Role of Krüppel-Like Factor 4 and its Binding Proteins in Vascular Disease

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Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor that plays a key role in cellular differentiation and proliferation during normal development and in various diseases, such as cancer. The results of recent studies have revealed that KLF4 is expressed in multiple vascular cell types, including phenotypically modulated smooth muscle cells (SMCs), endothelial cells and monocytes/macrophages and contributes to the progression of vascular diseases by activating or repressing the transcription of multiple genes via its associations with a variety of partner proteins. For example, KLF4 decreases the expression of markers of SMC differentiation by interacting with serum response factor, ELK1 and histone deacetylases. KLF4 also suppresses SMC proliferation by associating with p53. In addition, KLF4 enhances arterial medial calcification in concert with RUNX2. Furthermore, endothelial KLF4 represses arterial inflammation by binding to nuclear factor-κB. This article summarizes the role of KLF4 in vascular disease with a particular focus on in vivo studies and reviews recent progress in our understanding of the regulatory mechanisms involved in KLF4-mediated gene transcription.

Introduction

Vascular proliferative diseases, such as atherosclerosis and restenosis after percutaneous coronary intervention, are major causes of mortality in Westernized societies. Such conditions are recognized to be complex disorders involving multiple cell types, including smooth muscle cells (SMCs), endothelial cells (ECs), lymphocytes and monocytes/macrophages. For the advancement of therapeutic and preventive approaches, it is important to understand the molecular and cellular mechanisms underlying the progression of vascular diseases in each cell type.

The mammalian Krüppel-like factor (KLF) family is a subclass of zinc-finger containing DNA-bind-
during normal development\textsuperscript{11-15} as well as in various diseases, such as cancer\textsuperscript{13, 16, 17}. Although KLF4 is abundantly expressed in the colon, testis and lungs\textsuperscript{30}, it is also detectable in the vascular system. Indeed, human KLF4 cDNA was first cloned from a human umbilical vein EC cDNA library\textsuperscript{10}, and KLF4 has been shown to be constitutively expressed in ECs in the aorta and pulmonary arteries and veins\textsuperscript{18}. In contrast, KLF4 is not expressed in differentiated SMCs in adult vessels, although it is induced in phenotypically modulated SMCs following vascular injury\textsuperscript{19, 20}. Moreover, the results of recent studies have demonstrated that KLF4 is also expressed in monocytes/macrophages\textsuperscript{21-23}. As such, KLF4 is expressed in multiple cells that participate in the development of vascular diseases, although its role has not yet been fully determined. This review article summarizes the contribution of KLF4 to the development of vascular diseases, with a particular focus on in vivo studies, and considers recent advances in our understanding of the molecular mechanisms whereby KLF4 exerts multiple effects by interacting with various partner proteins. For a more comprehensive review of KLF family members and/or their role in transcriptional regulation in vascular diseases, the reader should refer to several excellent review articles\textsuperscript{1-4, 24-28}.

KLF4 in SMCs

Although the primary function of SMCs is contraction in order to maintain vessel tone, SMCs also retain considerable plasticity, allowing them to exert multiple effects in response to changes in local environmental cues\textsuperscript{26, 27}. For example, in association with vascular injury or atherosclerotic lesion formation, SMCs undergo a process often referred to as phenotypic modulation or phenotypic switching, characterized by a dramatic increase in the rate of proliferation, migration and synthesis of extracellular matrix proteins in addition to a decreased expression of SMC-specific/-selective differentiation markers. The results of studies conducted by our laboratory and others have revealed KLF4 to be a critical transcriptional regulator of phenotypic switching of SMCs by interacting with various proteins. The multiple roles of KLF4 in the functions of SMCs will be reviewed in this section.

KLF4 Suppresses the Expression of SMC Differentiation Markers by Interacting with ELK1, Serum Response Factor and/or p65

Differentiated SMCs express a unique repertoire of contractile proteins and signaling molecules, such as SM α-actin (ACTA2), SM-myosin heavy chain (MYH11), SM22α (TAGLN) and h1-calponin, in order to maintain vessel tone\textsuperscript{26, 27}. The transcription of these SMC differentiation marker genes is controlled by common cis-elements, including multiple CC(A/T)\textsubscript{6}GG (CArG) elements and a transforming growth factor-β control element (TCE)\textsuperscript{26-35}. For example, the Acta2 gene and Mphh11 gene have three CArG elements and a single TCE, respectively, whereas the Tagln gene contains two CArG elements and one TCE in its promoter-enhancer region. Studies using transgenic mice harboring the LacZ reporter gene driven by the promoter-enhancer regions of SMC differentiation marker genes have demonstrated that multiple CArG elements as well as TCE are required to recapitulate the expression patterns of the endogenous gene\textsuperscript{29-35}. The binding factor for CArG elements is serum response factor (SRF), which regulates the expression of SMC differentiation marker genes by cooperating with its co-activator, myocardin (MYOCD)\textsuperscript{36-39}, or its co-repressor, phosphorylated ELK1\textsuperscript{40-42}. While SRF is ubiquitously expressed and it itself does not activate the transcription of SMC differentiation marker genes in SMCs, MYOCD is selectively expressed in SMCs and cardiac muscle and induces the transcription of multiple CArG-containing SMC differentiation marker genes via homodimerization\textsuperscript{36-39, 43}. Of interest, Myocd-deficient mice exhibit no vascular SMC differentiation and die by embryonic day 10.5, although it is possible that this early embryonic lethality is secondary to defects in the extraembryonic circulation and/or pericardial effusion\textsuperscript{44}. In addition, studies using Myocd chimeric knockout mice have shown that MYOCD is dispensable for the development of vascular SMCs\textsuperscript{45, 46}. Studies conducted to date suggest that MYOCD is not necessarily required for SMC differentiation, although it plays a critical role in the expression of CArG element-containing SMC differentiation marker genes. On the other hand, ELK1 is expressed widely and phosphorylated in response to platelet-derived growth factor-BB (PDGF-BB) and/or oxidized phospholipids, thus mediating the repression of SMC differentiation marker genes in cultured SMCs\textsuperscript{40-42}. MYOCD and ELK1 have a structurally related SRF-binding motif and thus compete for the common docking region of SRF\textsuperscript{40}. The balance between MYOCD and phosphorylated ELK1 is one of the determinants regulating the transcriptional activity of SMC differentiation marker genes.

The trans-acting factor for TCE is KLF4. Adam \textit{et al.}\textsuperscript{40} identified KLF4 as a binding factor for the TCE located within the Tagln promoter using a yeast
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SMC promoter-enhancers in order to form a multi-protein complex, thereby inhibiting MYOCD- and SRF-dependent gene transcription (Fig. 1). The repressive effects of KLF4 on SMC differentiation markers have been confirmed in tamoxifen-inducible Klf4-deficient mice in vivo. Although the expression of ACTA2 and TAGLN is transiently decreased at early time points after carotid injury in wild-type mice, this decrease is delayed in Klf4-deficient mice20. In addition, the association between KLF4 and the CArG/TCE-containing promoter region of the Acta2 gene and Tagln gene is also transiently detectable in injured carotid arteries in wild-type mice20. These findings provide evidence that KLF4 is a transcriptional repressor of SMC differentiation markers in vivo. It is of interest to determine whether the deletion of ELK1 or HDACs also results in a similar vascular phenotype in mice with vascular injury.

KLF4 also represses the expression of MYOCD, a very potent SRF co-factor of SMC differentiation marker gene transcription19, 47. Interleukin-1β (IL-

one-hybrid screen. The overexpression of KLF4 decreases the expression of SMC differentiation marker genes, including Acta2, Myh11 and Tagln, in cultured SMCs34, 35. Moreover, PDGF-BB and oxidized phospholipids, respectively, activate the KLF4 expression, while small-interfering (si)RNA-induced knockdown of Klf4 attenuates the PDGF-BB- and oxidized phospholipid-induced suppression of SMC differentiation marker genes in cultured SMCs19, 47. The underlying mechanisms clarified to date include: (1) direct KLF4 binding to the TCE20, 35; (2) an interaction between KLF4 and SRF19; (3) an interaction between KLF4 and phosphorylated ELK142; (4) reduced SRF binding to CArG elements19; and (5) the recruitment of histone deacetylases (HDACs), including HDAC5 and HDAC2, to the promoter region of SMC differentiation marker genes via the association with KLF442. In response to stimuli eliciting SMC phenotypic switching, the aforementioned transcription factors, including KLF4, phosphorylated ELK1, SRF and HDACs, are likely to gather onto SMC promoter-enhancers in order to form a multi-protein complex, thereby inhibiting MYOCD- and SRF-dependent gene transcription (Fig. 1). The repressive effects of KLF4 on SMC differentiation markers have been confirmed in tamoxifen-inducible Klf4-deficient mice in vivo. Although the expression of ACTA2 and TAGLN is transiently decreased at early time points after carotid injury in wild-type mice, this decrease is delayed in Klf4-deficient mice20. In addition, the association between KLF4 and the CArG/TCE-containing promoter region of the Acta2 gene and Tagln gene is also transiently detectable in injured carotid arteries in wild-type mice20. These findings provide evidence that KLF4 is a transcriptional repressor of SMC differentiation markers in vivo. It is of interest to determine whether the deletion of ELK1 or HDACs also results in a similar vascular phenotype in mice with vascular injury.

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Fig. 1. KLF4 suppresses the expression of SMC differentiation markers by interacting with ELK1, SRF and HDACs

A: MYOCD, SRF and multiple CArG elements positively regulate the transcription of SMC differentiation marker genes in SMCs. SRF binds to the CArG element as a dimer, while MYOCD is a non-DNA binding co-factor for SRF. The homodimerization of MYOCD potently activates SMC differentiation marker genes. B: The transcription of SMC differentiation marker genes is negatively controlled by vascular injury in vivo and/or treatment with PDGF-BB and oxidized phospholipids in cultured SMCs. In response to stimuli eliciting SMC phenotypic switching, KLF4 is induced and binds to the TCE within the promoter region of SMC differentiation marker genes. At the same time, ELK1 is phosphorylated and competes with MYOCD for SRF binding. KLF4 physically binds to phosphorylated ELK1, SRF and HDAC2/5 and reduces the binding of SRF with CArG elements.
IL-1β is able to repress the Myocd expression and concomitantly decrease the expression of SMC differentiation markers in cultured SMCs. The effects of IL-1β on the Myocd expression have been shown to be mediated in part by KLF4, because: (1) IL-1β induces the KLF4 expression; and (2) the repressive effects of IL-1β on SMC differentiation marker genes as well as Myocd are attenuated in Klf4-deficient cultured SMCs. The Myocd promoter contains a consensus KLF4 binding site, 5’-(G/A)(G/A)GG(C/T)G(C/T)-3’49), and a consensus nuclear factor (NF)-κB binding site. The mutation of each of these elements abolishes the IL-1β-induced repression of Myocd transcription in cultured SMCs. Moreover, KLF4 and p65 are enriched within the Myocd promoter in injured carotid arteries, as determined using chromatin immunoprecipitation assays48. Of interest, the results of co-immunoprecipitation assays have shown that KLF4 interacts with p65, although these assays were performed by inducing the overexpression of proteins in non-SMCs51. As such, KLF4 represses the Myocd expression in concert with p65 following vascular injury in vivo and/or after IL-1β treatment in cultured SMCs in vitro (Fig. 2). At present, it remains unknown whether phosphorylated ELK1 and HDACs are involved in the regulation of Myocd gene transcription. In addition, although PDGF-BB reduces the Myocd expression in cultured SMCs19), it is unclear whether this reduction is also mediated by KLF4 and p65. Further studies are needed to clarify these questions.

KLF4 Inhibits SMC Proliferation by Interacting with p53

Because KLF4 has been shown to be a repressor of SMC differentiation markers and cellular proliferation is believed to be inversely related to cellular differentiation, KLF4 was initially hypothesized to be an inducer of SMC proliferation. However, of major interest, the analysis of conditional Klf4 knockout mice has revealed KLF4 to be a repressor of SMC proliferation in vivo20). Indeed, as compared to control mice, injury-induced neointimal formation is more significantly accelerated in tamoxifen-inducible Klf4 knockout mice. The enhanced neointimal formation observed in Klf4-deficient mice is due to increased proliferation rather than an altered rate of apoptosis, and the lack of induction of p21WAF1/Cip1 (CDKN1A), a cell cycle inhibitor, in the medial layer of SMCs is involved in this phenotype20). The antiproliferative effects of KLF4 are likely to be mediated primarily by CDKN1A, because: (1) KLF4 induces the expression of CDKN1A in cultured SMCs52) as well as in other cell types, such as HEK293 cells and HCT116 colon cancer cells53, 54); (2) KLF4 does not suppress cellular proliferation in Cdkn1a-deficient SMCs20); and (3) the overexpression of CDKN1A inhibits neointimal formation following vascular injury55, 56), whereas Cdkn1a ablation enhances neointimal formation in the carotid injury model57. KLF4 induces the CDKN1A expression by cooperating with p53 (Fig. 3). Indeed, there are two consensus KLF4 binding sites and a p53 binding site in the promoter-enhancer region of the Cdkn1a gene20). A distal KLF4 binding site is located within close proximity to the p53 binding site at the enhancer region, whereas a proximal KLF4 binding site is positioned within the promoter. The mutation of each of these elements decreases the transcriptional activity of the Cdkn1a gene, thus suggesting that these cis-elements are functional. In addition, the overexpression of KLF4 increases the binding
of p53 as well as that of KLF4 itself to the Cdkn1a promoter-enhancer in cultured SMCs. Moreover, the association of KLF4 and p53 with the Cdkn1a promoter-enhancer is increased in the carotid arteries following vascular injury. As such, KLF4 induces the CDKN1A expression by binding to the proximal and distal KLF4 binding sites and recruiting p53 to the distal p53 binding site in the Cdkn1a promoter-enhancer following vascular injury (Fig. 3). Of interest, a physical interaction between the zinc finger region of KLF4 and the N-terminal domain of p53 has been demonstrated using co-immunoprecipitation assays. Moreover, the overexpression of p53 decreases neointimal formation following carotid injury, whereas the loss of p53 accelerates the formation of neointima in a vein bypass graft model, femoral injury model and Apoe-knockout mice. As such, KLF4 functions not only as a suppressor of SMC differentiation, but also as an inhibitor of SMC proliferation by cooperating with p53. It is very intriguing that a single transcription factor can behave as a repressor of both SMC growth and differentiation. However, there are some circumstances under which differentiation and proliferation occur simultaneously in SMCs, as discussed previously.

For example, during late embryogenesis and postnatal development, SMCs exhibit an extremely high rate of proliferation, yet, at the same time, they demonstrate the most rapid rate of induction of SMC differentiation marker expression in the rat and chicken aorta. Currently, animal studies investigating SMC proliferation in conditional Klf4 knockout mice are limited to a vascular injury model. It is of significant interest to determine whether KLF4 also regulates SMC proliferation in other vascular disease models, such as that involving atherosclerotic lesion formation in Apoe-knockout mice.

KLF4 Contributes to SMC Calcification in Concert with RUNX2

Arterial medial calcification is frequently seen in patients with chronic kidney disease and is associated with an increased risk of cardiovascular disease and mortality. Although such calcification was previously thought to be a passive or degenerative process involving calcium phosphate precipitation in the vessels, it is now recognized to be a cell-regulated process with many similarities to the mechanisms underlying embryonic osteogenesis. During the process of calcification in the arteries, vascular SMCs change their phenotype into osteoblast-like cells by decreasing the expression of SMC differentiation markers and increasing the expression of osteogenic markers, such as osteopontin (SPP1), osteocalcin, alkaline phosphatase and RUNX2, thus secreting multiple factors required for extracellular matrix mineralization. Hyperphosphatemia is a critical factor promoting the change in the phenotype of SMCs into osteoblast-like cells in patients with chronic kidney disease. Indeed, treatment with phosphate binders, sevelamer hydrochloride and/or lanthanum carbonate effectively attenuates the formation of arterial medial calcification by decreasing the serum phosphate concentration in rats with chronic renal failure. Sevelamer hydrochloride has also been shown to delay the progression of coronary and aortic calcification in hemodialysis patients.

KLF4 has been demonstrated to contribute to the phenomenon of high phosphate-induced phenotypic switching of SMCs into osteoblast-like cells. In cultured rat aortic SMCs, a high phosphate concentration increases the expression of osteopontin, alkaline phosphatase and RUNX2 and decreases the expression of ACTA2 and TAGLN. A high phosphate concentration also induces the KLF4 expression. Of interest, the siRNA-mediated knockdown of Klf4 attenuates the high phosphate-induced suppression of SMC differentiation markers, as well as the high phosphate-induced increase in the expression of osteogenic markers and calcium deposition. In addition, KLF4 is induced in the calcified aorta of adenine-induced chronic renal failure rats. These findings suggest that KLF4 plays an important role in the high phosphate-induced phenotypic switching of SMCs into osteogenic cells. Regarding the underlying mechanisms, a high phosphate concentration has been shown to increase the binding of KLF4 and RUNX2 to the promoter region of SMC differentiation marker genes, including Acta2 and Tagln, as determined using chromatin immunoprecipitation assays. Of note, the overexpression of RUNX2 is capable of decreasing the expression of SMC differentiation markers by inhibiting the association of MYOCd with the SRF-CaRg elements in C3H10T1/2 cells. In addition, RUNX2 has been shown to interact with KLF4, as assessed according to co-immunoprecipitation assays. As such, it is likely that KLF4 and RUNX2 cooperatively inhibit the MYOCd-SRF-CaRg element-mediated transcription of SMC differentiation marker genes in response to a high phosphate concentration in SMCs. On the other hand, it remains unclear how KLF4 regulates the expression of osteogenic genes under high phosphate conditions. Although multiple consensus KLF4 binding sites have been found in the human SPP1 and murine Spp1 promoter-enhancer, they are located very far from the
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Fig. 4. KLF4 mediates the high phosphate-induced repression of SMC differentiation markers by cooperating with RUNX2

A high phosphate concentration induces the expression of KLF4 and RUNX2 in SMCs. KLF4 binds with the TCE, SRF and RUNX2. RUNX2 inhibits the formation of the MYOCDSRF complex. KLF4 induces dissociation between the SRF and CArG elements. Consequently, these factors cooperatively decrease the transcription of SMC differentiation marker genes.

RUNX2 binding site. In addition, it remains undetermined whether other osteogenic genes also contain multiple KLF4 binding sites in their promoter-enhancers. Further studies are therefore needed to determine whether RUNX2 and KLF4 cooperatively regulate the transcription of osteogenic genes in the context of the three-dimensional chromatin structure.

As discussed above, KLF4 plays multiple roles, including the repression of SMC differentiation markers, inhibition of SMC proliferation and induction of SMC calcification, by interacting with a diverse range of proteins in SMCs in vivo. In particular, it is interesting that a single transcription factor, KLF4, simultaneously functions as a repressor of SMC growth and differentiation. To date, the involvement of KLF4 in the SMC-induced migration and secretion of extracellular matrix proteins has been suggested by the results of in vitro experiments using cultured SMCs. Approaches using in vivo animal models are required to confirm and extend these in vitro findings.

KLF4 in ECs

Vascular ECs have a number of important functions, including the regulation of blood coagulation, recruitment of inflammatory cells and integration and transduction of humoral and mechanical signals to surrounding tissues. Endothelial dysfunction critically contributes to the pathogenesis of vascular disease. KLF4 is constitutively expressed in ECs, and the results of several recent studies have shown that it is a key determinant of vascular inflammation and thrombosis. Indeed, EC-specific Klf4 deletion, which is accomplished via VE-cadherin (Cdh5) promoter-dependent Cre-recombination of the Klf4 floxed allele and the reconstitution of hematopoietic cells by bone marrow transplantation, results in an increased area of atherosclerotic lesions as well as enhanced infiltration of CD45-positive inflammatory cells in Apoe-background mice fed a high-fat diet. In contrast, the EC-specific overexpression of KLF4 decreases the rate of atherosclerotic lesion formation in these mice. In addition, EC-specific Klf4-deficient mice are predisposed to early thrombosis, as assessed using in vivo carotid injury assays and in vitro fibrin clot formation assays. These findings provide convincing evidence that KLF4 plays anti-inflammatory and antithrombotic roles in ECs. Furthermore, the Tie2 (Tek) promoter-dependent deletion of Klf4 in ECs and some hematopoietic cells in mice results in enhanced neointimal formation following vascular injury. Although the injury-induced downregulation of SMC differentiation markers is unaffected by Klf4 deletion, the Tek promoter-dependent knockout of Klf4 increases the rate of cellular proliferation and enhances the recruitment of inflammatory cells, such as macrophages and T-lymphocytes, to injured arteries. The enhanced recruitment of inflammatory cells is likely to be due to the augmented induction of cell adhesion molecules, such as VCAM1 and E-selectin, in injured arteries in Klf4 conditional knockout mice. Consistent with these in vivo findings, the results of multiple studies have demonstrated that the overexpression of KLF4 increases the expression of anti-inflammatory and antithrombotic factors, including endothelial nitric-oxide synthase and thrombomodulin, whereas the knockdown of KLF4 leads to the enhancement of the tumor necrosis factor-α (TNFα)-induced expression of VCAM1 and tissue factor in cultured ECs. The KLF4-induced expression of thrombomodulin...
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increased endothelial barrier permeability. Moreover, the siRNA-induced global knockdown of Klf4 in mice augments lipopolysaccharide-induced lung injury and pulmonary edema75). However, it is possible that the phenotype observed in siRNA-treated animal models is confounded by the effects of Klf4 siRNA on cell types other than ECs. Therefore, further studies using EC-specific Klf4 knockout animals are needed to determine whether endothelial KLF4 plays a role in preventing vascular leakage in response to inflammatory stimuli.

Most recently, endothelial KLF4 has been shown to modulate pulmonary arterial hypertension76). Indeed, Cdh5 promoter-dependent Klf4 knockout mice exhibit elevation of the right ventricular and pulmonary arterial pressures as well as right ventricular hypertrophy following hypoxia. EC-selective Klf4 knockout also results in the increased expression of endothelin-1 and decreased expression of endothelial nitric-oxide synthase, endothelin receptor subtype B and prostacyclin synthase in the lungs. Importantly, the KLF4 expression is reduced in the lungs of patients with pulmonary artery hypertension76). As such, KLF4 is likely to be a novel transcriptional modulator of pulmonary artery hypertension.

Taken together, accumulating evidence suggests that endothelial KLF4 is required for the maintenance of a normal endothelial function and protection against pro-inflammatory and prothrombotic stimuli in the setting of vascular diseases.

KLF4 in Monocytes/Macrophages

KLF4 is not expressed in cell lines such as HL-60 (myeloid leukemia cell), HeL (erythrocyte), Jurkat (T cell), Raji (immature B cell) and U-266 (mature B cell), although it is expressed in monocyte/macrophage cell lines, including THP-1 (monocytic leukemia) and U937 (histiocytic leukemia)21). It is also expressed in human peripheral blood monocytes and murine peritoneal macrophages22). The forced expression of KLF4 in primary common myeloid progenitors and/or hematopoietic stem cells induces monocyte differentiation in clonogenic assays, whereas Klf4 deficiency inhibits monocyte, but increases granulocyte, differentiation21, 22). These findings suggest that KLF4 is a critical regulator of monocyte differentiation. Moreover, the results of recent studies have provided evidence that KLF4 regulates macrophage polarization23). The expression of KLF4 is robustly increased in macrophages following treatment with interleukin-4, a stimulus inducing the expression of the macrophage M2 (anti-inflammatory) subtype.

Fig. 5. KLF4 attenuates the inflammation-related induction of the VCAM1 gene by inhibiting the binding of p65 to the VCAM1 promoter

Inflammatory signals, such as TNFα, induce the phosphorylation of p65, after which phosphorylated p65 binds to the VCAM1 promoter to activate transcription in ECs. However, KLF4 limits inflammation by inhibiting the binding of p65 to the VCAM1 promoter via the association between KLF4 and p65.

has been shown to be mediated in part via the association of KLF4 with its co-activator, p30073). In contrast, KLF4 represses the TNFα-induced expression of VCAM1 via the inhibition of p65 binding to the VCAM1 promoter due to the association between p65 and KLF4 (Fig. 5). In response to TNFα stimulation, p65, a component of NF-κB, is phosphorylated and subsequently translocated from the cytoplasm to the nucleus, where it engages DNA to initiate transcription of the VCAM1 gene. Mechanistic studies have revealed that KLF4 has no effect on the phosphorylation or translocation of p65, although it hampers the binding of p65 to the VCAM1 promoter, as determined using chromatin immunoprecipitation assays74). Because KLF4 is able to bind to p6553), the formation of the p65-KLF4 complex is likely to be a key event preventing the inflammatory process. However, the results of previous studies have failed to demonstrate that KLF4 interferes with p65 binding to the NF-κB binding site based on electrophoresis mobility shift assays18). Electrophoresis mobility shift assays do detect biochemical binding between DNA and proteins, although they do not necessarily reflect DNA-protein binding within intact chromatin. Most likely, factors other than double-stranded DNA, KLF4 and p65, such as the higher order chromatin structure, are required for KLF4 to inhibit p65 binding to DNA in ECs.

Endothelial KLF4 also has the potential to contribute to the maintenance of the endothelial barrier function, in that KLF4 is required for the VE-cadherin expression75). In cultured human lung microvascular ECs, the siRNA-induced depletion of KLF4 results in the decreased expression of VE-cadherin and increased endothelial barrier permeability. Moreover, the siRNA-induced global knockdown of Klf4 in mice augments lipopolysaccharide-induced lung injury and pulmonary edema75).
myeloid KLF4 is an essential regulator of vascular inflammation.

**Conclusion and Perspectives**

Multiple lines of evidence indicate that KLF4 plays various roles in the progression of vascular disease by associating with a variety of proteins in multiple cell types. KLF4 exerts multiple effects, including an antiproliferative effect, anti-inflammatory effect, antithrombotic effect and de-differentiation effect, by cooperating with various binding proteins, rather than exhibiting the simple presence or absence of a single transcription factor (Fig. 6). Although most of the functions of KLF4 are accomplished by binding to the consensus cis-regulatory elements located within the promoter-enhancer regions of target genes, it is interesting to note that the anti-inflammatory effects of KLF4 in ECs are independent from the KLF4 binding site. Nevertheless, KLF4 has its partner proteins to...
carry out its functions. Further studies are needed to determine whether a single KLF4 molecule simultaneously exerts multiple effects by forming a combinatorial multi-protein complex, such as KLF4, p53 and p65. In addition, some functions of KLF4 are redundant with those of other KLF family members, including KLF11\(^7\) and KLF15\(^7\). It is of significant importance to determine whether KLF4 interacts with these KLF family members to exert their overlapping functions.

Recently, a number of epigenetic studies have provided evidence that changes in the chromatin structure affect gene transcription. It is highly possible that alterations in epigenetic modifications, such as histone modification patterns and DNA methylation patterns, are involved in the KLF4-dependent regulation of target genes, as KLF4 has been shown to contribute to the reprogramming of multiple somatic cells into pluripotent stem cells\(^7,\,8\). In this regard, the relationships between KLF4 and epigenetic factors in vascular cells are poorly understood at present. Further studies are thus needed to determine whether KLF4 has the ability to change the chromatin structure in order to regulate gene transcription in the vessels.

Finally, it is hoped that further clarification of the molecular mechanisms underlying the functions of KLF4 will contribute to the advancement of therapeutic and preventive strategies for treating multiple vascular diseases.

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**Conflicts of Interest**

None.

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