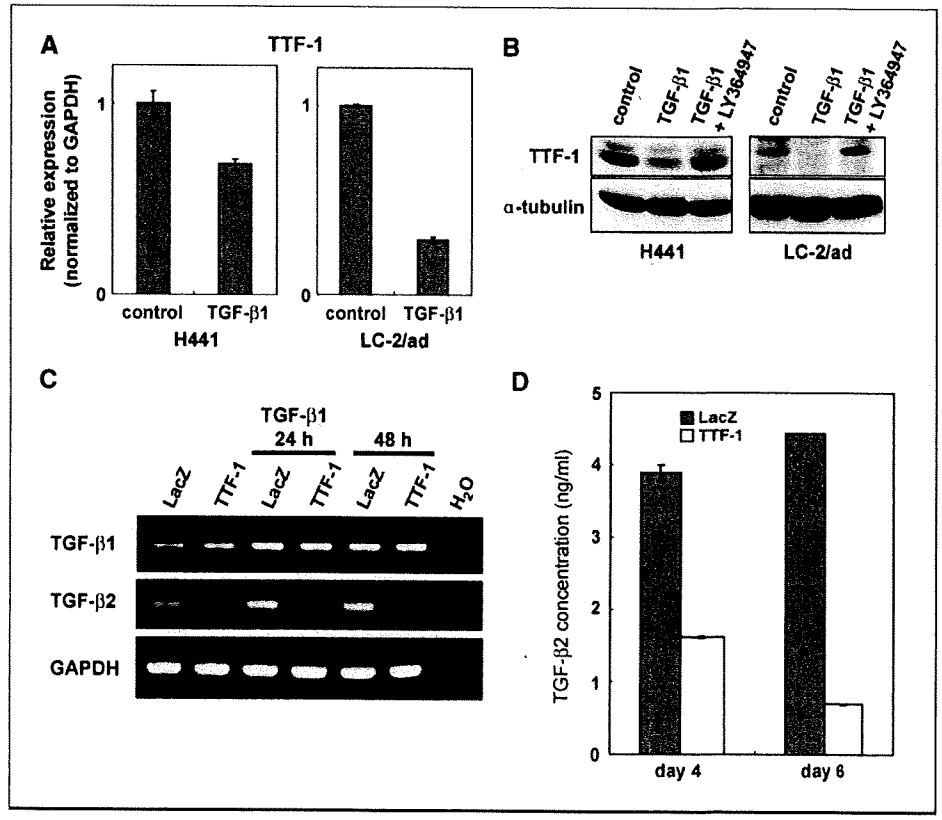

Figure 5. Exogenous TGF-β down-regulates TTF-1 and TTF-1 down-regulates TGF-β2 in lung adenocarcinoma cells. *A*, quantitative PCR of TTF-1. H441 or LC-2/ad cells were treated with or without TGF-β1 for 72 h. *Bars*, SD. *B*, immunoblotting of TTF-1. H441 or LC-2/ad cells were treated with or without TGF-β1 and LY364947 for 72 h. *C*, semiquantitative RT-PCR. A549 cells infected with Ad-LacZ or Ad-TTF-1 were incubated in the presence or absence of TGF-β1 for additional 48 h. *D*, ELISA for TGF-β2. A549 cells infected with Ad-LacZ or Ad-TTF-1 were incubated in serum free media for 48 h (4 d after infection). The cells were incubated for another 48 h in the replaced serum-free media (6 d after infection). *Bars*, SD.

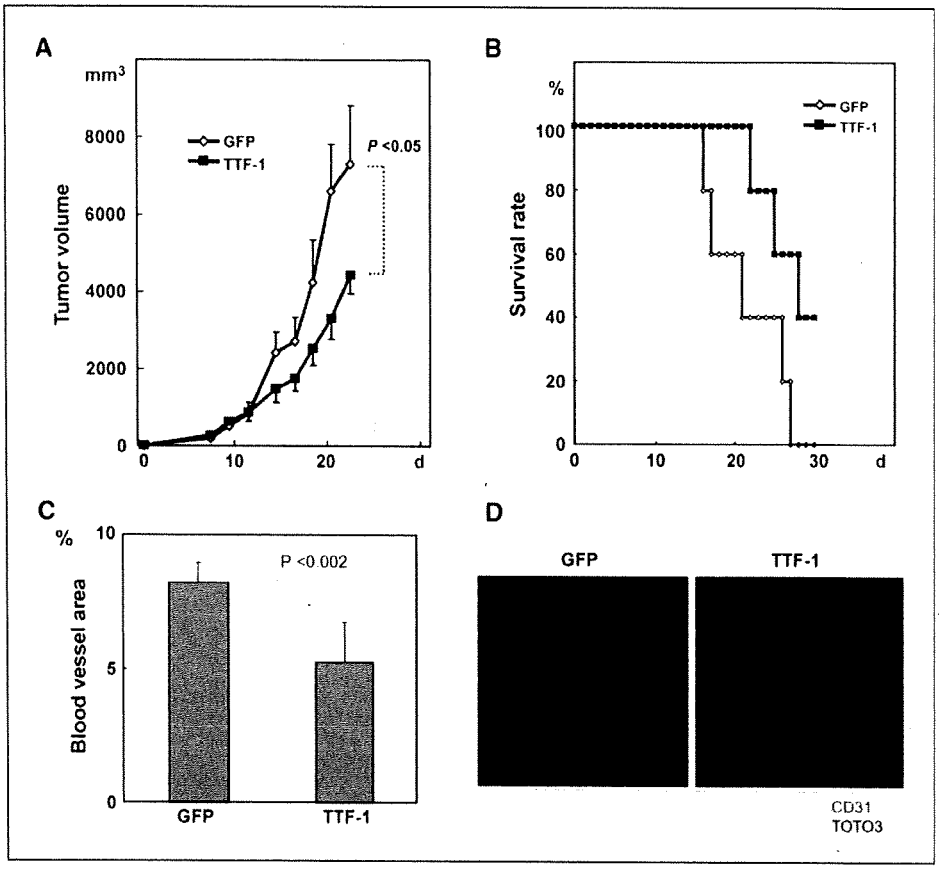

Figure 6. Growth retardation of TTF-1 expressing tumor. *A*, LLC cells infected with retroviruses encoding GFP or TTF-1 were s.c. inoculated into C57/BL6 mice ($n = 5$ for each group). $P < 0.05$ (multivariate ANOVA). *Bars*, SE. *B*, survival rate of the mice bearing GFP-expressing or TTF-1–expressing tumors. $P = 0.068$, log-rank test. *C*, blood vessel density in tumors derived from GFP-expressing or TTF-1–expressing LLC cells. The percentage of the area positively stained for CD31 was measured from randomly selected five fields in each mouse. The value indicates the average of five mice in each group. *P* value was calculated by Student's *t* test. *Bars*, SD. *D*, representative photographs of immunohistochemical staining of CD31 (*red*) in each tumor. *Blue*, TOTO3 (nuclei).

non-TRU type by Yatabe and colleagues, the majority of TTF-1–positive cases showed TRU morphology. Conversely, most of adenocarcinomas with TRU morphology were TTF-1 positive (29). These observations suggest that loss of TTF-1 expression is associated with poor differentiation of adenocarcinomas. Therefore, we believe that recent data showing the oncogenic role of TTF-1 do not exclude the possibility that TTF-1 might act as a tumor suppressor in another subset of lung adenocarcinomas, possibly combined with the mutation or amplification of other oncogenes.

We found that TGF-β suppresses the expression of TTF-1, and this effect was inhibited by LY364947. Expression of TTF-1 might be sustained by the feed-forward mechanism through binding of TTF-1 to its own promoter (30). We have also shown that TTF-1 can attenuate TGF-β signaling by down-regulation of TGF-β2. TGF-β signaling is often positively modulated through the induction of TGF-β ligands of different isoforms (31). Thus, enhancement of autocrine TGF-β signaling may accelerate the decrease of TTF-1 expression, and conversely, TTF-1 may attenuate autocrine TGF-β signaling. Because TTF-1 exerts a tumor suppressive effect through inhibition of EMT, these findings delineate a novel pathway that TGF-β accelerates lung cancer progression.

Three isoforms of TGF-β ligands show different expression profiles during lung branching morphogenesis. Whereas TGF-β1 expression is prominent throughout the mesenchyme, TGF-β2 is mainly localized to the epithelium of the developing distal airways. TGF-β2 may be critical for determining the epithelial cell behavior in a cell autonomous fashion. TTF-1 is expressed at the tip of the developing distal airway and may play a role in the maintenance of the epithelial polarity. Reciprocal regulations between TTF-1 and TGF-β signaling, involved in lung branching morphogenesis, may be recapitulated in lung adenocarcinoma cells.

Loss of TTF-1 expression may be associated with poor differentiation of adenocarcinomas, and our results showed that TTF-1 inhibits EMT and invasiveness of lung adenocarcinoma cells. Some clinical studies showed that TTF-1 positivity is a good prognostic indicator in patients with non-small cell lung cancer. Taken together, our present study sheds light on the new functional aspect of TTF-1, which can inhibit cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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COUP-TFII regulates the functions of Prox1 in lymphatic endothelial cells through direct interaction

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During embryonic lymphatic development, Prox1 homeobox transcription factor is expressed in a subset of venous blood vascular endothelial cells (BECs) in which COUP-TFII orphan nuclear receptor is highly expressed. Prox1 induces differentiation of BECs into lymphatic endothelial cells (LECs) by inducing the expression of various LEC markers including vascular endothelial growth factor receptor 3 (VEGFR3). However, the molecular mechanisms of how transcriptional activities of Prox1 are regulated are largely unknown. In the present study, we show that COUP-TFII plays important roles in the regulation of the function of Prox1. In BECs and LECs, Prox1 promotes the proliferation and migration toward VEGF-C by inducing the expression of cyclin E1 and VEGFR3, respectively. Gain-of-function studies showed that COUP-TFII negatively regulates the effects of Prox1 in BECs and LECs whereas loss-of-function studies showed that COUP-TFII negatively and positively regulates Prox1 in BECs and LECs, respectively. We also show that endogenous Prox1 and COUP-TFII physically interact in LECs and that both Prox1 and COUP-TFII bind to the endogenous cyclin E1 promoter. These results suggest that COUP-TFII physically and functionally interact during differentiation and maintenance of lymphatic vessels.

Introduction

Lymphatic vascular systems play critical roles in the maintenance of tissue fluid homeostasis and the mediation of the afferent immune response. Defects in the lymphatic systems result in lymphedema. In pathological situations, they serve as routes of the metastatic spread of malignant tumors to regional lymph nodes. Because of such clinical relevance, understanding of the molecular mechanisms that govern lymphangiogenesis is crucial (Karpanen & Alitalo 2008).

Numerous groups have shown that activation of signaling pathways via vascular endothelial growth factor receptor 3 (VEGFR3) by VEGF-C/D plays central roles in the formation of lymphatic systems. Genetic ablation of *Vegf-c* gene leads to the lack of lymphatic formation (Karkkainen *et al.* 2004). Additionally, expression of VEGF-C under skin-specific promoter induces hyperplasia of cutaneous lymphatic vessels (Jeltsch *et al.* 1997; Veikkola *et al.* 2001). Furthermore, inhibition of VEGFR3 signals via VEGFR3-Fc trap leads to diminishment of lymphatic vessels (He *et al.* 2002).

However, lymphangiogenesis is not regulated only by VEGFR3 signaling pathways. Recent reports have shown that integrin α9/β1 complexes serve as receptors for VEGF-C/D to regulate cell migration (Vlahakis *et al.* 2005). Furthermore, receptor tyrosine kinases including Tie2 (Morisada *et al.* 2005), fibroblast growth factor receptor 3 (FGFR3: Shin *et al.* 2006), platelet-derived growth factor receptor β (PDGFRβ: Cao *et al.* 2004) and hepatocyte growth factor receptor (HGFR: Kajiya *et al.* 2005) have been implicated in lymphangiogenesis. Therefore, if there are transcription factors that regulate these multiple lymphangiogenic signals, such master regulators can be ideal candidates as targets of anti-lymphangiogenesis therapies.

During embryonic lymphatic development, a homeobox transcription factor Prox1 has been shown to play important roles in the differentiation of venous endothelial cells

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into lymphatic endothelial cells (LECs; Oliver 2004). At 9.5 dpc of mouse development, Prox1 starts to become expressed specifically in a subpopulation of endothelial cells located on one side of the anterior cardinal vein. At this stage, venous endothelial cells express CD34, a blood vascular endothelial cell (BEC) marker, and low level of VEGFR3, whose expression becomes restricted to LEC at later stages. Upon Prox1 expression, expression of BEC markers decreases while expression of LEC markers, such as podoplanin and VEGFR3, increases. These Prox1 expressing cells start sprouting from veins and migrate towards mesenchymal cells expressing VEGF-C. Importantly, in Prox1 deficient mice, the migration of LECs is arrested, leading to complete lack of lymphatic systems (Wigle & Oliver 1999; Wigle *et al.* 2002).

Being a transcription factor, Prox1 regulates the expression of various target genes. When Prox1 was adenovirally transduced into human dermal microvascular endothelial cells (HDMECs), expression of LEC-specific genes was up-regulated (Petrova *et al.* 2002). Although Prox1-mediated induction of LEC-specific genes was not observed in non-BECs, Prox1 was capable of inducing the expression of cyclin E1 and E2 in various cell types. These results suggest that Prox1 may induce cell proliferation and differentiation of BECs into the LECs.

We recently examined the effects of Prox1 on the migration of two types of endothelial cells, mouse embryonic stem cell-derived endothelial cells and human umbilical vein endothelial cells (HUVECs) (Mishima *et al.* 2007). Prox1 induces the expression of VEGFR3 and integrin α9, which results in the endothelial migration towards VEGF-C. Furthermore, when Prox1 expression was knocked-down in human dermal LECs (HDLECs), expression of VEGFR3 and integrin α9 was attenuated with decrease in the migration towards VEGF-C. These results suggest that Prox1 serves as a master regulator in the differentiation and maintenance of LECs.

However, the molecular mechanisms of how Prox1 regulates the transcription of its target genes have been poorly understood. Shin and colleagues showed that Prox1 directly binds to the FGFR3 promoter to induce its expression in endothelial cells (Shin *et al.* 2006). Prox1 has also been shown to bind to the βB1-crystallin promoter to regulate its expression in lens epithelium (Cui *et al.* 2004). While various transcription factors are involved in the regulation of βB1-crystallin expression, the roles of Prox1 binding proteins in the Prox1-mediated transcriptional regulation have not yet been elucidated.

Qin *et al.* showed that Prox1 binds liver receptor homologue-1 (LRH-1/NR5A2), a member of *fushi tarazu* factor 1 subfamily of orphan nuclear receptors, which positively regulates the expression of cholesterol 7-α-hydroxylase (cyp7a1) in liver. Prox1 negatively regulates the transcriptional activities of LRH-1 by sequestrating LRH-1 proteins from cyp7a1 promoter (Qin *et al.* 2004). The suppression of the transcriptional activities of LRH-1 by Prox1 does not require the DNA binding domain of Prox1. These results suggest that other nuclear receptor family members may also physically and functionally interact with Prox1.

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are orphan members of the steroid/thyroid hormone receptor superfamily. Two genes termed COUP-TFI (also known as EAR3/NR2F1) and COUP-TFII (also known as ARP-1/NR2F2) are closely related members and are expressed in various organs. COUP-TFs play important roles in the regulation of organogenesis, neurogenesis, and cellular differentiation during embryonic development. In blood vessels, COUP-TFII is specifically expressed in venous but not in arterial endothelium (You *et al.* 2005). Targeted disruption of COUP-TFII in endothelial cells results in the acquisition of arterial characteristics in mutant veins, suggesting that COUP-TFII has a critical role in maintaining vein identity. As lymphatic vessels are originated from veins, ablation of COUP-TFII in endothelial cells causes the decrease in Prox1-expressing cells (Srinivasan *et al.* 2007). However, the roles of COUP-TFII in LECs have not yet been elucidated.

In the present study, we found that COUP-TFII is expressed in LECs. By gain- and loss-of-function analyses, we showed that COUP-TFII suppresses the transcriptional activities of Prox1 to induce VEGFR3 and cyclin E1 in HUVECs, which leads to the inhibition of Prox1-mediated induction of endothelial cell proliferation and migration towards VEGF-C. Interestingly, both gain- and loss-of-function of COUP-TFII in HDLECs suppressed the expression of VEGFR3 and cyclin E1, suggesting that endogenous level of COUP-TFII is required to maintain the characteristics of LECs. Furthermore, we showed that COUP-TFII physically interacts with Prox1 and that both COUP-TFII and Prox1 bind to the cyclin E1 promoter. These results suggest that COUP-TFII regulates the transcriptional activities of Prox1 in LECs via physical interaction.

Results

COUP-TFII is expressed in human LECs

We first studied the expression of COUP-TFII in BECs and LECs using HUVECs and HDLECs by Western blot analysis (Fig. 1A). While COUP-TFII protein was detected in both HUVECs and HDLECs, its expression level was lower in HDLECs, in which Prox1 was expressed.

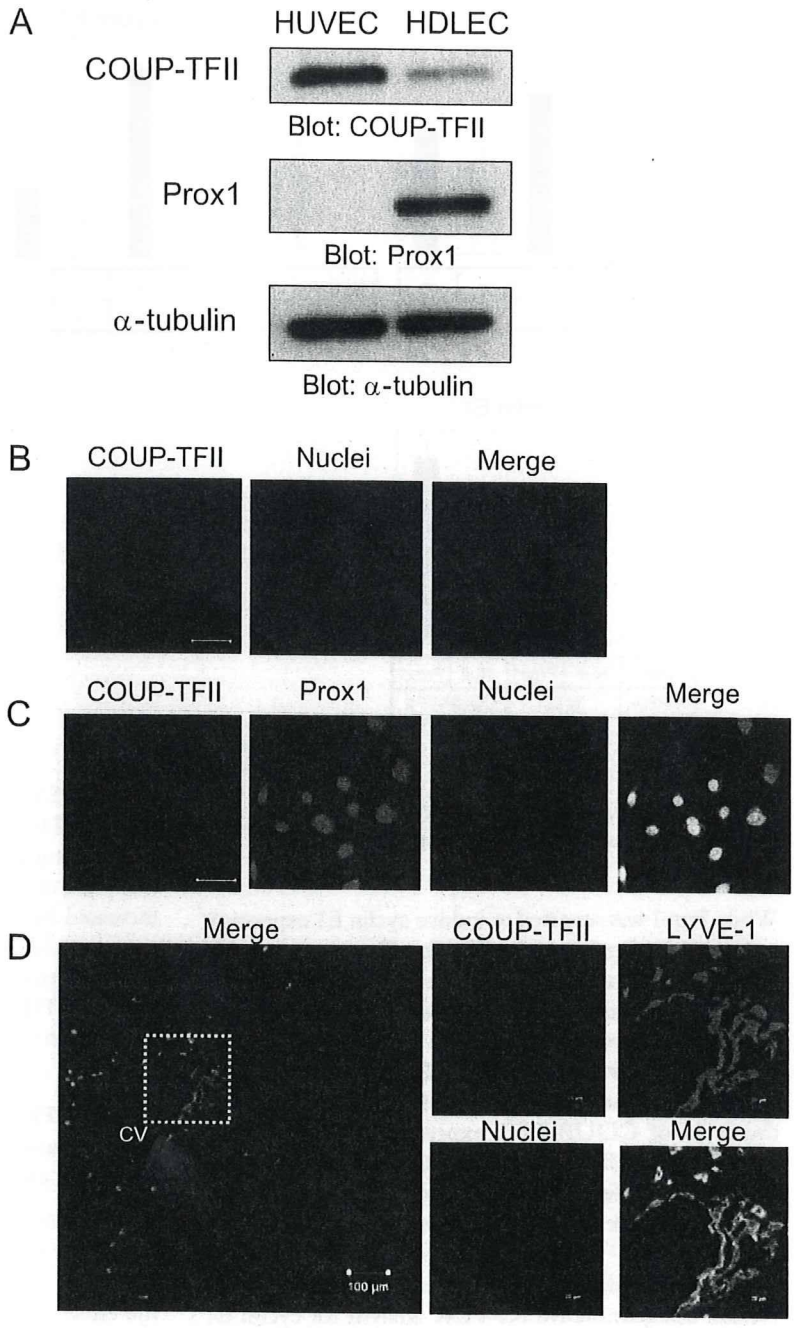

Figure 1 COUP-TFII is expressed in BECs and LECs. (A) Western blot analyses for COUP-TFII (top panel) and Prox1 expressions (middle panel) in HUVECs and in HDLECs. α-tubulin was used as a loading control (bottom panel). (B–C) Immunostaining of HUVECs (B) and HDLECs (C) was carried out for COUP-TFII (red) and Prox1 (green) with nuclear staining by TOTO3 (blue). COUP-TFII and Prox1 were co-localized to nuclei in HDLECs (see Merge). Scale bars, 50 μm. (D) Immunohistochemistry was carried out for COUP-TFII (red) and LYVE-1 (green) with nuclear staining by TOTO-3 (blue) using transverse sections at the level of the heart of 11.5 dpc mouse embryo. Right small panels are magnified images of the boxed area of the left large panel. CV; cardinal vein. Scale bars, 100 μm (left panel) and 20 μm (right four panels).

We also observed that COUP-TFII protein was localized to the nuclei of HUVECs (Fig. 1B) and co-localized with Prox1 in HDLECs (Fig. 1C). The specificity of anti-COUP-TFII antibody used was confirmed using the HUVECs whose expression of COUP-TFII was knocked down by siRNA (Fig. S1 in Supporting Information).

Furthermore, we examined the COUP-TFII expression in embryos. At 11.5 dpc of mouse development, LECs that are positive for LYVE-1, a LEC marker, sprout out from cardinal veins (Fig. 1D) (Oliver 2004). We observed that these sprouting LECs express COUP-TFII (Fig. 1D). These results suggest that COUP-TFII is temporally and spatially co-localized with Prox1 in LECs.

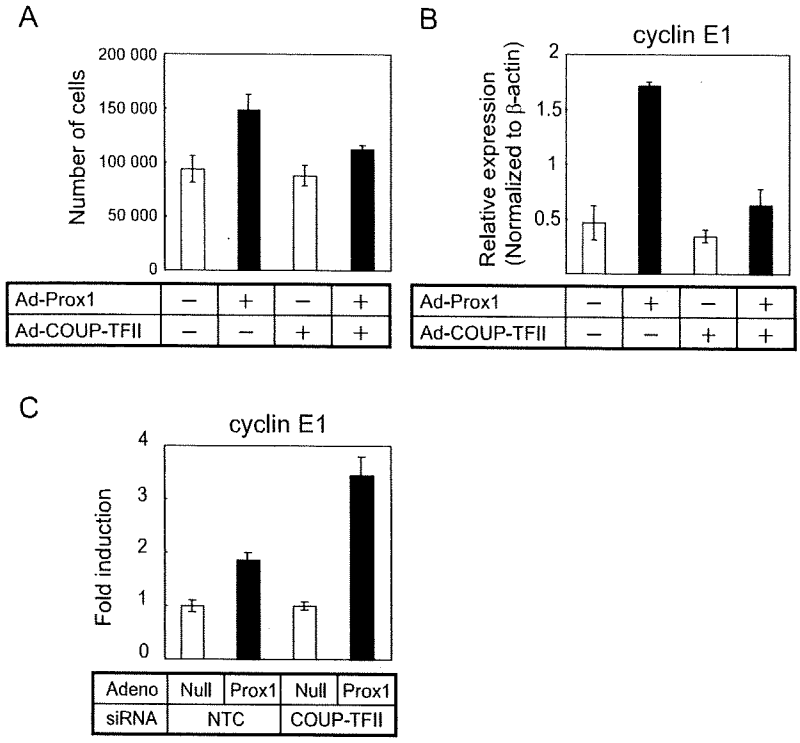

Figure 2 COUP-TFII suppresses Prox1-induced cell proliferation through the regulation of cyclin E1. (A) Numbers of cells were counted 48 h after infection of HUVECs with adenovirus coding for Prox1 (Ad-Prox1) in combination with that for COUP-TFII (Ad-COUP-TFII). Each value represents the mean of triplicate determinations; Bars, SD. (B–C) Effects of gain- and loss-of-function of COUP-TFII on the expression of cyclin E1 in HUVECs. HUVECs were infected with Ad-Prox1 in combination with Ad-COUP-TFII (B) or siRNAs for COUP-TFII (C), followed by quantitative RT-PCR analysis for cyclin E1. Non-coding adenovirus (Null) and siRNA carrying scrambled sequences (NTC) were used as negative controls. Bars, SD.

COUP-TFII suppresses Prox1-induced cell proliferation through the regulation of cyclin E1 expression

While Prox1 was reported to induce cyclin E1 expression in HDMECs (Petrova *et al.* 2002), its effects on endothelial cell proliferation have not yet been examined. When Prox1 was expressed in HUVECs using adenovirus, it significantly increased cell number (Fig. 2A). In order to examine the effect of COUP-TFII on Prox1-mediated promotion of endothelial cell proliferation, we increased the level of COUP-TFII expression by adenovirus coding for COUP-TFII. While COUP-TFII expression itself did not affect cell proliferation, elevated cell proliferation by Prox1 was significantly repressed by COUP-TFII (Fig. 2A).

In order to dissect the molecular mechanisms, we carried out quantitative RT-PCR analysis for cyclin E1 (Fig, 2B) and E2 (Fig. S2 in Supporting Information). In accordance with the result of cell proliferation, COUP-TFII significantly suppressed the cyclin E1 expression induced by Prox1 (Fig. 2B), which was also confirmed for cyclin E2 expression (Fig. S2A in Supporting Information).

We next examined whether endogenous COUP-TFII is necessary to suppress Prox1-mediated induction of cyclin E1 expression by knocking down endogenous COUP-TFII expression using siRNA (Fig. S1 in Supporting Information). As shown in Fig. 2C, the induction of cyclin E1 expression by Prox1 was significantly increased by the loss of COUP-TFII expression, which was also confirmed for cyclin E2 expression (Fig. S2B in Supporting Information). These findings suggest that COUP-TFII suppresses Prox1-induced cell proliferation by interfering with cyclin E expression.

COUP-TFII suppresses Prox1-mediated endothelial cell migration towards VEGF-C by regulating VEGFR3 expression

We recently showed that Prox1 induces endothelial cell migration towards VEGF-C through up-regulation of VEGFR3 expression (Mishima *et al.* 2007). To examine the effect of COUP-TFII on the Prox1-mediated promotion of endothelial chemotaxis towards VEGF-C, we carried out chamber migration assays using HUVECs. As shown in Fig. 3A, COUP-TFII significantly suppressed the chemotaxis towards VEGF-C enhanced by Prox1.

In consistent with the results of the chamber migration assay, COUP-TFII suppressed the VEGFR3 mRNA expression induced by Prox1 (Fig. 3B). This result was further confirmed at protein level (Fig. 3C). In addition,

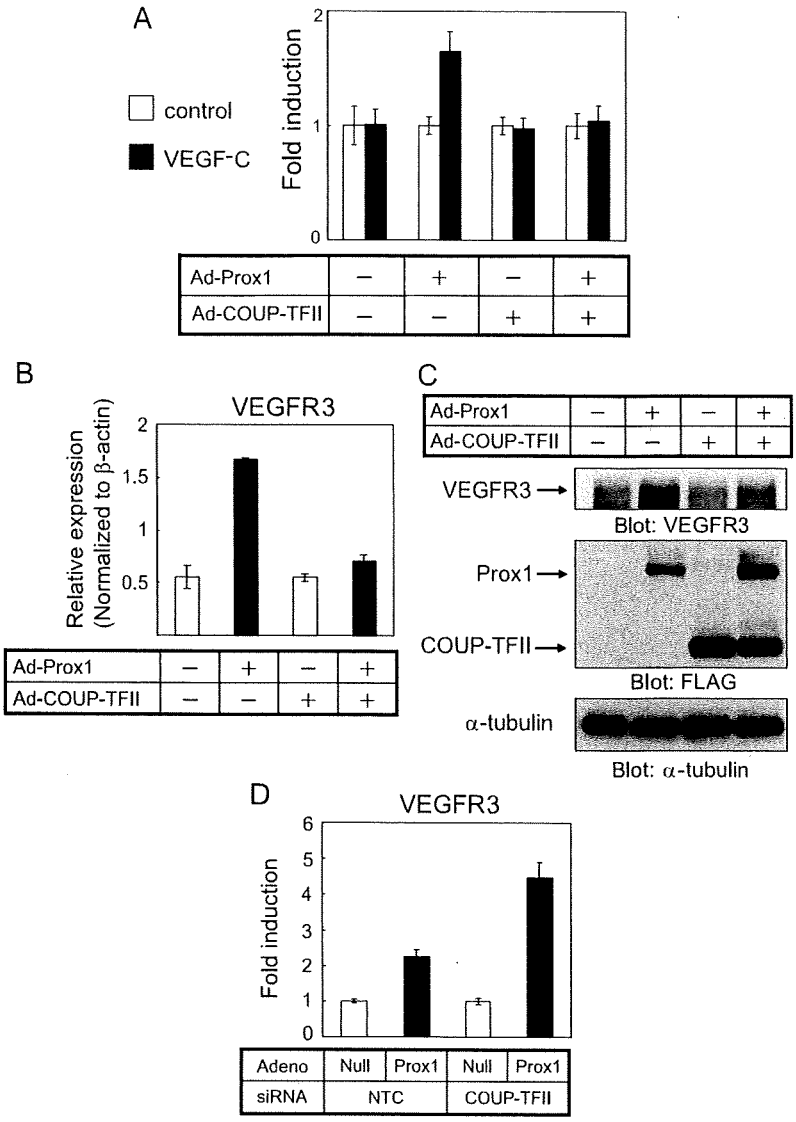

Figure 3 COUP-TFII represses Prox1-induced endothelial cell migration towards VEGF-C via regulation of VEGFR3 expression. (A) Effect of COUP-TFII on the Prox1-induced endothelial cell migration towards VEGF-C. Migration of HUVECs infected with indicated adnoviruses was measured by Boyden chamber assay as described previously (Mishima *et al.* 2007). Relative migration towards VEGF-C is shown as a ratio of the number of migrated cells in the presence of VEGF-C against that in the absence of VEGF-C. Bars, SD. (B–C) Effect of gain-of-function of COUP-TFII on the Prox1-induced expression of VEGFR3. HUVECs were infected with indicated adenoviruses, followed by quantitative RT-PCR (B) and Western blotting (C: top panel) for VEGFR3. Western blotting was also carried out for FLAG-tagged Prox1 and COUP-TFII transduced by adenoviruses (middle panel). α-tubulin was used as a loading control (bottom panel). (D) Effect of loss-of-function of COUP-TFII on the Prox1-induced expression of VEGFR3. HUVECs were infected with adenovirus coding for Prox1 (Ad-Prox1) or non-conding adenovirus (Ad-Null) in combination with siRNA for COUP-TFII or negative control siRNA (NTC), followed by quantitative RT-PCR for VEGFR3. Bars, SD.

when COUP-TFII expression was knocked down in HUVECs, induction of VEGFR3 expression by Prox1 was enhanced (Fig. 3D). These results suggest that COUP-TFII suppresses Prox1-induced endothelial chemotaxis towards VEGF-C by regulating VEGFR3 expression.

Endogenous level of COUP-TFII expression in HDLECs is required to maintain the expression of VEGFR3

We next investigated the roles of COUP-TFII in the LECs in which endogenous Prox1 is expressed. When the level of COUP-TFII expression was elevated in HDLECs, both proliferation (Fig. 4A) and chemotaxis towards VEGF-C (Fig. 4C) were suppressed with concomitant decrease in the expression of cyclin E1 (Fig. 4B) and VEGFR3 (Fig. 4D). These findings were consistent with the results observed in HUVECs. However, when COUP-TFII was knocked down in HDLECs, their migration towards VEGF-C and VEGFR3 expression were attenuated (Fig. 4E,F), whereas neither cell number nor cyclin E1 expression changed (data not shown). These results suggest that COUP-TFII differentially functions between HDLECs and HUVECs.

We further examined whether COUP-TFII alters the endogenous Prox1 expression in LECs. As shown in Fig. 4G, endogenous Prox1 expression was significantly decreased when the level of COUP-TFII expression was

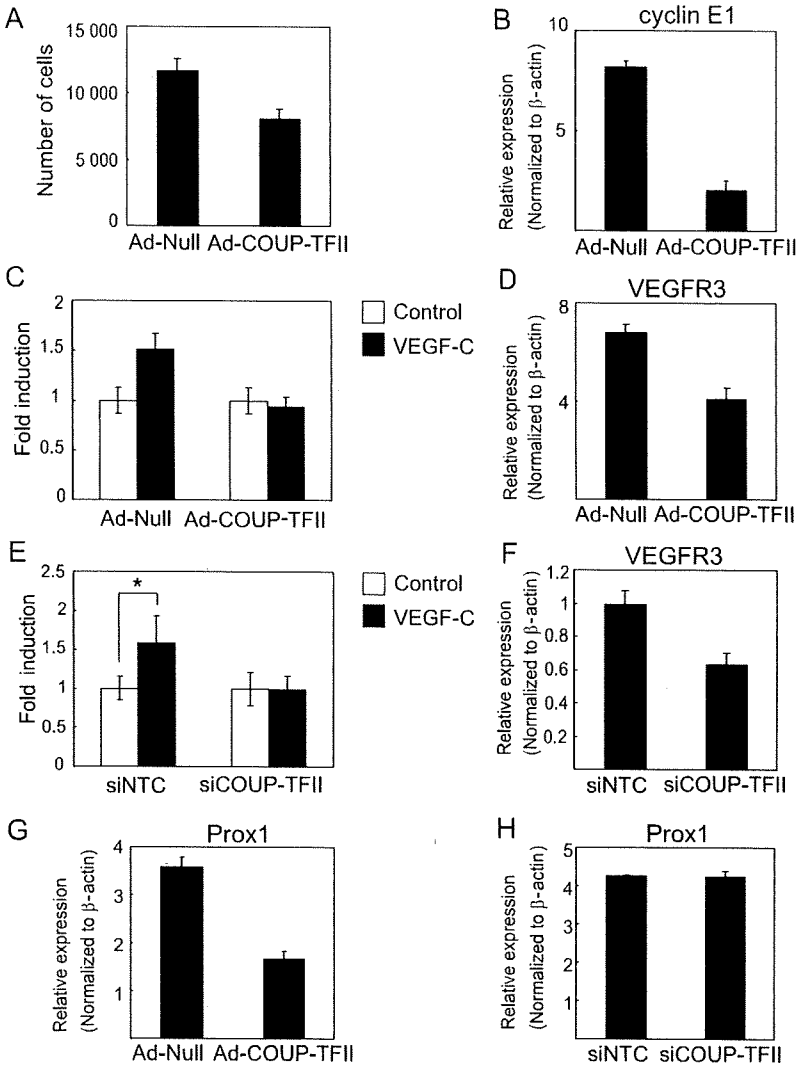

Figure 4 Endogenous level of COUP-TFII expression plays important roles in the maintenance of characteristics of HDLECs. (A–D) HDLECs infected with non-coding adenovirus (Ad-Null) or adenovirus coding for COUP-TFII (Ad-COUP-TFII) were subjected to cell proliferation assay (A), chamber migration assay using VEGF-C as an attractant (C) and quantitative RT-PCR analyses for cyclin E1 (B) and VEGFR3 (D). (E, F) HDLECs transfected with control siRNA (siNTC) or siRNA for COUP-TFII (siCOUP-TFII) were subjected to chamber migration assay using VEGF-C as an attractant (E) and quantitative RT-PCR analyses for VEGFR3 (F). In (C) and (E), relative migration towards VEGF-C is shown as a ratio of the number of migrated cells in the presence of VEGF-C against that in the absence of VEGF-C. (G, H) Expression of endogenous Prox1 expression was determined in the HDLECs infected with Ad-COUP-TFII (G) or transfected with siCOUP-TFII (H). $*P < 0.01$. Bars, SD.

elevated in HDLECs, while the loss of COUP-TFII expression did not alter the Prox1 expression (Fig. 4H). These results suggest that excessive level of COUP-TFII may interfere with the functions of Prox1 directly by inhibiting the transcriptional activities of Prox1 and indirectly by suppressing the endogenous Prox1 expression.

COUP-TFII interacts with Prox1 in HDLECs

Previous reports that LRH-1 and SF-1, members of nuclear receptor superfamily, bind Prox1 (Qin *et al.* 2004; Steffensen *et al.* 2004) prompted us to examine whether COUP-TFII and Prox1 interact. We carried out co-immunoprecipitation experiments with cell lysates prepared from the HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII (Fig. 5A). When the lysates were subjected to immunoprecipitation, we detected COUP-TFII in the immunoprecipitates pulled down with anti-Prox1 antibody, which indicates that Prox1 is capable of interacting with COUP-TFII in HUVECs.

Next, we examined whether endogenous Prox1 and COUP-TFII interact in HDLECs using the Duolink *in situ* proximity ligation assay (PLA). This method enables us to monitor subcellular localization of endogenous protein–protein interactions at single molecule resolution (Söderberg *et al.* 2006, 2008). In the HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII, we detected a number of strong fluorescence signals in the presence of specific antibodies, which indicates the interaction between Prox1 and COUP-TFII in the

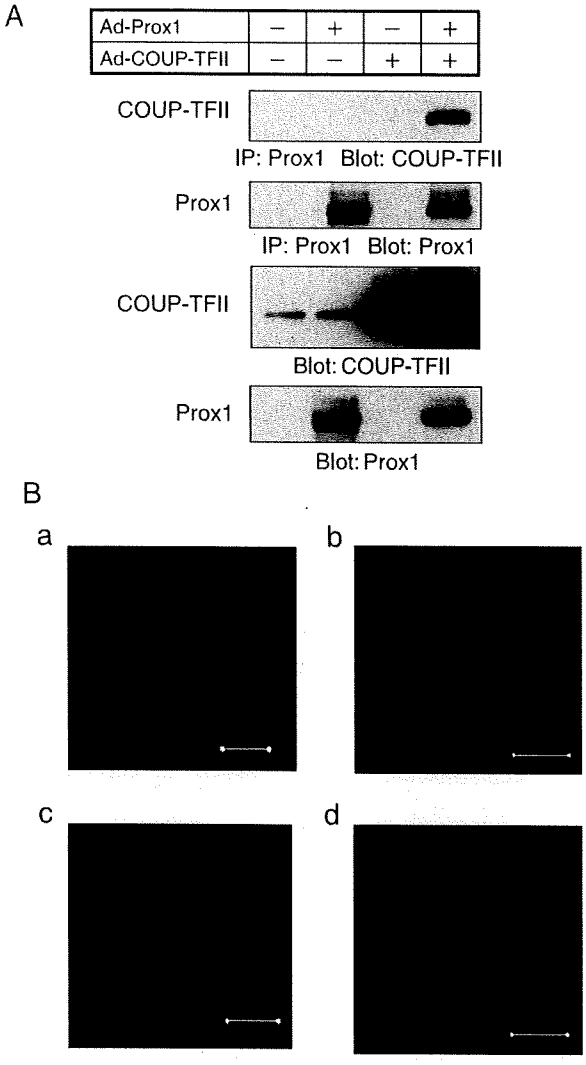

Figure 5 COUP-TFII physically interacts with Prox1. (A) Co-immunoprecipitation of COUP-TFII with Prox1 in HUVECs. HUVECs infected with adenovituses coding for Prox1 (Ad-Prox1) and COUP-TFII (Ad-COUP-TFII) were subjected to immunoprecipitation with anti-Prox1 antibody, followed by immunoblotting with anti-COUP-TFII antibody (top panel). Expression of Prox1 and COUP-TFII was also confirmed. (B) Interaction of endogenous Prox1 and COUP-TFII in HDLECs. PLA was carried out to detect the proximal location of Prox1 and COUP-TFII (shown as red dots) as described in Experimental Procedures. All samples were counterstained with TOTO3 (blue) to visualize nuclei. (a–c) HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII (a), native HDLECs (b) and native HUVECs (c) were subjected to PLA after treating with antibodies for Prox1 and COUP-TFII. Note that specific interaction between Prox1 and COUP-TFII is detected in the nuclei only when Prox1 and COUP-TFII are present. (d) Native HDLECs were subjected to PLA without treating with antibodies for Prox1 and COUP-TFII. Scale bars, 10 μm.

nuclei (Fig. 5Ba), whereas no signals were detected in native HUVECs in the presence of specific antibodies (Fig. 5Bc) or in HDLECs in the absence of specific antibodies (Fig. 5Bd). In the native HDLECs, we could detect definite fluorescence signals restricted to the nuclei in the presence of specific antibodies (Fig. 5Bb), suggesting that endogenous Prox1 and COUP-TFII interact in the nuclei of HDLECs. In order to examine the specificity of the signals, we knocked down COUP-TFII expression by siRNA in HDLECs and carried out PLA (Fig. S3 in Supporting Information). The fluorescence signals seen in the HDLECs transfected with control siRNA were significantly decreased by knocking down COUP-TFII expression. These results allowed us to conclude that endogenous COUP-TFII interacts with Prox1 in the nuclei of HDLECs.

COUP-TFII and Prox1 bind to the cyclin E1 promoter

Petrova *et al.* showed that Prox1 activates cyclin E1 promoter whereas Prox1 DNA binding mutant does not (Petrova *et al.* 2002), suggesting that Prox1 may regulate the transcription of cyclin E1 via direct binding to the cyclin E1 promoter. Because COUP-TFII suppresses Prox1-induced cyclin E1 expression and physically interacts with Prox1, we examined whether Prox1 and COUP-TFII bind to the endogenous cyclin E1 promoter in intact chromatin.

Cross-linked chromatin samples prepared from HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII were subjected to chromatin immunoprecipitation (ChIP) assays (Fig. 6). The cyclin E1 promoter region

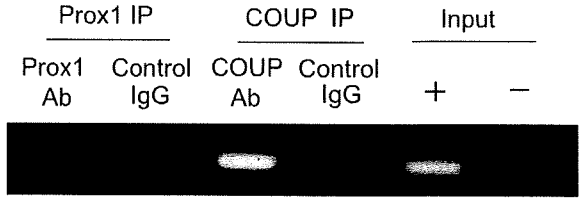

Figure 6 COUP-TFII and Prox1 directly bind to the cyclin E1 promoter. HUVECs infected with adenoviruses coding for Prox1 and COUP-TFII were subjected to ChIP assay. PCR was carried out to detect the cyclin E1 promoter containing putative binding sequences for Prox1 and COUP-TFII. Prox1 Ab and COUP Ab lanes show amplification of target sequences within the immunoprecipitates (IP) using antibodies for Prox1 and COUP-TFII, respectively. Control IgG lanes show PCR amplification of samples precipitated with corresponding control IgG antibodies. Input lanes show amplification of total input DNA (+) or no DNA (–).

containing putative binding consensus sequences for Prox1 and COUP-TFII was pulled down with antibodies for Prox1 and COUP-TFII, suggesting that both Prox1 and COUP-TFII bind to the cyclin E1 promoter.

Discussion

In the present study, we show that COUP-TFII regulates the transcriptional activities of Prox1. In BECs, both gain- and loss-of-function studies showed that COUP-TFII negatively regulates Prox1 to induce the expression of cyclin E1 and VEGFR3. COUP-TFII has been reported to negatively regulate the transcription via binding to the promoter and recruitment of co-repressor complexes containing N-CoR, SMRT and histone deacetylase (HDAC) (Park *et al.* 2003). The present findings that COUP-TFII and Prox1 physically and functionally interact may suggest that positive transcriptional regulation by Prox1 is repressed by the co-repressor complexes that are recruited to the promoter by COUP-TFII.

We also found that endogenous level of COUP-TFII in LECs is required to maintain the expression of VEGFR3, which is not consistent with the results observed in HUVECs. This difference may be caused by the cell type specific contexts of expression of other transcription factors. Additionally, elevation of the level of COUP-TFII expression in LECs also decreased the expression of VEGFR3. We also found that both gain- and loss-of-function of COUP-TFII decreased the expression of LEC markers including integrin α9 and podoplanin and BEC markers including VE-cadherin and VEGFR2 (Fig. S4). These results suggest that a certain range of COUP-TFII expression is required to maintain the expression of a group of LEC and BEC markers. Similar phenomenon is observed in the relationship between the expression of Oct3/4 and maintenance of pluripotency of mouse embryonic stem cells (Niwa *et al.* 2000).

While endothelial markers examined so far appear to require endogenous level of COUP-TFII in HDLECs, we found that not all of endothelial markers are regulated by COUP-TFII in a similar manner. Ablation of COUP-TFII gene in endothelial cells allowed the ectopic expression of ephrin B2 and Neuropilin 1 (NRP1), both of which are arterial endothelial cell markers, in veins (You *et al.* 2005). In accordance with the previous result, COUP-TFII expression decreased ephrin B2 expression while loss-of-COUP-TFII expression increased it in HDLECs (Fig. S4E in Supporting Information). However, to our surprise, both gain- and loss-of-function studies showed that COUP-TFII positively regulates NRP1 expression in HDLECs (Fig. S4F in Supporting Information). Molecular mechanisms of how COUP-TFII differentially regulates the transcription of various target genes between BECs and LECs remain to be investigated in the future.

During embryonic lymphatic development, Prox1 expressing BECs in veins differentiate into LECs and sprout out to form primary lymphatic plexus (Oliver 2004). While we showed that COUP-TFII is expressed in HDLECs at lower level than in HUVECs, it remains to be elucidated how COUP-TFII expression is regulated during lymphatic development in embryos. Loss-of-function studies show that COUP-TFII negatively and positively regulates the VEGFR3 expression in BECs and LECs, respectively. These results may suggest that COUP-TFII maintains the identity of venous endothelial cells by inhibiting Prox1-mediated VEGFR3 expression in BECs, but aids in maintaining VEGFR3 expression in LECs. This differential transcriptional regulation by COUP-TFII may play important roles in segregating lymphatics from veins during formation of primary lymphatic plexus.

As Prox1 plays critical roles in the formation and maintenance of lymphatic vessels (Mishima *et al.* 2007), regulation of transcriptional activities of Prox1 will aid in manupilating lymphangiogenesis in pathological situations. While ligands for COUP-TFII receptor have not yet been identified, better understanding of the regulation of the COUP-TFII may be useful for developing therapeutic strategies to treat lymphedema and tumor lymphangiogenesis in the future.

Experimental procedures

Cell culture and adenovirus production

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 coding for mouse Prox1 and mouse COUP-TFII were generated and used as described (Shirakihara *et al.* 2007).

RNA interference and oligonucleotides

siRNAs were introduced into cells using HiperFect reagent (QIAGEN) according to the manufacturer's instructions. The COUP-TFII siRNA (SI00128814) and the negative control siRNA were obtained from QIAGEN.

Immunohistochemistry and Western blot analysis

Immunostaining was carried out with anti-Prox1 (1 : 100 dilution; Abcam), anti-COUP-TFII (1 : 100 dilution; Perseus Proteomics)

and anti-LYVE-1 (1 : 200 dilution; Abcam) antibodies, followed by counterstaining with TOTO3 (Invitrogen–Molecular Probes). 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. Antibodies to FLAG and α-tubulin for Western blot analysis were obtained from SIGMA. Antibody to human VEGFR3 for Western blot analysis was obtained from Santa Cruz. Western blot analysis was carried out as described (Watabe *et al.* 2003).

Proximity ligation assay (PLA)

The Duolink *in situ* PLA kits were purchased from Olink (http://www.olink.com/). Fixation of the cells, blocking of non-specific binding of antibody, and immunostaining using anti-Prox1 (Abcam) and anti-COUP-TFII (Perseus Proteomics) were carried out as described above. Subsequently, a pair of secondary antibodies conjugated with oligonucleotides (PLA probes) was 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, 2008). Nuclei counterstaining and analysis of the images were carried out as described earlier.

Isolation of RNA and quantitative RT-PCR

Total RNAs were extracted from HUVECs and HDLECs using the RNeasy Mini Kit (QIAGEN). First-strand cDNAs were synthesized by SuperScriptIII reverse transcriptase (Invitrogen) using random hexamer primers according to the manufacturer's instruction. Quantitative RT-PCR analyses were carried out using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR master mix (Applied Biosystems). All expression data were normalized to those for β-actin. In the cases when siRNAs and adenoviruses were simultaneously used, expression data are presented as a ratio of the expression level in the samples infected with adenovirus coding for Prox1 against those with non-coding adenovirus. The primer sequences are available online as indicated in Table S1 in Supporting Information.

Chamber migration assay

Migration assay was carried out as described previously (Mishima *et al.* 2007). As a chemoattractant, 100 ng/mL and 300 ng/mL of recombinant VEGF-C (Calbiochem) were used for HDLECs and HUVECs, respectively.

ChIP assays

HUVECs infected with adenoviruses were fixed by adding formaldehyde and harvested. In order to precipitate Prox1 and COUP-TFII, anti-Prox1 antibody (R&D) and anti-COUP-TFII (Perseus Proteomics) were used. PCR of the cyclin E1 promoter containing putative binding sites for Prox1 and COUP-TFII was done using immunoprecipitated chromatin with the following pair of oligonucleotide primers:

5′-ACCAGCCTGAGCAACATAGCA-3′ and 5′-CAGTGAG ACCCCCATTTCTACA-3′.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Efficacy of knockdown of COUP-TFII expression in HUVECs.

Figure S2 COUP-TFII suppresses Prox1-induced cyclin E2 expression in HUVECs.

Figure S3 Interaction of endogenous Prox1 and COUP-TFII in HDLECs.

Figure S4 Endogenous level of COUP-TFII expression plays important roles in the maintenance of characteristics of HDLECs.

Table S1 Primers used for RT-PCR

Additional Supporting Information may be found in the online version of the article.

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