Table 2. Percentage of apoptotic cells in various hematopoietic cell populations

Marker	Genotype	
	PSF1$^{+/+}$	PSF1$^{+/-}$
Normal state		
Lin$^-$Kit$^+$Sca1$^+$	5.4 ± 4	9.3 ± 0.4
Lin$^-$Kit$^+$	4.5 ± 3	8.1 ± 0.2
Lin$^-$	7.0 ± 5	12 ± 0.6
5-FU treated*		
Lin$^-$Kit$^+$Sca1$^+$	5.2 ± 2	7.4 ± 0.5
Lin$^-$Kit$^+$	4.2 ± 2	5.1 ± 0.7
Lin$^-$	4.7 ± 2	5.9 ± 1

*BM cells were collected from mice 6 days after treatment with 5-FU.

Table 3. Relative mRNA expression in transfected cells

Average of relative expression	Plasmid used for transfection	
	Flag-PSF1, VSVG-PSF2, HA-PSF3, Myc-SLD5 (GINS)	VSVG-PSF2, HA-PSF3, Myc-SLD5 (G-NS)
VSVG-PSF2	1	1.05
HA-PSF3	1	1.14
Myc-SLD5	1	1.14

incomplete GINS complex, along with the destabilization of other GINS components in PSF1$^{+/-}$ HSCs. Finally, we concluded that PSF1 is expressed in proliferating HSCs and is essential for BM regeneration and regulation of stem cell pool size (Figure 6B).

increased compared with the analogous populations derived from wild-type mice; however, none of these differences was statistically significant. In addition, no obvious S-phase arrest was found in PSF1$^{+/-}$ HSCs (Figure 5A). We also examined the expression of ATM, ATR, XCCR1, Brca2, and p21 in both wild-type and PSF1$^{+/-}$ CD34-KSL cells by qRT-PCR; however, no obvious differences were found (data not shown). These data suggested that haploinsufficiency does not lead to abnormal DNA replication or increased activation of DNA damage checkpoint.

Taken together, these data indicate that both alleles of PSF1 are essential for promoting HSC cycling and that this requirement is limited to HSCs.

PSF1 regulates molecular stability of other GINS components in mutual manner

To address whether the silencing of one of the GINS components, PSF1, affects the cellular stability of other GINS components, we performed ectopic expression of all GINS component with or without PSF1 (Figure 6). For the evaluation of the transfection efficiency by plasmids, the amounts of overexpressed gene transcripts were quantified by real-time PCR; no significant differences were found between GINS and G-NS condition for VSVG-PSF2, HA-PSF3, and Myc-SLD5 expression (Table 3). These data indicated that transfection efficiencies of all plasmids were almost equivalent between GINS and G-NS conditions. When all GINS components (PSF1, PSF2, PSF3, and SLD5) were cotransfected, a stable "GINS" complex was formed. However, lack of PSF1 led to destabilization of PSF2, PSF3, and SLD5 (G-NS; Figure 6A). These data suggest that lack of PSF1 results in the formation of an
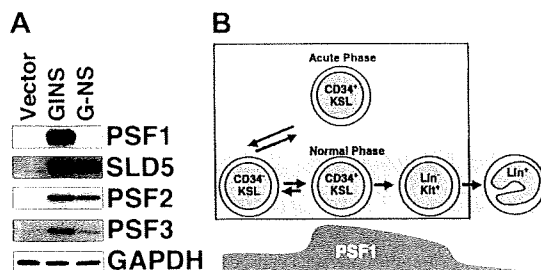

Figure 6. PSF1 mutually regulates molecular stability of other GINS components. (A) Western blot analysis of GINS components ectopically expressed on NIH3T3 cells. Cells were cotransfected with VSVG-PSF2, HA-PSF3, and Myc-SLD5 in the presence (GINS) or absence (G-NS) of Flag-PSF1 or empty vector (Vector). The blots were probed with specific antibodies as indicated. GAPDH was used as a loading control. (B) Scheme of PSF1 expression in the course of HSC differentiation. The level of PSF1 expression is represented by the dark gray area. Both alleles of PSF1 are essential for populations in the light gray area.

Discussion

In this study, we showed that PSF1 is highly expressed in proliferating HSCs, and haploinsufficiency of PSF1 caused severe delay in induction of HSC proliferation during ablated BM reconstitution and disrupted pool size maintenance of HSCs throughout life. In addition, we showed that PSF1 regulates protein stability of other GINS components.

During embryogenesis, PSF1$^{-/-}$ embryos show severe growth defect in the inner cell mass, that is, the pluripotent stem cells.[18] This observation raises the possibility that PSF1 could regulate the proliferation and/or pool size for other tissue stem cells. Recently, stem cells were identified in the small intestine.[30] Crypt base columnar cells are stem cells and can be visualized by continuous bromodeoxyuridine incorporation study. Our preliminary experiment showed that the number of crypt base columnar cells also decreased in adult PSF1$^{+/-}$ mice compared with adult wild-type mice (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Further experiments may help establish the function of PSF1 in the regulation of cell proliferation and/or the pool size of various tissue stem cells.

So far, a multiplicity of molecules have been studied for their role in cell-cycle progression, including extrinsic factors, such as Notch and sonic hedgehog, Wnt3a, etc, and intrinsic factors, such as Bmi1, PTEN, p21, p18, and others.[31] However, the mechanism of DNA replication in HSCs has not been elucidated. Although the essential role of PSF1 in DNA replication has been reported in yeast,[8] its function in mammalian cells has not been clearly understood. We previously reported that PSF1 was essential for cell division of totipotential embryonic stem cells by gene-targeting studies and showed that PSF1 was highly expressed in adulthood in BM, testis, and ovary, where cell division of stem cells is actively induced in the adult. Here we reported that PSF1 is essential for acute proliferation of HSCs. Taken together, it is clear that PSF1 plays important roles in cell proliferation of the stem cell system. Moreover, we and other groups isolated mammalian PSF2, PSF3, and SLD5, which together make up the GINS complex, and the roles of these GINS component have been reported in cell division.[7,8] In this report, we found that PSF1 expression was weak in slow cycling CD34$^-$ LT-HSCs and high in cycling CD34$^+$ ST-HSC. Therefore, the GINS complex is likely to closely associate with cell cycle of HSCs. At present, molecules affecting PSF1 expression in dormant HSCs have not been isolated; however, proliferating HSCs after BM ablation by 5-FU almost exclusively express PSF1 at high levels. This suggested that PSF1 expression is

inductively, but not intrinsically, regulated in HSCs affected by exogenous molecules produced from cells responding to BM suppression. At present, although it is not clear whether PSF1 plays a role in DNA replication of HSCs, isolation of molecules affecting PSF1 expression in HSCs may contribute to the understanding of process of self-renewal in HSCs.

It was reported that "GINS" replication complex, which is composed of PSF1, PSF2, PSF3, and SLD5, interacts with CDC45 and MCM complex and is involved in the initiation of DNA replication in lower eukaryote.[14-17] To determine whether haploinsufficiency of *PSF1* impairs DNA replication at stem cell level resulting in reduced pool size of HSCs, we examined the expression levels of DNA-damage checkpoint genes, such as ATM, ATR, XCCR1, BRCA2, and p21 in HSCs. No significant differences were found between CD34⁻ KSL cells derived from young and old *PSF1*$^{+/+}$ and *PSF1*$^{+/-}$ BM (data not shown). These data suggested that the decreased pool size of HSC population in *PSF1*$^{+/-}$ BM is not induced by activation of DNA damage checkpoint.

In this study, haploinsufficiency of PSF1 severely suppressed BM reconstitution by delaying the proliferation of the HSC population. Based on our result, there are 2 possibilities to explain this suppression not only by gene-dose effect, but also other processes. As one possibility, PSF1 may bring about the molecular stability of DNA replication proteins. Overexpression studies suggested that PSF1 regulates stable expression of other GINS components (Figure 6A). Thus, it is probable that lower expression of PSF1 in HSCs of *PSF1*$^{+/-}$ mice may lead to down-regulation of SLD5, PSF2, and PSF3 in HSCs. Therefore, incompletely formed GINS complex may have a dominant negative effect and/or induce instability of other DNA replication complexes, such as CDC45 or MCMs. Another possibility is that PSF1 may induce HSC specific gene expression for effective engraftment capacity. It was reported that HSCs shift gene expression and engraftment phenotype with cell cycle transit.[32] Compared with HSCs from wild-type mice, HSCs obtained from 5-FU-injected *PSF1*$^{+/-}$ mice expressed a lower level of Mac-1, which appeared to be expressed in cycling stem cells and to be involved in cell adhesion (Figure 5A).[26] Thus, haploinsufficiency of *PSF1* may affect HSC properties. As it is thought that DNA replication of important genes for cell function occurs in the early period of the S phase, it is possible that PSF1 regulates the expression of several genes involved in the formation of the BM stem cell niche through DNA replication specifically in HSCs. In addition, loss of PSF1 causes abnormality of chromatin segregation in mice and nematodes (M.U., N.T., unpublished data,

May 1, 2007). It has been also reported that PSF2 depletion inhibits the transition of metaphase to anaphase through the suppression of the attachment of tubulin to the kinetochore.[33] These data suggest that the GINS complex might have roles in other biologic processes.

It is also known that, after chemotherapy with anticancer drug, some patients have prolonged BM suppression for unknown reasons.[34] Moreover, BM dysfunction resulting in pancytopenia is observed with aging in elderly people for unknown reasons. Haploinsufficiency of *PSF1* in mice induced delay of BM recovery after 5-FU treatment and attenuated the number of HSCs with aging. Therefore, attenuation of PSF1 expression in HSCs may cause prolonged BM suppression after chemotherapy and pancytopenia with aging. So far, the association of stem cell division with DNA replication proteins in hematopoietic disorders has not been reported. It is intriguing to analyze the relationship of hematopoietic diseases and DNA replication protein, such as PSF1. Based on our analysis, identification of *PSF1*-dependent genes probably sheds light on the mechanism of DNA replication in HSCs and ontogeny of hematopoietic disorders.

Authorship

Contribution: M.U. and N.T. designed the research, analyzed data, and wrote the paper; K.S. and M.A. helped generate PSF1 mutant mice; and M.I. helped generate anti-PSF1 antibody.

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Theme Issue Article

Maturation of blood vessels by haematopoietic stem cells and progenitor cells: Involvement of apelin/APJ and angiopoietin/Tie2 interactions in vessel caliber size regulation

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Summary

Apelin is a recently-isolated bioactive peptide from bovine gastric extract. The gene encodes a protein of 77 amino acids, which can generate two active polypeptides, long (42–77) and short (65–77). Both peptides ligate and activate APJ, a G protein-coupled receptor expressed in the cardiovascular and central nervous systems. Although an essential role for the apelin/APJ system in blood vessel formation has been reported in *Xenopus*, its precise function in mammals is unclear. Blood vessel tube formation is accomplished by two main mechanisms: 1) single cell hollowing, in which a lumen forms within the cytoplasm of a single endothelial cell (EC), and 2) cord hollowing in which a luminal cavity is created *de novo* between ECs in a thin cylindrical cord. Molecular control of either single cell or cord hollow-ing has not been precisely determined. Angiopoietin-1 (Ang1) has been reported to induce enlargement of blood vessels. Apelin is produced from ECs upon activation of Tie2, a cognate receptor of Ang1, expressed on ECs. It has been suggested that apelin induces cord hollowing by promoting proliferation and aggregation/assembly of ECs. During angiogenesis, haematopoietic stem cells (HSCs) and progenitor cells (HPCs) are frequently observed in the perivascular region. They produce Ang1 and induce migration of ECs, resulting in a fine vascular network. Moreover, HSCs/HPCs can induce apelin production from ECs. Therefore, this review article posits that HSCs/HPCs regulate caliber size of blood vessels via apelin/APJ and Angiopoietin/Tie2 interactions.

Blood vessel size determination

The formation of blood vessels is initiated by the assembly of endothelial cells (ECs), or EC progenitors, and their subsequent tube formation. This process is termed vasculogenesis and is followed by angiogenesis, which results in the emergence of new vessels through the sprouting and elongation from, or the remodeling of, preexisting vessels (1). In both processes, to maintain the structural stability of nascent EC tubes, mural cells (MCs) such as smooth muscle cells and pericytes are recruited around the forming tube and adhere to ECs.

Many genes and molecules involved in these processes have been identified (2–10), with vascular endothelial growth factor (VEGF) mainly playing a role in the development and tube formation of ECs. The ECs forming the tube recruit supporting MCs by releasing PDGF-BB (11). MCs subsequently adhere to

ECs resulting in the formation of a structurally stable blood vessel. It has been reported that this cell adhesion between ECs and MCs is induced when angiopoietin-1 (Ang1), produced by MCs, stimulates Tie2, a receptor tyrosine kinase on ECs (12–15). Therefore, Ang1 is involved in the maturation process of blood vessels. One of these maturation processes for blood vessel formation is adjustment of caliber size, which is very important to supply oxygen and nutrient adequately to tissues. Understanding the process of caliber size regulation is crucial for developing improved clinical approaches to treat cancer and hypoxic disease. However, the molecular mechanisms of blood vessel caliber size determination are not yet clearly understood.

Tube formation is a fundamental mechanism for organ and tissue-generation in most major organs, such as the lung and kidney, as well as the vasculature. The molecular mechanisms involved in tube generation in general are not perfectly under-

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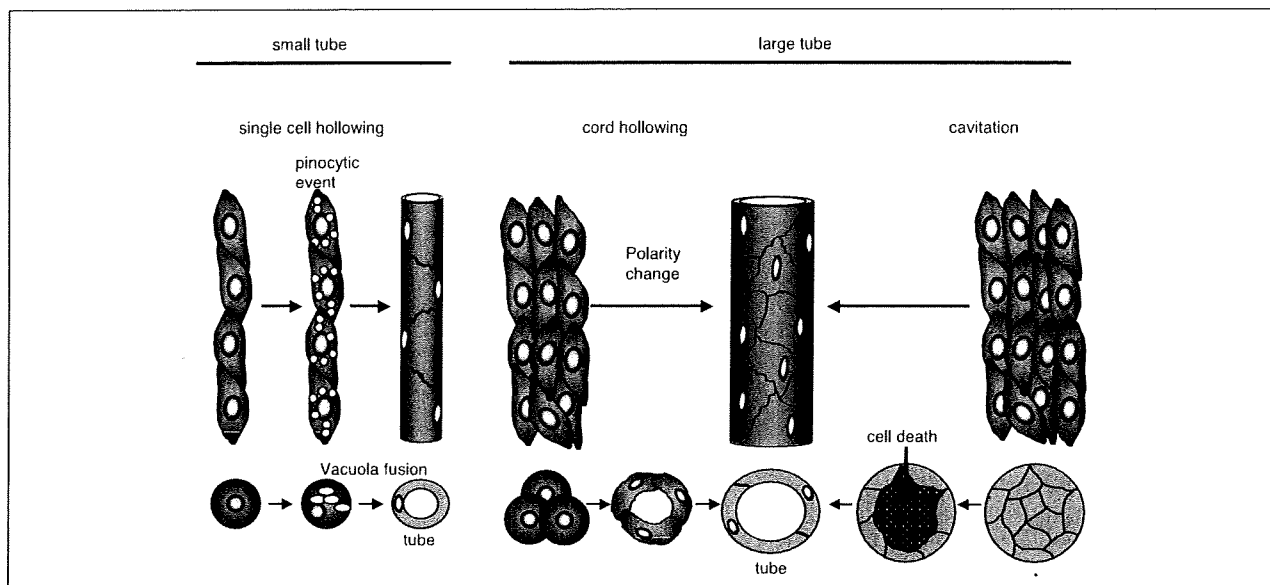E-mail: ntakaku@biken.osaka-u.ac.jp

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Figure 1: Tube formation in the vascular system. Schematic representation of tube formation observed in the vascular system. In single-cell hollowing, several vacuoles generated in the cytoplasm of an endothelial cell (EC) fuse with each other by pinocytic events, forming a tube within the cell. This then connects with the tube in an adjacent EC, resulting in formation of a narrow capillary tube. On the other hand, ag-gregated ECs change their polarity into either apical or basal orientation and gradually form tubes, resulting in the generation of enlarged blood vessels (cord hollowing). When ECs in the center of the aggregate are eliminated by apoptosis or differentiation into another lineage (i.e. hae-matopoietic cells), a tube will be formed (cavitation).

stood; however, anatomical observations of tube morphogenesis by epithelial cells have been well-described and show that tube development can occur in many different ways (16). Based on previous observations of tube formation in general, tubes in the vasculature might be generated by the following steps (Fig. 1). 1) single-cell hollowing: a lumen forms within the cytoplasm of a single EC; 2) cord hollowing: a luminal cavity is created *de novo* between ECs in a thin cylindrical cord; 3) cavitation: the central cells of a column composed of assembled ECs or endothelial progenitors are eliminated, forming a luminal cavity (this process may be less likely in angiogenesis, but has been observed in blood islands of the yolk sac); and 4) Wrapping or intussuscep-tion: an EC sheet invaginates and curls until the edges of the in-vaginating region meet and seal.

Recently, Kamei et al. (17) clearly demonstrated activity of the single-cell hollowing system for blood vessel formation in Zebrafish. Their in-vivo imaging technique showed that intracel-lular and intercellular fusion of endothelial vacuoles drives vas-cular lumen formation. Folkman and Haudenschild (18) de-scribed „longitudinal vacuoles" that „appeared to be extruded and connected from one cell to the next" in EC culture experi-ments *in vitro*. Therefore, single-cell hollowing may be able to construct capillaries of narrow caliber. However, the size of a single EC is limited, so single-cell hollowing cannot give rise to larger vessels. Thus, the cord hollowing system is required for constructing larger vessels. Identification of molecules utilised in cord hollowing but not in single-cell hollowing would there-fore lead to a better understanding of how blood vessel caliber size is determined.

For cord hollowing, ECs once assembled and aggregated gradually manifest polarity, with luminal and apical regions. In-vivo experiments using zebrafish showed that the EC-derived se-creted factor Egfl7 had a crucial role in proper lumen formation after aggregation of endothelial progenitors by regulating their polarity (19). Although the mechanism by which Egfl7 regulates lumen size has not yet been elucidated because of the difficulty of isolating its receptor, these findings imply that tube formation by single-cell hollowing does not occur in areas where ECs need to generate larger tubes.

Depending on the degree of tissue demand for oxygen, one EC starts to sprout from pre-existing vessels for the generation of small-sized capillaries by single-cell hollowing, but under severe hypoxia, several ECs assemble in one sprouting point from pre-existing vessels and generate larger vessels by a cord hollowing mechanism. Therefore, in the initiation of cord hollowing, sev-eral ECs/EC progenitors are required. Thus, for cord hollowing, on sensing hypoxia, ECs need to proliferate and assemble to con-stitute large cylinders, whereas for single-cell hollowing, prolif-eration is not required (Fig. 1).

Tie2 activation induces apelin in ECs

The Ang1/Tie2 and VEGF/VEGFR systems are potent regu-lators influencing caliber size determination in blood vessels. Transgenic overexpression of Ang1 in keratinocytes induces en-larged blood vessel formation in the dermis (20) and adminis-tration of a potent Ang1 variant was also reported to result in en-largement of blood vessels (21, 22). Therefore, knowledge of the precise molecular mechanism of Ang1/Tie2 induction of blood

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vessel enlargement would facilitate our understanding of the process of caliber size determination during angiogenesis.

On the other hand, VEGFs and their cognate receptors (VEGFRs), play central roles in the proliferation of ECs under physiological conditions (23); however, in contrast to Ang1, transgenic overexpression of VEGF in keratinocytes induces formation of a greater number of blood vessels in the dermis, but these were reported to be exclusively of very small caliber (20).

Both VEGF and Ang1 are required for the process of angiogenesis. What happens when both Ang1 and VEGF are overexpressed? Double transgenic mice expressing both these factors in keratinocytes had blood vessels in the dermis larger than wild-type mice but smaller than mice transgenic for Ang1 alone (20). Therefore, the relative amounts of Ang1 and VEGF may alter the caliber size of blood vessels and molecules affected by VEGFR. Hence, Tie2 on ECs must be involved in the regulation of caliber size in blood vessels.

Genes upregulated following Ang1 binding to Tie2 on ECs have been identified by the subtraction method. In this way, the apelin gene was isolated from human umbilical venous endothelial cells (HUVECs) (24). Of many proangiogenic cytokines, such as Ang1, VEGF, bFGF, PDGF-BB, and EGF, it was found that apelin expression was upregulated in HUVECs only by Ang1 and bFGF (Table 1).

Apelin, a ligand for APJ, was recently isolated as a bioactive peptide from bovine gastric extract. The apelin gene encodes a protein of 77 amino acids, which can generate two active polypeptides: the long (42–77) and the short (65–77) forms of apelin (25–27), which both activate APJ. Apelin mRNA and protein are highly expressed in the lung and mammary gland. However, the distribution of the different molecular forms of apelin differs among tissues: apelin molecules with sizes close to apelin-36 (long forms) are major components in the lung, testis, and uterus, but both long and short (approximating apelin-13) forms are detected in the mammary gland (26).

APJ is a G protein-coupled receptor, reportedly expressed in the cardiovascular and central nervous systems (28, 29). In the brain, APJ expression is observed in neurons (30) as well as in oligodendrocytes and astrocytes (31). In the brain, the apelin/ APJ system plays a role in maintaining body fluid homeostasis and regulating the release of vasopressin from the hypothalamus (32). In the cardiovascular system, APJ is expressed in the endothelial lineage in various species of amphibians, as well as in mice and humans (29, 33, 34). In the latter two, the expression of the receptor has also been detected by immunocytochemistry in vascular smooth muscle cells and cardiomyocytes (35). Apelin/ APJ function in cardiomyocytes is thought to associate with a very strong inotropic activity (36, 37). The function of apelin/ APJ in the EC lineage is reported to be associated with the hypotensive activity of apelin (38), as the activation of APJ leads to nitric oxide (NO) production by the ECs (39), and this possibly plays a role in the relaxation of the smooth muscle cell.

Using morpholino antisense oligonucleotides (MO), requisite roles of the apelin/APJ system have been reported in the cardiovascular system of *Xenopus laevis* (40, 41) and Zebrafish (42). *Xenopus apelin* (*Xapelin*) was detected in the region around the presumptive blood vessels during early embryogenesis and overlapped with the expression of *Xmsr*, the *Xenopus* homolog of

Table 1: Apelin and APJ expression on HUVECs stimulated by angiogenic cytokines.

	Apelin	APJ
Ang1	⇑	–
VEGF-A	–	⇑
bFGF	⇑	–
PDGF-BB	–	–
EGF	–	–

⇑ induced, – :not induced.

Table 2: APJ expression on endothelial cells from different tissues.

E10.5 AGM	+++
E10.5 Yolk Sac	++
E10.5 head	++
E10.5 heart	+/-
adult heart	+/-
adult liver	+/-
adult tumor	+

E10.5: embryonic day 10.5, adult: 8 weeks-old. +: positive, +/-:weak.

APJ. Overexpression of *Xapelin* disorganised the expression of the endothelial precursor cell marker *XlFli* at the neurula stage. Knock down of *Xapelin or* Xmsr induced abnormal heart morphology and attenuated the expression of *Tie2*, resulting in the disruption of blood vessel formation in the posterior cardinal vein, intersomitic vessels, and vitelline vessels. In contrast, apelin protein has been shown to induce angiogenesis in the chicken chorioallantoic membrane assay (41).

APJ expression on EC lineage cells and the phenotype of apelin-deficient mice

APJ expression is observed in the EC lineage in mammals; however, when apelin first becomes expressed by ECs and which ECs express its receptor APJ is not clear. During early embryogenesis, compared to ECs from other tissues, such as yolk sac, head region, and heart at the same stage (E10.5), ECs from the the AGM region [Aorta-Gonad-Mesonephros region followed by para-aortic splanchnopleural mesoderm (P-Sp) region at embryonic day (E) 10.5 to E11.5], in which angiogenesis is actively taking place, strongly express APJ (Table 2). However, in the adult, ECs from heart and liver express it only very weakly, but ECs of blood vessels in tumors have been reported to express APJ more strongly (43).

During early embryogenesis at E8.5–9.5, APJ is expressed on ECs sprouted from the dorsal aorta. However, it is not on ECs of the dorsal aorta constructed by vasculogenesis processes. ECs sprouted from dorsal aorta form intersomitic vessels and most express APJ at E8.5; however, APJ expression is observed on ECs in the migrating tip region of intersomitic vessels at E9.5 (24). Therefore, taken together, these expression profiles suggest that APJ is expressed by ECs during angiogenesis but not vasculogenesis. In the neonate,
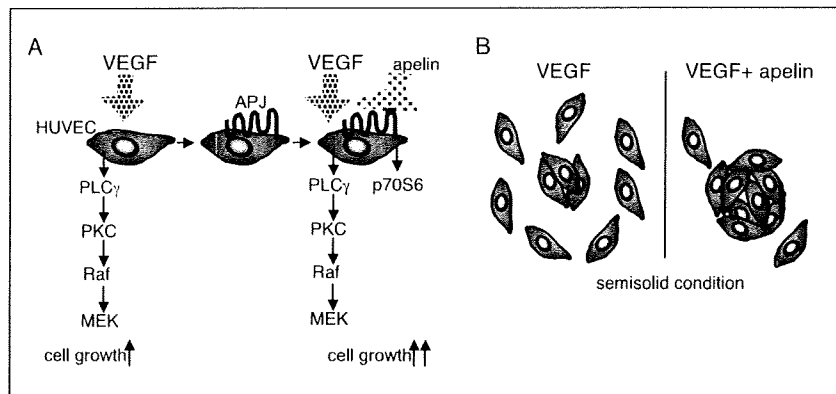
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Figure 2: Effect of VEGF and apelin on proliferation and assembly of ECs.
A) VEGF activates the PLCγ-PKC-Raf-I-MEK-MAP kinase pathway through its cognate receptor. VEGF also induces expression of the apelin receptor APJ on HUVECs. Apelin then activates p70S6 kinase. Therefore, VEGF and apelin coordinately enhance the proliferation of HUVECs. B) Apelin function in spheroid formation by ECs. In semisolid culture media, HUVECs pre-stimulated with VEGF generate larger spheroids in the presence of apelin than with VEGF alone.

APJ expression is observed in ECs of blood vessels in the dermis, but gradually disappears with maturity. These expression patterns strongly suggest that APJ plays a spatio-temporal role for the maturation of blood vessels by transient expression on ECs where angiogenesis is taking place. Generally, apelin-deficient mutant animals appear healthy as adults, but although body size and number of somites was similar between wild-type and apelin mutant embryos at E9.5, the caliber of intersomitic vessels was narrower in the apelin-deficient embryos (24). Moreover, the blood vessels observed in the trachea, dermis, heart and other organs were narrower than those in wild-type mice after birth. Therefore, it is suggested that apelin regulates caliber size of blood vessels.

Coordinate effect of apelin with VEGF for the proliferation of HUVECs

It is well known that VEGF induces proliferation of HUVECs. However, apelin alone is not so effective in this respect. Because APJ expression is observed in ECs during angiogenesis, it is pos-

sible that apelin cannot function in the absence of VEGF, which is upregulated during angiogenesis in response to tissue hypoxia. HUVECs do not constitutively express APJ strongly; however, it is greatly upregulated on stimulation with VEGF (Table 1). Therefore, in the presence of VEGF, HUVECs can respond to apelin effectively. Indeed, apelin alone does not affect proliferation of HUVECs, but in the presence of VEGF, it enhances their proliferation to VEGF (24).

It has been reported that VEGF-A-induced activation of the Raf-1–MEK–MAP kinase pathway mediated mainly by activation of PLCγ and subsequent stimulation of PKC (particularly PKCβ) resulted in the proliferation of ECs (44, 45). Recently, it has been reported that apelin activates p70S6 kinase for cell-cycle progression (46). Therefore, VEGF and apelin may coordinately induce proliferation of HUVECs (Fig. 2).

Alone among the proangiogenic cytokines, such as Ang1, bFGF, PDGF-BB, and EGF, VEGF induces APJ expression on HUVECs (Table 1). Of course, other molecules may also affect APJ expression on ECs; however, it is very interesting that VEGF affects APJ expression, suggesting a close relationship between the APJ/apelin system and tissue hypoxia in which angiogenesis is induced.
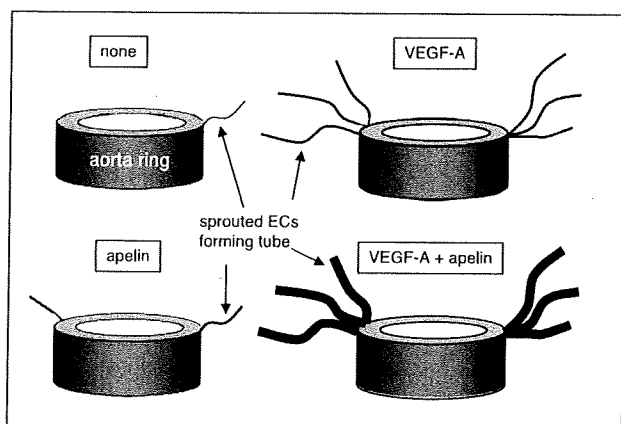

Figure 3: Apelin and VEGF together induce enlarged capillary tube formation in the aorta ring assay. Apelin alone does not induce capillary tube formation in the aorta ring assay. However, in the presence of VEGF, it induces larger capillary tubes than VEGF alone.

Apelin regulates cell assembly in spheroids

Spheroid models of cells have been widely used in tumor and embryonic stem cell studies of cellular differentiation, cell-cell interactions, and hypoxia responses, and were recently utilised to induce proliferation of neural stem cells. Based on these studies, Korff and Augustin (47) developed a spheroid culture system of ECs, such as HUVECs or bovine aortic ECs (BAECs) and showed that these three-dimensional spheroid EC models are useful for the analysis of differentiated cell function. In this culture system, ECs are suspended in culture medium containing 20% methocel, seeded into non-adhesive bacteriological dishes and cultured. Under these conditions, suspended ECs aggregate spontaneously within 4 hours to form cellular aggregates of varying size and cell number (24). Therefore, molecules affecting cell-to-cell assembly can induce larger spheroids in this culture system. When HUVECs were pretreated with VEGF for the induction of APJ and maintained in this spheroid culture system in the presence of apelin, this agent induced the formation of

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Figure 4: Apelin is involved in the regulation of blood vessel caliber size. Endothelial sprouts from pre-existing vessels express APJ following stimulation with VEGF. During angiogenesis, when the Tie2 agonist, Ang1, stimulates ECs sprouted from vessels, apelin expression is induced in these cells. VEGF and apelin coordinately enhance proliferation and assembly of ECs, resulting in the formation of larger tubes. VEGF continuously stimulates ECs during angiogenesis; however, once VEGF expression is reduced in the foci, APJ expression is down-regulated in ECs and caliber size regulation is finalised.
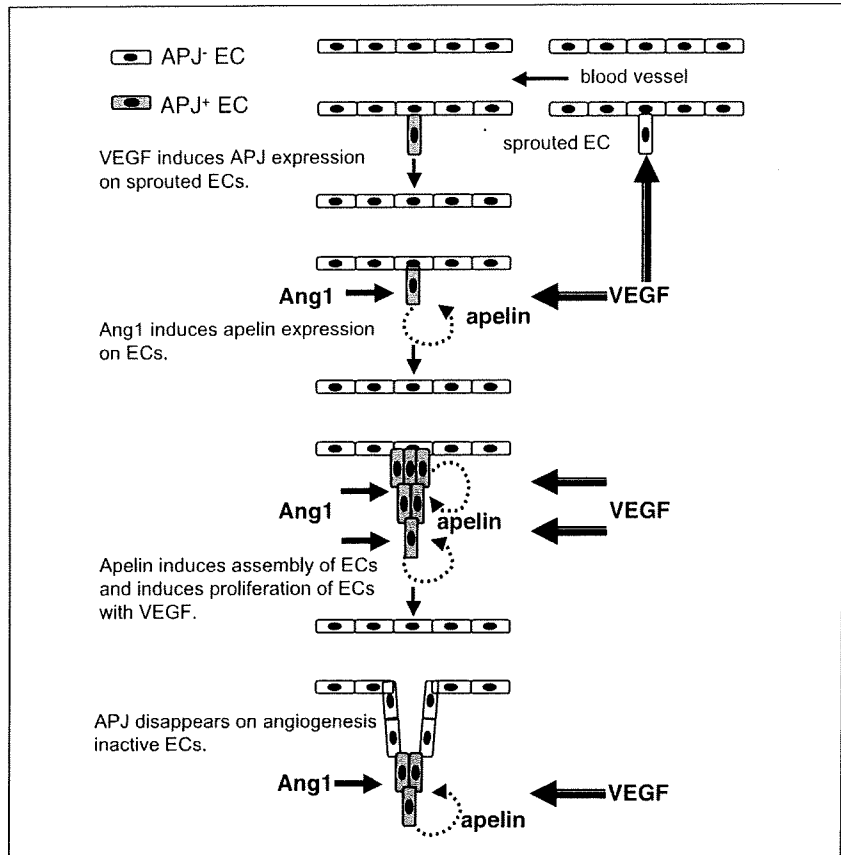
larger spheroids than VEGF alone (Fig. 2). Induction of APJ on Ba/F3 hematopoietic cells (pro-B lymphocyte cell line), also facilitated their aggregation upon stimulation with apelin (Kidoya and Takakura unpublished data). These data indicate that apelin acts on cell-to-cell aggregation or assembly.

When ECs are cultured on Matrigel, a solid gel of basement membrane proteins, they rapidly align and form hollow tube-like structures. Grant et al. (48) first reported this effect of Matrigel. In the original study, the authors reported that tube formation is a multi-step process induced by laminin and that laminin-derived synthetic peptides can induce single-cell hollowing. However, in a similar culture system, Kamei et al. (17) induced cord hollowing to create enlarged tubes. Therefore, this culture system can be utilised to examine whether a certain molecule regulates capillary caliber size. HUVECs cultured on Matrigels in the presence of apelin generate larger tube-like structures than when they are cultured in the presence of VEGF (24). Therefore, this indicates that apelin is involved in cord hollowing.

Apelin induces formation of large tubes in the aorta ring assay ex vivo

The aorta ring assay, first reported by Nicosia and Madri in 1987 (49), can be employed to explore the roles of angiogenesis-related molecules ex vivo. This report described the utilization of rat aorta

"rings" as explants. Several subsequent studies modified this method; now most researchers culture aorta rings in a three-dimensional (3-D) extracellular matrix, such as type I collagen or Matrigel. Under these culture conditions, ring explants generate capillary-like endothelial sprouts in vitro. Thus, this culture system mimics sprouting angiogenesis from pre-existing blood vessels.

Upon addition of proangiogenic factors or anti-angiogenic factors, formation of capillary-like tubes is affected (Fig. 3). In the absence of growth factors, very small numbers and very short capillary-like structures are observed. However, upon addition of VEGF, capillary-like tubes radially sprout from the aorta ring. In this 3-D system, apelin alone does not induce abundant capillary-like tube formation, but in the presence of VEGF, the caliber size of the capillary tube is enlarged by apelin. In culture there is of course no blood flow through capillary-like tubes, showing that the effect of apelin on capillary enlargement is independent of blood flow.

Apelin acts as a potent caliber size regulator by inducing cord hollowing

Given the expression of APJ on ECs and the function of apelin, the role of this molecule in inducing enlarged blood vessels by promoting proliferation and cell-to-cell aggregation/assembly may be as follows (Fig. 4). Upon stimulation by VEGF, ECs
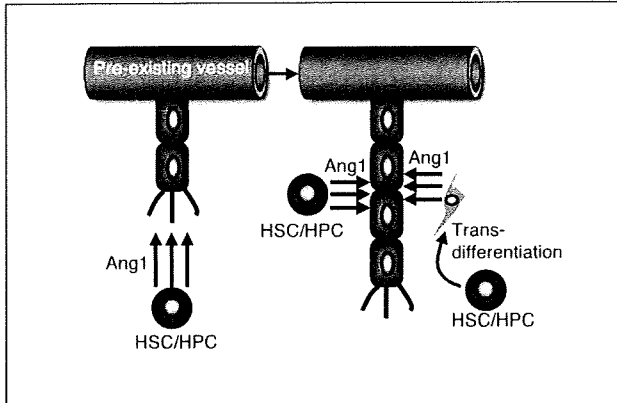
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Figure 5: Function of HSCs/HPCs and production of Ang I. Haematopoietic stem cells (HSCs) and progenitor cells (HPCs) migrate into avascular regions and produce Ang I, which then induces chemotaxis of ECs and determines the migration direction of EC sprouting. Therefore, in this case, HSCs/HPCs act as proangiogenic accessory cell components. Moreover, HSCs/HPCs located at perivascular regions differentiate into mural-like cells. Both HSCs/HPCs and such mural-like cells produce Ang I and then induce apelin expression in ECs to regulate vascular diameter.

progenitor cells (HPCs) also produce Ang1 (51). HSCs/HPCs migrate into avascular areas before ECs, so Ang1 from these cells can induce angiogenesis by promoting EC chemotaxis (51). Moreover, HSCs/HPCs induce the enlargement of blood vessels observed in the fibrous cap surrounding tumors (52) and Ang1 from HSCs/HPCs in embryos, as well as adults, facilitates structural stability of newlydeveloped blood vessels as a physiological function during angiogenesis (53). Indeed, AML1/RUNX1 mutant embryos that lack HSCs have unstable blood vessels which frequently rupture (53–55). These findings support the notion that HSCs play an important role in structural stabilization of blood vessels. HSCs/HPCs are suggested to give rise to MCs (53, 56), which are a major source of Ang1. Therefore, it is possible that Ang1 from the HSC/HPC population, frequently observed in ischemic regions, and from MCs differentiated from HSCs/HPCs, is the source of Tie2 activation during angiogenesis (Fig. 5). Ang1 produced in this manner then induces the production of apelin from ECs.

Conclusions

Control of blood vessel caliber changes is an important mechanism influencing blood pressure and flow, especially for larger vessels, and is a fundamental event for supplying oxygen and nutrients in smaller vessels. The apelin/APJ system may be involved in the size-sensing mechanism of blood vessels. Knockingout the apelin gene suggests that molecular cues other than apelin can rescue narrow caliber size blood vessels by compensational upregulation, because in the early stage of embryogenesis the narrow caliber of intersomitic vessels, observed in apelin mutant embryos, was rescued in the later stage (24). To further clarify the size-sensing mechanism of blood vessels, isolation of upregulated molecules responsible for such compensation of blood vessel caliber in apelin mutant embryos will be required.

Recently, therapeutic angiogenesis using genes or cytokines such as VEGF, HGF, etc. and cells from bone marrow or peripheral blood has been applied to the clinical management of ischemic patients (57). For the development of ideal therapeutic angiogenesis modulators, molecules controlling the caliber size of newly developing blood vessel would be the preferred choice. Apelin could be one candidate for such a modulator.

sprouted from pre-existing vessels express APJ. Subsequently, Ang1 or bFGF stimulates such sprouted ECs to express apelin. In the presence of both VEGF and apelin, EC proliferation is enhanced more than in the presence of VEGF alone. They then adhere and form contacts with each other through junctional proteins, and construct enlarged blood vessels. Upon stimulation of APJ by apelin, junctional proteins such as Claudin-5 and VE-Cadherin are upregulated in HUVECs (24). When VEGF ceases to affect ECs, APJ expression is lost, and the modification of caliber size is finalised. As described above (Fig. 1), the single-cell hollowing system generates narrow capillaries and the cord hollowing system is responsible for the production of larger blood vessels. Therefore, apelin may function in the later, cord hollowing system and be involved in the size determination of blood vessels during angiogenesis.

Haematopoietic stem cells are candidate sources of Ang I for the production of apelin during angiogenesis

Ang1 is usually produced from MCs in cells composing blood vessels (50). However, haematopoietic stem cells (HSCs) and

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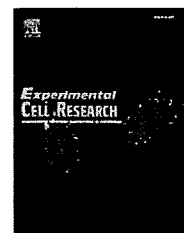
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Lipid rafts serve as signaling platforms for Tie2 receptor tyrosine kinase in vascular endothelial cells

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ARTICLE INFORMATION

ABSTRACT

The Tie2 receptor tyrosine kinase plays a pivotal role in vascular and hematopoietic development. The major intracellular signaling systems activated by Tie2 in response to Angiopoietin-1 (Ang1) include the Akt and Erk1/2 pathways. Here, we investigated the role of cholesterol-rich plasma membrane microdomains (lipid rafts) in Tie2 regulation. Tie2 could not be detected in the lipid raft fraction of human umbilical vein endothelial cells (HUVECs) unless they were first stimulated with Ang1. After stimulation, a minor fraction of Tie2 associated tightly with the lipid rafts. Treatment of HUVECs with the lipid raft disrupting agent methyl-β-cyclodextrin selectively inhibited Ang1-induced Akt phosphorylation, but not Erk1/2 phosphorylation. It has been reported that inhibition of FoxO activity is an important mechanism for Ang1-stimulated Tie2-mediated endothelial function. Consistent with this, we found that phosphorylation of FoxO mediated by Tie2 activation was attenuated by lipid raft disruption. Therefore, we propose that lipid rafts serve as signaling platforms for Tie2 receptor tyrosine kinase in vascular endothelial cells, especially for the Akt pathway.
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Introduction

Angiogenesis, the outgrowth of novel blood vessels from pre-existing ones, is essential for a number of physiological processes such as embryonic development, organ formation, tissue regeneration, and tissue remodeling [1]. However, under pathological conditions, uncontrolled angiogenesis sustains the progression of many diseases, including diabetic retinopathy, psoriasis, rheumatoid arthritis, and tumor growth [1]. In the latter condition, numerous studies have provided evidence that tumor growth and metastasis are angiogenesis dependent [2]. Therefore, it is necessary to analyze the molecular mechanism of angiogenesis to develop angiogenesis disrupting agents.

Angiopoietin-1 (Ang1) is the ligand of the endothelial tyrosine kinase receptor Tie2 [3]. Mice lacking Ang1 die during embryogenesis (E12.5) showing a poorly remodeled and immature vasculature with defects in endothelial cell (EC) adhesion to mural cells [4]. Ang1 is a potent and unique angiogenic protein that induces EC migration

and survival. The Ang1-Tie2 system seems to have different functions for blood vessels depending on the situation of ECs. Recently, it has been suggested that Tie2 activation by Ang1 induces phosphorylation of Akt rather than Erk when EC-EC contact is established, resulting in quiescence of blood vessel formation [5]. On the other hand, when ECs are migrating and proliferating, Tie2 mainly activates the Erk pathway rather than Akt, resulting in progression of angiogenesis. Therefore, utilization of the Akt or Erk pathway is key for explaining the different functions of Tie2 in blood vessel formation. However, how such signaling diversity occurs by a single Tie2 receptor is not fully elucidated.

Plasma membrane lipid raft domains, which contain high concentrations of cholesterol and sphingolipids [6,7], are known to function as centers for the assembly of signaling complexes. Such assembly is suggested to facilitate both specificity and the rate of signaling events, and they have become a central facet of signaling research since the function of many receptors and their

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downstream effectors are dependent on lipid rafts [8–11]. The presence of receptor and effector proteins in lipid rafts, as well as the ability of lipid rafts to enhance receptor signaling [12], has led to the concept of a signalosome where proteins are localized together to facilitate receptor signaling following agonist exposure [8,13]. For some receptors, lipid raft complexes appear to play an inhibitory role [12], although this need not conflict with signal facilitation if inhibition is directed at the unstimulated receptor.

Here, we hypothesize that Ang1-Tie2 could mediate different biologic effects under the influence of lipid rafts. We show here that lipid rafts are essential components for Tie2-Akt pathway activation, but not Tie2-Erk pathway activation. These data suggest that lipid rafts serve as signaling platforms for the Tie2 receptor tyrosine kinase and could mediate different biologic effects in vascular ECs.

Materials and methods

Reagents and antibodies

Recombinant human angiopoietin-1 (Ang1) was purchased from R&D systems (Minneapolis, MN). Monoclonal anti-caveolin-1 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-Tie2 antibody was purchased from Upstate (Lake Placid, NY). Monoclonal anti-transferrin receptor antibody was purchased from Zymed (South San Francisco, CA). Monoclonal anti-GAPDH antibody was purchased from Chemicon (Temecula, CA). Monoclonal anti-Na$^-$/K$^+$ ATPase beta 1 subunit antibody was purchased from NOVUS Biologicals (Littleton, CO). Polyclonal anti-Akt, anti-phospho-Akt (Ser473), anti-Erk1/2, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-FoxO1, anti-phospho-FoxO1 (Thr24), anti-phospho-FoxO1 (Ser256), anti-phospho-FoxO1 (Ser319), anti-FoxO3a, anti-phospho-FoxO3a (Ser253), and anti-phospho-FoxO3a (Ser318/321) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-phospho-Tie2 (Tyr992) antibody was purchased from R&D systems. Horseradish peroxidase (HRP)-coupled anti-mouse and anti-rabbit Ig were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA). Alexa Fluor 488-coupled anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR).

Cell culture

Human umbilical vein ECs (HUVECs) were purchased from Kurabo (Kurashiki, Japan) and maintained per manufacturer's instructions. For Ang1 stimulations, cells were starved in RPMI1640 medium containing 0.1% bovine serum albumin (BSA; Sigma, St Louis, Missouri) for 3 h in parallel with untreated cells and then stimulated with Ang1 as indicated in the figure legend. For methyl-beta-cyclodextrin (mβCD; Sigma) treatment, mβCD was added to the culture media and incubated at 37 °C as indicated in the figure legend before Ang1 was added to conditioned media.

Lipid raft isolation

Lipid rafts were isolated by two different methods. First, Opti-Prep gradient centrifugation using the 1% Triton X-100 method was performed using a previously described protocol with some modifications [14,15]. HUVECs grown to confluence in 10 cm dishes were used. After washing with PBS, cells were scraped and precipitated by centrifugation. Precipitate was lysed in 140 μL lysis buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 12.5 mM EDTA, 1% Triton X-100) for 30 min on ice. The lysate was added to four volumes of 50% Opti-Prep in the same lysis buffer and placed at the bottom of an ultracentrifuge tube. A 0–40% discontinuous Opti-Prep gradient was formed above the sample (0.3 mL lysis buffer without Opti-Prep, 1 mL 30% Opti prep in lysis buffer) and centrifuged at 55,000 rpm for 2 h in a TLS-55 rotor (Beckman Instruments, Fullerton, CA). Ten 0.2 mL fractions were gently collected from the top of the gradient. Second, centrifugation with 1% Triton X-100 was performed using a previously described protocol with modifications [16]. All steps were performed in a 4 °C cold room and on ice. Briefly, HUVECs grown in a six-well plate were lysed with 0.5 mL of lysis buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 12.5 mM EDTA, 1% Triton X-100), collected in plastic tube, and lysed on ice for 30 min. Lysate and insoluble material were centrifuged for 30 min in a microfuge at 12,000 ×g at 4 °C. The supernatant was collected (soluble fraction). Buffer was again added to the pellet and centrifuged for 10 min in a microfuge at 12,000 ×g at 4 °C. The supernatant was discarded. The pellet was solubilized in 0.1 mL of RIPA buffer (10 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1 mM EDTA-2Na).

Immuno-blotting

The proteins were separated by electrophoresis on 7.5% or 10% polyacrylamide gels containing SDS. The proteins were transferred by electroblotting to polyvinylidene difluoride membranes, and the membranes were subsequently incubated with the primary antibody. Proteins were detected with HRP-coupled secondary antibodies after extensive washing using ECL reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Caveolin-1 was used as a marker for the lipid raft fraction. The Na$^+$/K$^+$ ATPase beta 1 subunit and transferrin receptor were used as markers for the non-raft fraction. GAPDH was used as an internal control. Representative data from more than three independent experiments is shown.

Immunocytochemistry

HUVECs were fixed with 4% paraformaldehyde in PBS and permeabilized with methanol. Next, the cells were incubated with anti-FoxO1 antibody, then with Alexa Fluor 488-coupled anti-rabbit IgG, and examined by fluorescence microscopy.

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Student's t-test was used for statistical analysis. Differences were considered statistically significant if the P-value was less than 0.05.

Results

Localization of Tie2 in lipid rafts

One line of research suggests that Tie2 localizes to the caveolae, a subset of lipid rafts, of ECs [17]. However, how localization of Tie2 is affected in the presence or absence of Ang1 has not been

investigated. Therefore, we analyzed the precise location of Tie2 on ECs and determined whether it is affected by Ang1 stimulation. We examined the subcellular localization of Tie2 using the gradient centrifugation method with 1% Triton X-100, the most popular method for identifying lipid raft-associated proteins [14]. As shown in Fig. 1A, most Tie2 was detected in the non-lipid raft fraction with very little in the lipid raft fraction in the absence of Ang1 stimulation. However, treatment with Ang1 resulted in an increase of Tie2 in the lipid raft fraction (although not in very large amounts). Localization of Tie2 into the raft fraction following Ang1 stimulation was prevented by mβCD treatment (Fig. 1B), suggesting that Tie2-raft interaction is induced by the binding of Ang1 to Tie2.

Akt and Erk pathway regulation by Tie2

As reported above, we found that a minor fraction of Tie2 tightly associates with lipid rafts after stimulation with Ang1. Next, we examined whether phosphorylated Tie2 is found in lipid rafts. As was shown in Fig. 1, the amount of Tie2 localizing to lipid rafts is low. This made it impossible to detect phosphorylated Tie2 in the lipid rafts following their isolation using the gradient centrifugation method with 1% Triton X-100 (data not shown). Therefore, we used an alternative method for the isolation of insoluble proteins in cold non-ionic detergents [16]. As shown in Fig. 1, Tie2 is almost undetectable in the insoluble fraction (lipid raft fraction) before Ang1 stimulation. However, Tie2 became detectable in lipid rafts from 15 to 60 min after stimulation with Ang1, peaked and decreased again by 120 min (Figs. 2A, B). On the other hand, we found Tie2 in the soluble fraction before stimulation with Ang1, the amount of which was largely unaffected by Ang1, except at 120 min (Figs. 2A, B). This attenuation of Tie2 in the soluble
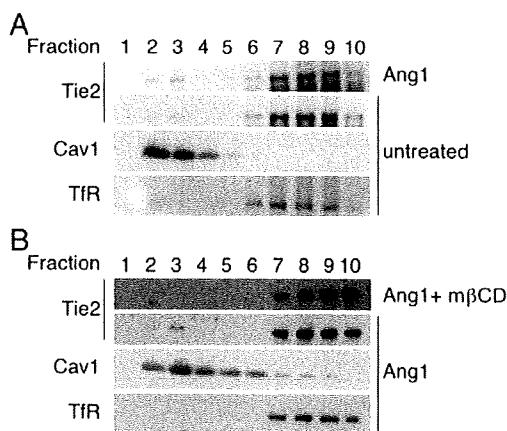

Fig. 1 – Ang1 stimulation induces localization of Tie2 in lipid rafts. (A) HUVECs were incubated with (Ang1) or without (untreated) Ang1 (200 ng/mL) for 30 min. (B) HUVECs were incubated with (Ang1 + mβCD) or without (Ang1) mβCD (10 mM) for 30 min and then incubated with Ang1 (200 ng/mL) for 30 min. In all experiments, HUVECs were fractionated by Opti-Prep gradient centrifugations with 1% Triton X-100. Fractions were collected and subjected to immuno-blotting with antibodies directed against Tie2, caveolin-1 (Cav1), or transferrin receptor (TfR). Cav1 and TfR were used as markers for lipid raft and non-raft-membrane fractions, respectively.
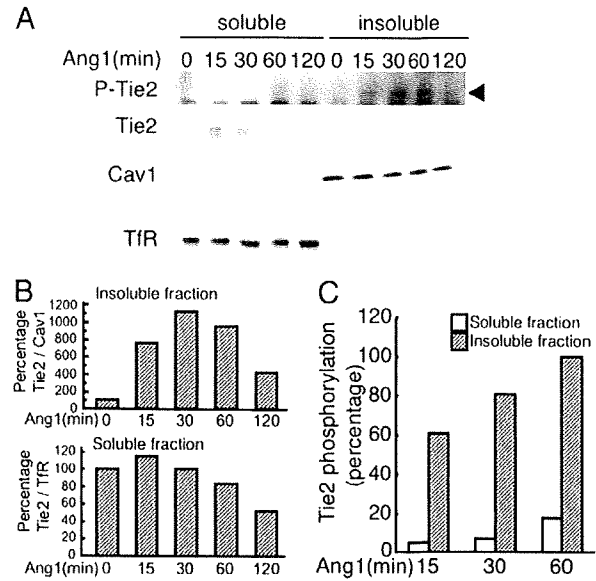

Fig. 2 – Phospho-Tie2 tightly interacts with lipid rafts. (A) HUVECs were incubated with Ang1 (200 ng/mL) for several minutes as indicated. Total cell lysates were fractionated into 1% Triton X-100 insoluble and soluble fractions. Each fraction was subjected to immuno-blotting with antibodies directed against Tie2, phospho-Tie2 (P-Tie2), caveolin-1 (Cav1), or transferrin receptor (TfR). Cav1 and TfR were as markers used for lipid raft and non-raft-membrane fractions, respectively. (B) Tie2 and Cav1, and TfR observed in (A) were quantified. The amount of Tie2 is expressed as the ratio of Tie2 to TfR or Cav1 compared to the value obtained for Ang1 untreated cells in the soluble or insoluble fractions, respectively. (C) Tie2 and P-Tie2 observed in (A) were quantified. Tie2 phosphorylation represents the ratio of phosphorylated Tie2 to total Tie2 normalized to the value obtained for the insoluble fraction stimulated for 60 min.

fraction might be caused by its down modulation at a later time point after stimulation. Tie2 phosphorylation in lipid rafts was strongly induced 30–60 min after Ang1 stimulation and subsequently decreased again (Fig. 2A). Taking account of the low amount of Tie2 protein in the insoluble compared to the soluble fraction, it appears that phospho-Tie2 tightly interacts with EC lipid rafts (Figs. 2A, C).

Next, we examined whether Tie2 expression on ECs is affected by the presence or absence of lipid rafts. As shown in Figs. 3A and B, lipid rafts disrupted by mβCD reduced the amount of Tie2 in HUVECs weakly. The difference is significant but not large. This suggested that Ang1 stimulation on ECs induces Tie2 activation even in the presence of mβCD. Indeed, treatment of HUVECs with mβCD did not inhibit Ang1-induced Tie2 phosphorylation (Fig. 3C).

Next, we examined whether disruption of lipid raft formation affects Tie2 downstream signaling. Both Akt and Erk were phosphorylated upon Ang1 treatment in the absence of mβCD (Fig. 3D). Interestingly, treatment of HUVECs with mβCD selectively inhibited Ang1-induced Akt phosphorylation but not Erk1/2 phosphorylation (Fig. 3D). In the Tie2 signaling
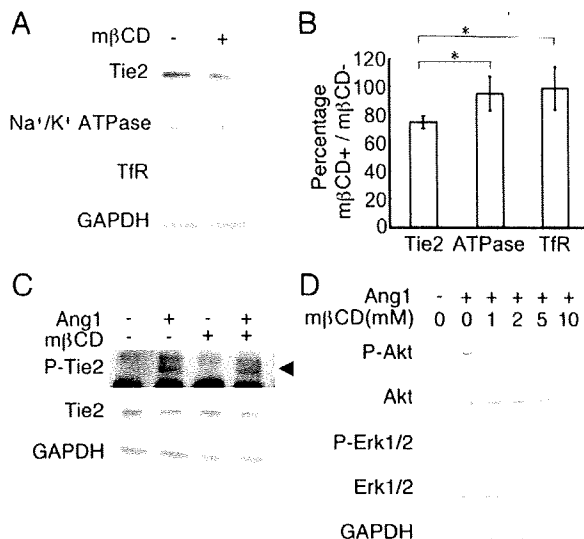
A

mβCD   -   +

Tie2

Na⁺/K⁺ ATPase

TfR

GAPDH

B

C

Ang1   -   +   -   +
mβCD   -   -   +   +
P-Tie2
Tie2
GAPDH

D

Ang1   -   +   +   +   +   +
mβCD(mM)   0   0   1   2   5   10

P-Akt

Akt

P-Erk1/2

Erk1/2

GAPDH

Fig. 3 – Lipid rafts serve as a signaling platform for the Tie2-Akt pathway. (A) HUVECs were incubated with or without mβCD (5 mM) for 3 h, and total protein was subjected to immuno-blotting with the indicated antibodies. **(B)** Tie2, Na^+/K^+ ATPase, and transferrin receptor (TfR) observed in (A) were quantified. All data were normalized to the GAPDH density and the ratio of mβCD-treated data to untreated data is presented as a percentage. ($n > 3$), *$P < 0.05$. **(C)** HUVECs were incubated with or without mβCD (10 mM) for 15 min after serum starvation and then cultured in the presence or absence of Ang1 (200 ng/mL) for 15 min. **(D)** HUVECs were incubated with or without mβCD (1–10 mM) for 15 min after serum starvation and then stimulated with Ang1 (200 ng/mL) for 15 min. Total protein was subjected to immuno-blotting with the indicated antibodies.

pathway, Akt and Erk are suggested to be important for cell survival [18] and for cell migration and proliferation [19, 20], respectively. Therefore, our data suggest that lipid rafts may serve as a signaling platform for Tie2 in ECs, especially for the Akt pathway.

Activation and subcellular localization of FoxO are affected by disruption of lipid rafts

Recently, the forkhead box-containing O subfamily (FoxO), comprised of members such as FoxO1 and FoxO3a, has been reported to be important for negatively regulating vessel formation [21]. Further, inhibition of FoxO1 activity has been shown to be an important mechanism for Ang1-Tie2 mediated endothelial function [22]. It has been reported that FoxO is phosphorylated by Akt, inducing exclusion of FoxO from the nucleus and resulting in prevention of transcriptional regulation by FoxO [19,20,23–26]. Thus, we examined whether disruption of lipid rafts affects FoxO phosphorylation as mediated by Ang1 stimulation of Tie2 on HUVECs. As shown in Fig. 4A, Ang1 induced phosphorylation of FoxO1 and FoxO3a, and phosphorylation of FoxO1 and FoxO3a was attenuated by mβCD treatment. Furthermore, nuclear export of FoxO1 by Ang1 was suppressed by mβCD treatment (Figs. 4B and C). These findings

indicate that lipid raft disruption inhibits FoxO inactivation in relation to Tie2 activation.

Discussion

Regulation of the Tie2 receptor has been suggested to be one way of inhibiting angiogenesis in a variety of diseases. However, it is still controversial as to whether Ang1 is a proangiogenic or anti-angiogenic factor. Within the Tie2-mediated signaling pathway, Akt and Erk have been suggested to be important regulators of angiogenesis [27,28]. Due to the diverse signaling network downstream of Akt and differences observed in short-term versus long-term Akt activation, its role in pathophysiological processes remains elusive. For example, whereas short-term Akt activation in the heart resulted in increased angiogenesis, chronic activation led to decreased angiogenesis and increased fibrosis [29,30]. In terms of the Tie2 signaling pathway, activation of Akt through Tie2 activation induces cell survival [18]. Therefore, this pathway is thought to contribute to the maintenance of vascular quiescence or stability. In contrast to this Akt pathway, activation of Erk is suggested to be important for cell migration and proliferation [19,20]. Recently, it has been reported that Tie2 activation at cell–cell or cell–substratum contacts leads to preferential activation of Akt or Erk, respectively [5,31]. Although Tie2 is a context-dependent regulator of blood vessel formation, how Akt or Erk activation is altered by Tie2 has not been determined so far.

Plasma membrane lipid raft domains, which contain high concentrations of cholesterol and sphingolipids, are known to function as centers for the assembly of signaling complexes. Such assembly is suggested to facilitate both specificity and the rate of signaling events, and it has become a central facet of signaling research as the function of many receptors and their downstream effectors are dependent on rafts [8–11]. Our present data show that raft disruption inhibits Akt phosphorylation but not Erk phosphorylation. We found that a subset of Tie2 becomes insoluble upon Ang1-stimulation. These results indicate that Tie2 localization or interaction on/with lipid rafts is key for regulation of Akt phosphorylation via Tie2 activation. Regulatory mechanisms, i.e. the joint strength between Tie2 and lipid rafts, types of lipid rafts or types of associated proteins with lipid rafts, may promote Tie2 localization to the raft domain. However, at present, the precise molecular mechanism regulating Tie2 localization to rafts is not known. Although the in vivo relevance of these findings remains to be proven, our experiments suggest that lipid rafts serve as signaling platforms for Tie2, separately controlling the Akt and Erk pathways, and that lipid rafts are important for signal transduction of Akt via Tie2 phosphorylation.

Several lines of research demonstrated that lipid raft disruption reduced basal Akt phosphorylation levels in tumor cells and that treatment with cholesterol-depleting agents such as mβCD induced apoptosis [31–34]. Moreover, it has been reported that lipid rafts contain junction proteins, such as annexin and VE-cadherin, in vascular ECs and that those proteins are recruited when cell-to-cell contact is established [35]. Cell–cell contact is one situation when cell apoptosis is inhibited, and Tie2 located on the cell–cell boundary preferentially induces Akt phosphorylation rather than Erk phosphorylation upon stimulation with Ang1 [5,31]. Akt, as described above, is important mediator for cell survival for ECs [18]. Therefore, when cell–cell contact is
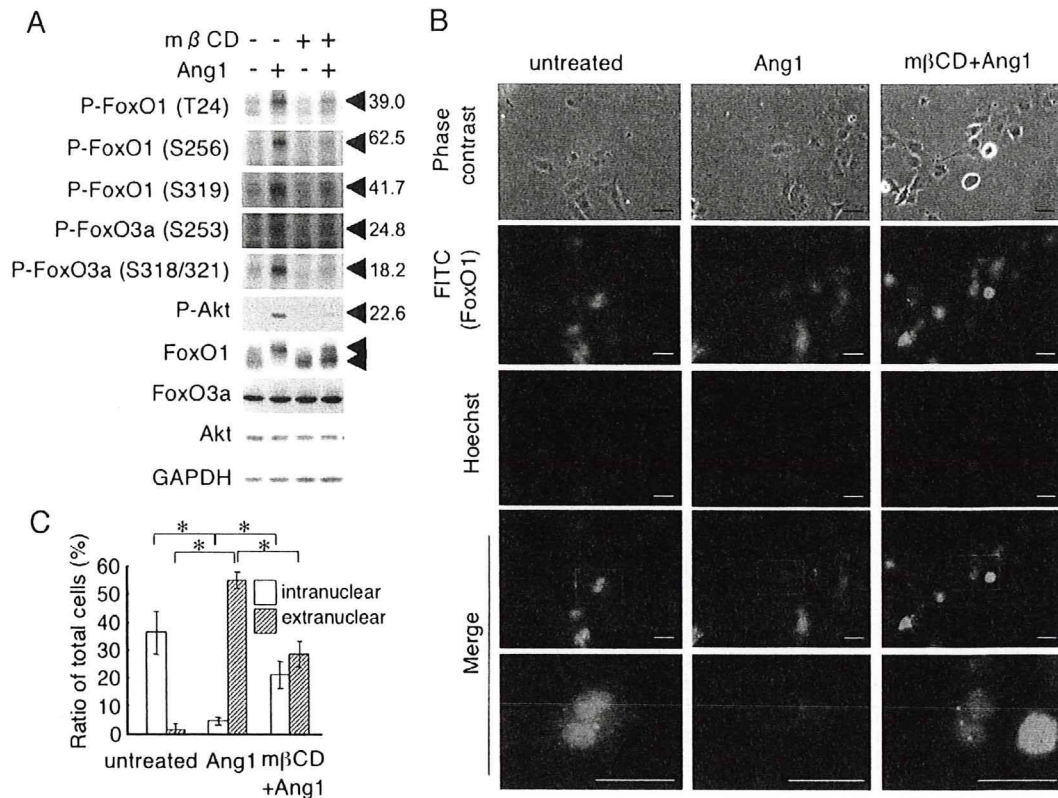
Fig. 4 – Lipid rafts serve as a signaling platform for the Tie2-FoxO pathway. (A) HUVECs were incubated with or without mβCD (10 mM) for 15 min and then stimulated with Ang1 (200 ng/mL) for 15 min. Total protein was subjected to immuno-blotting with the indicated antibodies. The density ratio is shown on the right side of each picture. To calculate the density ratio, the ratio of phosphorylated protein versus total protein was calculated for FoxO1, FoxO3a, and Akt protein. The ratio of Ang1-treated/mβCD-treated data to Ang1-treated/mβCD-untreated data is presented as a percentage for each. (B) HUVECs were incubated with or without mβCD (10 mM) for 15 min after serum starvation and then stimulated with Ang1 (200 ng/mL) for 30 min. Immunocytochemistry was performed after each treatment. Bottom panels show high power views indicated by a dashed box in each upper panel. Bar indicates 50 μm. (C) Quantitative evaluation of FoxO1 localization observed in (B). FoxO1 localization at the intranuclear or extranuclear part of the cell was scored and expressed as a percentage relative to total number of cells scored ($n = 3$), *$P < 0.01$.

established, recruitment of Akt to raft domain may be a key for regulation of EC survival via Tie2 activation.

At present, which molecules allow Tie2 to localize to lipid rafts are not known. One candidate is caveolin-1 because it is a major structural protein of caveolae, a subdomain of lipid rafts, and because the cytoplasmic domain of Tie2 contains a binding motif for caveolin-1 [36]. However, more precise analyses to identify the molecules that recruit Tie2 to lipid rafts will help to elucidate this mechanism.

In the present findings, we have provided evidence for the existence of a novel mechanism able to promote Akt signal transduction via Tie2 activation. Further precise molecular analyses of how Tie2 and Akt tether to lipid rafts may shed light on the mechanisms behind Tie2-dependent vascular quiescence and angiogenesis.

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 $\alpha9/\beta1$ 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.
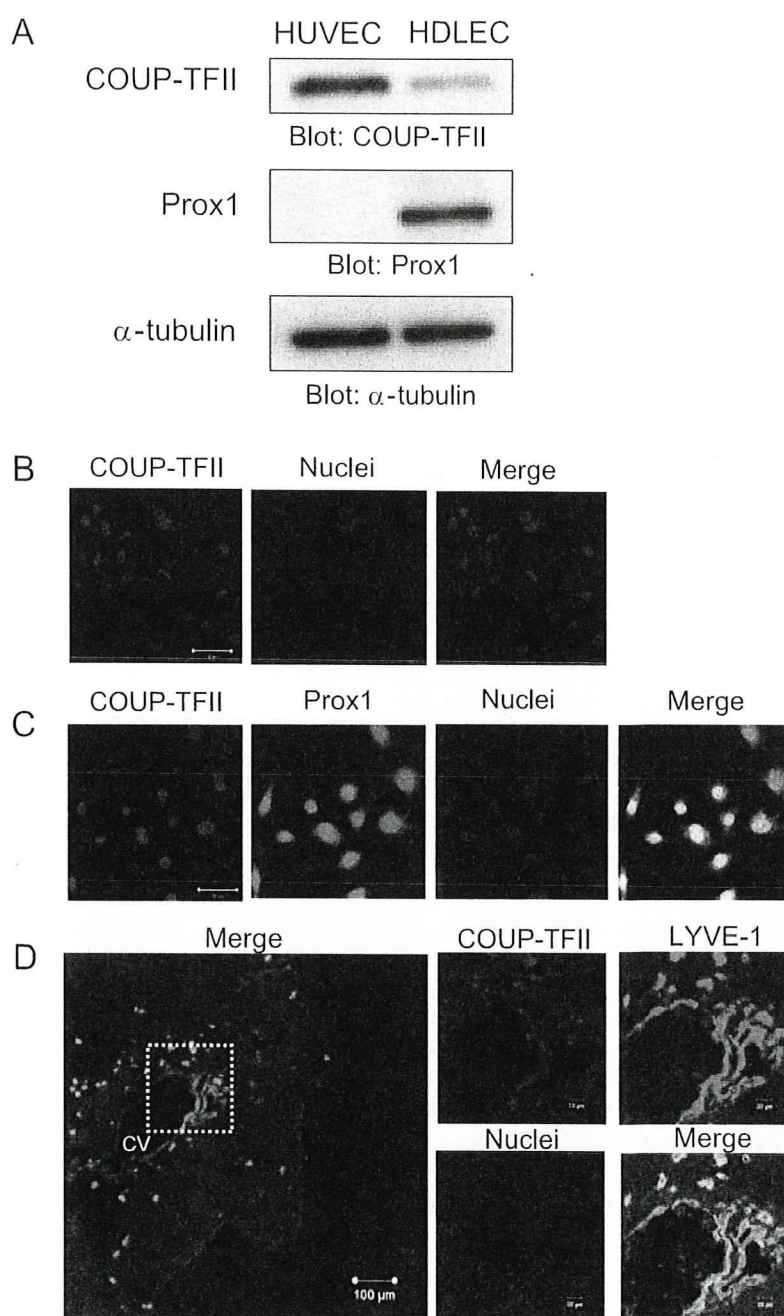
Figure 1 COUP-TFII is expressed in BECs and LECs. (A) Western blot analyses for COUP-TFII (top panel) and Prox1 expressions (middle panel) in HUVECs and in HDLECs. α-tubulin was used as a loading control (bottom panel). (B–C) Immunostaining of HUVECs (B) and HDLECs (C) was carried out for COUP-TFII (red) 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.
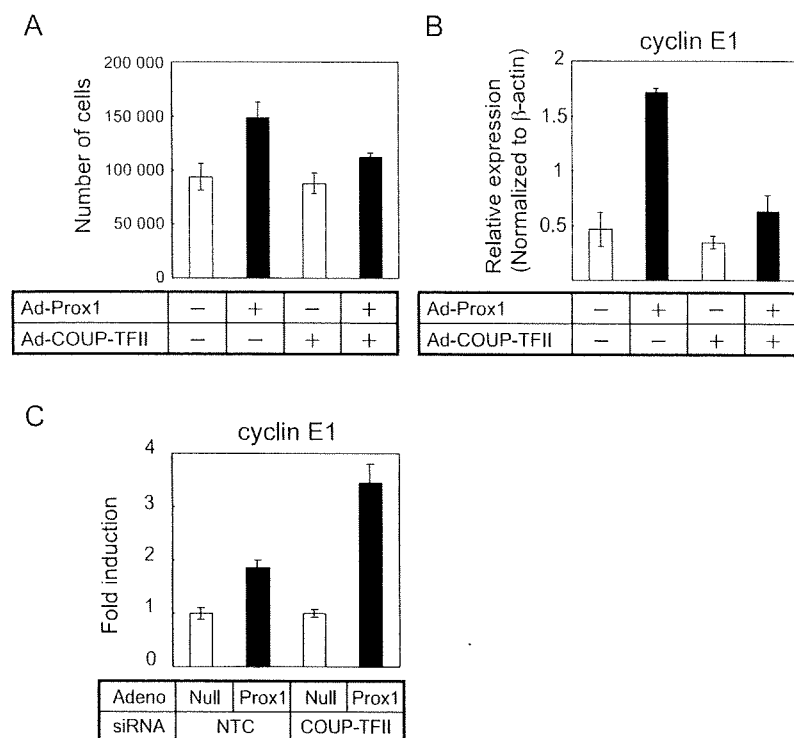
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Figure 2 COUP-TFII suppresses Prox1-induced cell proliferation through the regulation of cyclin E1. (A) Numbers of cells were counted 48 h after infection of HUVECs with adenovirus coding for Prox1 (Ad-Prox1) in combination with that for COUP-TFII (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,