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Review

Krüppel-like factors and vascular wall homeostasis

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Cardiovascular diseases (CVDs) are major causes of death worldwide. Identification of promising targets for prevention and treatment of CVDs is paramount in the cardiovascular field. Numerous transcription factors regulate cellular function through modulation of specific genes and thereby are involved in the physiological and pathophysiological processes of CVDs. Although Krüppel-like factors (KLFs) have a similar protein structure with a conserved zinc finger domain, they possess distinct tissue and cell distribution patterns as well as biological functions. In the vascular system, KLF activities are regulated at both transcriptional and posttranscriptional levels. Growing *in vitro, in vivo*, and genetic epidemiology studies suggest that specific KLFs play important roles in vascular wall biology, which further affect vascular diseases. KLFs regulate various functional aspects such as cell growth, differentiation, activation, and development through controlling a whole cluster of functionally related genes and modulating various signaling pathways in response to pathological conditions. Therapeutic targeting of selective KLF family members may be desirable to achieve distinct treatment effects in the context of various vascular diseases. Further elucidation of the association of KLFs with human CVDs, their underlying molecular mechanisms, and precise protein structure studies will be essential to define KLFs as promising targets for therapeutic interventions in CVDs.

Keywords: endothelial cells, vascular smooth muscle cells, inflammation, shear stress, atherosclerosis, vascular injury, drug development

Introduction

Cardiovascular disease (CVD) ranks the number 1 cause of death worldwide with an estimated 17.5 million people dying from CVD in 2012, according to the World Health Organization. The presence of one or more high risk factors, such as hypertension, diabetes, hyperlipidemia, and smoking, contributes to the pathophysiological process of CVDs. CVDs are complicated genetic and environmental factors-driven diseases. Identification of promising targets for prevention and treatment of CVDs is assiduously pursued in the cardiovascular field. Vascular wall cells, such as endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), are strictly regulated to maintain blood vessel structure and functions. Injury or inflammatory mediators destroy blood vessel homeostasis leading to vascular cell dysfunction and resulting in blood vessels narrowing (e.g. atherosclerosis, restenosis), blockage (thrombosis), regeneration (angiogenesis), stiffening (hypertension), or expansion and rupture (aneurysms). Recent studies suggest that the Krüppel-like factor (KLF) family plays a critical role in the maintenance of body homeostasis including cardiovascular, immune, digestive, respiratory, and hematopoietic systems.

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The first member of KLFs, KLF1 (EKLF), was identified in 1993 in erythroid cells, where KLF1 binds to the β-globin promoter (Miller and Bieker, 1993). To date, 18 KLFs (from KLF1 to KLF18) have been identified with distinct tissue distribution patterns and functions (McConnell and Yang, 2010; Pei and Grishin, 2013). KLFs have a conserved protein structure among human, mouse, and rat and amongst themselves. Three conserved Cys2His2-type zinc fingers in the carboxy-terminus of the KLF proteins bind to GC-rich sites in the promoter of target genes. Transactivation domain and transrepression domain are located at the amino-terminus of KLF proteins (Figure 1A) (McConnell and Yang, 2010). Although the similarity in DNA-binding ability leads to overlapping regulation of genes, KLF members have different biological functions and exhibit distinct phenotypes in various diseases, mostly resulting from their N-terminal sequences, which provide unique protein interaction motifs and posttranslational modification sites (McConnell and Yang, 2010). By regulation of gene expression, KLFs are involved in numerous biological processes (Figure 1B). Based on the published HPA RNA-seq from 95 human individuals representing 27 normal different tissues (Fagerberg et al., 2014), we performed additional analysis to identify the top KLF-expressing tissues, and summarized the findings in Table 1.

KLFs are critical regulators of vascular homeostasis, and some KLFs exhibit beneficial effects on prevention or inhibition

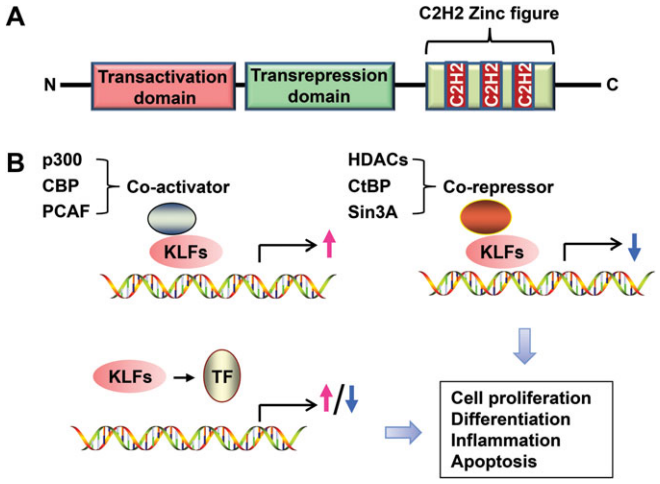

Figure 1 Schematic representation of KLF functional domains and gene regulation. (**A**) The transactivation and transrepression domains are located at the N-terminus of KLF proteins. Three consecutive zinc finger motifs are located at the C-terminus. (**B**) Diagram illustrating the regulatory patterns for KLFs in gene transcription. KLFs induce or repress gene expression in cooperation with co-activators or co-repressors or through interaction with other specific transcription factors. TF, transcription factor; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; HDACs, histone deacetylases; CtBP, C-terminal-binding protein; Sin3A, SIN3 transcription regulator family member A.

Table 1 Tissue distribution of KLFs.

Human KLFs	Top 3 expressing tissues (RPKM)	Heart (RPKM)
KLF1	Bone marrow, placenta, spleen	N/A
KLF2	Fat, ovary, bone marrow	4.09
KLF3	Colon, gall bladder, esophagus	4.69
KLF4	Colon, esophagus, skin	9.10
KLF5	Skin, esophagus, colon	0.79
KLF6	Bone marrow, gall bladder, esophagus	13.44
KLF7	Bone marrow, endometrium, fat	1.51
KLF8	Skin, fat, esophagus	0.54
KLF9	Fat, gall bladder, liver	16.83
KLF10	Bone marrow, gall bladder, lung	10.54
KLF11	Testis, lung, fat	6.47
KLF12	Lymph node, spleen, brain	2.17
KLF13	Bone marrow, lung, thyroid	11.78
KLF14	Testis, adrenal gland, fat	N/A
KLF15	Fat, ovary, kidney	2.13
KLF16	Brain, colon, spleen	0.73
KLF17	Testis, endometrium, esophagus	0.003

Summary of the KLF abundance as calculated here from the HPA RNA-seq analysis of normal tissue samples from 95 human individuals representing 27 different tissues (Fagerberg et al., 2014). The reads per kilobase per million mapped reads (RPKM) for KLFs in the top 3 KLF-expressing tissues and heart are listed in the table. N/A, not available.

of vascular diseases. Dysfunction of ECs and VSMCs induced by pathologic stimuli initiates or exacerbates various vascular diseases. To further underscore the significance of KLFs in these cells, we determined the mRNA abundance of KLFs in human coronary artery endothelial cells (HCAECs) and human aortic smooth muscle cells (HASMCs), introduced in Table 2. Herein,

Table 2 KLF abundance in HCAECs and HASMCs.

Human KLFs	(KLF/GAPDH) × 10^4 in HCAECs	(KLF/GAPDH) × 10^4 in HASMCs
KLF1	N/A	N/A
KLF2	93.46	25.14
KLF3	89.76	357.97
KLF4	0.79	184.43
KLF5	3.42	51.33
KLF6	549.30	424.61
KLF7	41.39	176.19
KLF8	0.49	2.75
KLF9	49.33	559.83
KLF10	167.61	176.32
KLF11	2.00	63.30
KLF12	30.91	226.97
KLF13	28.89	368.51
KLF14	0.11	N/A
KLF15	2.42	3.27
KLF16	95.04	58.03
KLF17	N/A	0.72

Cultured human coronary artery endothelial cells (HCAECs) and human aortic smooth muscle cells (HASMCs) were subjected to RNA-sequencing analysis when grown at 90% confluence. The fragments per kilobase of transcript per million mapped reads (FPKM) for KLFs were normalized with those of GAPDH. N/A, not available.

we summarize and discuss evidence underscoring the essential role of the KLF family in the regulation of vascular wall biology, specifically in ECs and VSCMs, and underlying mechanisms of KLF functions in vascular diseases and highlight the potential for KLFs as therapeutic targets in prevention and treatment of vascular diseases.

Roles of KLFs in ECs

Healthy endothelium is critical to maintain normal vascular homeostasis. Vascular ECs can respond to various physiological or pathophysiological signals by dynamic changes in a wide range of factors that regulate cellular adhesion, vascular inflammation, thromboresistance, vascular tone, and EC-dependent regulation of smooth muscle cell homeostasis. Prolonged and/or repeated exposure to CVD risk factors ultimately leads to EC dysfunction. KLFs, such as KLF2, KLF4, KLF6, and KLF11, exert distinct biological functions in ECs (Figure 2).

KLF2 and ECs

The last decades have witnessed growing research on KLF2 in relation to EC biology. KLF2 was first identified in 1995, using the zinc finger region of erythroid Krüppel-like factor (EKLF, KLF1) as the hybridization probe (Anderson et al., 1995), and first termed as lung Krüppel-like factor (LKLF), since it was found to be primarily expressed in lung (Anderson et al., 1995). Nonetheless, as early as E9.5, KLF2 is expressed in the mouse embryo vascular ECs. *Klf2*$^{-/-}$ mice die on E12.5 to E14.5 because of intra-embryonic hemorrhages (Kuo et al., 1997). Despite normal vascularization and angiogenesis, *Klf2*$^{-/-}$ mice embryos display impaired smooth muscle cell migration and blood vessel maturation (Wu et al., 2008). Mouse embryos with EC-specific loss of KLF2 die due to loss of normal vessel tone and lethal heart failure (Lee et al., 2006). Mice with KLF2 hemizygous deficiency (*Klf2*$^{+/-}$) on an

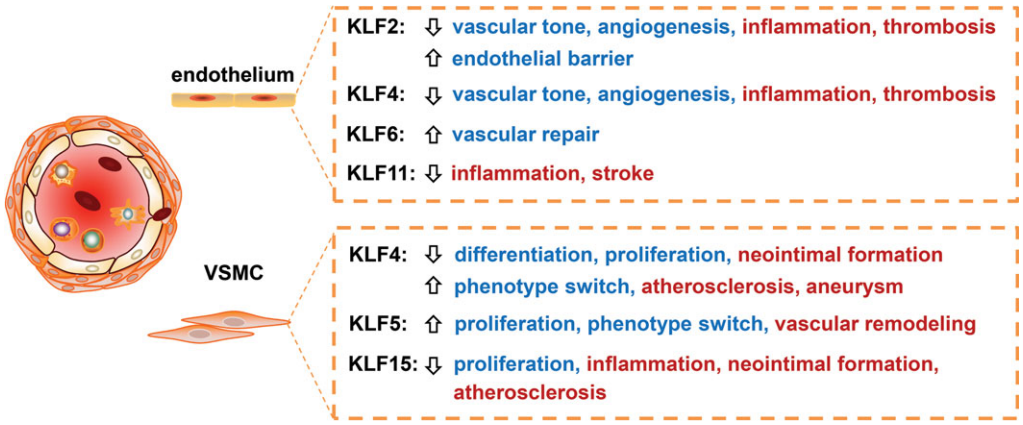

Figure 2 The roles of KLFs in vascular wall biology. KLFs regulate inflammation, proliferation and differentiation in ECs and VSMCs, and are further involved in various vascular diseases, underscoring an important role of KLFs in maintaining vascular homeostasis.

ApoE$^{-/-}$ background exhibit augmented atherosclerosis, attributed mostly to enhanced macrophage lipid uptake (Atkins et al., 2008).

KLF2 and inflammation. EC activation is an early stage in atherosclerosis, the major cause of heart attacks, strokes, and peripheral vascular disease (Davignon and Ganz, 2004). Microarray profiling shows that KLF2 overexpression confers anti-inflammatory and anti-thrombosis properties in ECs (Parmar et al., 2006). KLF2 induces endothelial nitric oxide synthase (eNOS) and simultaneously inhibits proinflammatory cytokine-induced adhesion molecules (E-selectin and VCAM1) through recruitment of co-activator p300/cyclic AMP response element-binding protein (CBP) to reduce NF-κB activity (SenBanerjee et al., 2004). Besides the NF-κB pathway, KLF2 could suppress constitutive proinflammatory transcription via inhibition of ATF2 phosphorylation (Fledderus et al., 2007) and Jun NH2-terminal kinase (JNK) (Boon et al., 2010), thus controlling the contribution of cytoskeletal rearrangements to EC inflammation. In addition, KLF2 inhibits inflammation through upregulation of anti-inflammatory genes in a laminar flow-dependent fashion. KLF2 mediates laminar flow-dependent upregulation of ENTPD1, which protects *ApoE*$^{-/-}$ mice from atherosclerosis (Kanthi et al., 2015). KLF2 upregulates PPAP2B, a laminar flow sensitive and anti-inflammatory gene in ECs that preserves integrity of the endothelial layer (Wu et al., 2015). The presence of antiphospholipid antibody is the marker for the antiphospholipid syndrome Lupus, characterized by high risk of thrombosis. Antiphospholipid antibody activates EC via inhibition of KLF2 and KLF4, with ensuing NF-κB activation (Allen et al., 2011).

KLF2 and vascular tone. EC is the key cell type controlling vascular tone *in vivo*. The KLF2–eNOS pathway mediates amelioration of vasospasm after subarachnoid hemorrhage (SAH) in rats treated with scutellarin, a flavonoid extracted from the traditional Chinese herb *Erigeron breviscapus* (Li et al., 2016a), providing an experimental basis for clinical use of scutellarin treatment in SAH patients. In the apelin-null mice, which develop more severe pulmonary artery hypertension (PAH), KLF2 and eNOS were decreased in the lung, implicating the KLF2–eNOS pathway in the pathological progression of PAH (Chandra et al., 2011).

KLF2 and angiogenesis. Endothelial survival, permeability, migration, and proliferation contribute to angiogenesis. KLF2 inhibits angiogenesis by multiple signaling pathways such as inhibition of transcription of VEGF receptor 2 (VEGFR2) (Bhattacharya et al., 2005) and hypoxia-inducible factor 1 (HIF-1) (Kawanami et al., 2009). KLF2 inhibits proliferation, migration, and tube formation in human liver sinusoidal ECs via suppression of ERK1/2 pathway (Zeng et al., 2015). Consistent with *in vitro* data, KLF2 heterozygous mice (*Klf2*$^{+/-}$) show increased microvessel density in the brain (Kawanami et al., 2009). Vascular ECs are highly glycolytic and have relatively low oxygen demand. During angiogenesis, EC changes from a quiescent to a metabolically active phenotype, relying on glycolysis as energy source (De Bock et al., 2013). KLF2 inhibits glycolysis by downregulation of key glycolytic enzymes such as 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase-3 (PFKFB3), phosphofructokinase-1, and hexokinase 2 (Doddaballapur et al., 2015), thus further inhibiting angiogenesis. However, in the zebrafish model, KLF2 is required for angiogenesis during aortic arch development (Meadows et al., 2009; Renz et al., 2015), indicating a distinct role of KLF2 in vascular development and postnatal regeneration.

KLF2 and thrombosis. The endothelium is an important barrier to maintain blood fluidity. In ECs, KLF2 inhibits pro-thrombotic factors such as plasminogen activator inhibitor 1 (PAI-1) and tissue factor (TF), and upregulates anti-thrombotic factor thrombomodulin (TM) under inflammatory conditions (Lin et al., 2005; Boon et al., 2007). Inhibition of the inhibitory kappa-B kinase-β (IKKβ) increases thrombomodulin (TM) expression via induction of KLF2 (Pathak et al., 2014). Patients with multiple myeloma, receiving proteasome inhibitors as a part of the chemotherapeutic regimen, appear to be at lower risk for thromboembolic events (Musallam et al., 2009). Mechanistically, proteasome inhibitors increase TM by inducing KLF2 and KLF4, independently of the NF-κB pathway (Hiroi et al., 2009; Nayak et al., 2014), making KLF2 and KLF4 potential targets for inhibition of thrombosis.

KLF2 and endothelial barrier. The pathological process in ischemic stroke shares many similarities with ischemic heart attack. KLF2 reduces infarction size by improving blood–brain barrier function in the focal cerebral ischemia mouse model (Shi et al., 2013). Indeed, KLF2 increases the expression of the key junction protein occludin and phosphorylation of myosin light chain, to preserve endothelial barrier and vascular integrity (Lin et al., 2010).

Other functions of KLF2 in ECs. Autophagy is of importance in maintaining cellular homeostasis. A positive loop between autophagy and KLF2 in ECs has been discovered, in which KLF2 improves microvascular function after acute liver injury induced by cold storage and warm reperfusion (Guixe-Muntet et al., 2017). In addition, KLF2 inhibits complement-mediated lysis (Kinderlerer et al., 2008) and suppresses oxidized LDL-induced apoptosis (Wang et al., 2006), indicating a protective role of KLF2 in ECs. A recent study reported that gain of MEKK3–KLF2/4 function causes cerebral cavernous malformations (CCM) in a neonatal mouse model of CCM disease (Zhou et al., 2016), suggesting that MEKK3–KLF2/4 signaling is a causal mechanism for CCM pathogenesis. These studies implicate a distinct role of KLF2 in vascular and cerebrovascular development.

KLF4 and ECs

KLF4 was first identified as gut-enriched Krüppel-like factor (GKLF) (Yet et al., 1998). KLF4 mRNA is abundant in mouse gastrointestinal and skin epithelial cells and induces growth arrest in numerous cell types (Shields et al., 1996). KLF4-deficient mice exhibit neonatal death due to loss of the skin barrier function (Segre et al., 1999). However, KLF4 is also expressed in the vascular wall, including ECs and VSMCs, and plays a critical role in vascular wall biology. Noteworthy, KLF4 is one of the 'Yamanaka factors' (along with Oct3/4, Sox2, and c-Myc) used to induce pluripotency in both mouse and human somatic cells (Takahashi and Yamanaka, 2006).

KLF4 and vascular protection. Similar to KLF2, KLF4 confers an anti-inflammatory and vasoprotective phenotype in ECs by inhibiting NF-κB activation (Hamik et al., 2007), and inducing eNOS expression (Shen et al., 2009; Mun and Boo, 2012). EC-specific *Klf4*$^{-/-}$ mice exhibit increased atherosclerosis and thrombosis (Yoshida et al., 2014), aggravated LPS-induced lung injury and pulmonary edema through impaired endothelial barrier (Cowan et al., 2010), and deleterious PAH from increased endothelin-1 and decreased eNOS (Shatat et al., 2014).

KLF4 and angiogenesis. KLF4 inhibits angiogenesis and endothelial proliferation via increasing miR-15a in both ECs and VSMCs (Zheng et al., 2013). However, sustained expression of KLF4 promotes ineffective angiogenesis leading to impaired tumor growth by activating the Notch signaling pathway (Hale et al., 2014). In retinal microvascular ECs, KLF4 promotes angiogenesis via activation of VEGF signaling (Wang et al., 2015). Thus, KLF4 may regulate angiogenesis in a cell type- and expression level-dependent manner.

KLF4 and thrombosis. KLF4 has protective effects on thrombosis through transcriptional regulation of pro- and anti-thrombotic genes. Overexpression of KLF4 increases TM and eNOS expression but suppresses PAI-1 and TF expression under inflammatory conditions (Zhou et al., 2012; Stavrou et al., 2015). Mechanistically, KLF4 works synergistically with p300 to activate the TM promoter and, thereby, increasing transcription (Zhou et al., 2012). In addition to KLF2 and KLF4, whether other KLFs are involved in thrombosis remains to be investigated.

KLF6 and ECs

KLF6 transactivates the expression of a considerable number of genes involved in the TGFβ pathway, e.g. endoglin, collagen 1, urokinase-type plasminogen activator, TGFβ receptor type 1, and MMP14 (Botella et al., 2002; Gallardo-Vara et al., 2016), and colocalizes with endoglin in the vascular endothelium following carotid balloon injury in rats (Botella et al., 2002). Interestingly, KLF6 interacts with specificity protein 1 (Sp1) to cooperatively transactivate common target genes. Vascular injury triggers KLF6 nuclear translocation and cooperation with Sp1 to upregulate activin receptor-like kinase 1 (ALK1) and consequently induce endothelial activation (Garrido-Martín et al., 2013). TGFβ, in turn, enhances the interaction between KLF6 and Sp1 by inhibiting KLF6 RNA alternative splicing that functionally antagonizes full-length KLF6 (Botella et al., 2009), leading to an increase in growth-inhibitory KLF6 activity. In addition, disruption of Sp2/KLF6 repression complex is required for farnesoid X receptor to increase EC migration (Das et al., 2006).

KLF11 and ECs

KLF11 was cloned as a Sp1-like transcription factor and is involved in cell growth and differentiation (Cook et al., 1998). In population studies, KLF11 mutation causes maturity-onset diabetes of the young type 7 (MODY7) (Neve et al., 2005). Indeed, recent studies by others and us provide evidence on the critical role of KLF11 in EC homeostasis. Proinflammatory stimuli, like TNFα and LPS, increase KLF11 expression in vascular ECs (Fan et al., 2012). KLF11 potently inhibits inflammation by suppressing the NF-κB pathway (Fan et al., 2012) and downregulating endothelin-1 in ECs (Glineur et al., 2013). *Klf11*$^{-/-}$ mice display exacerbated endothelial inflammation represented by increased leukocyte–endothelial recruitment and upregulated proinflammatory adhesion molecules (Fan et al., 2012). We also found that KLF11 deficiency aggravates ischemic stroke in a mouse middle cerebral artery occlusion model. KLF11 facilitates PPARγ-mediated inhibition of pro-apoptotic miR-15a in cerebral vascular ECs (Yin et al., 2013). In addition, KLF11 antagonizes caveolin-1 transcription induced by Sp1/sterol-responsive element-binding protein (SREBP) during cholesterol depletion in ECs, suggesting a role of KLF11 in cholesterol metabolism in ECs (Cao et al., 2005).

Roles of KLFs in VSMCs

VSMC is the major cell type in the vascular wall, controlling vascular tone and maintaining vascular wall homeostasis. It displays plasticity, switching from a quiescent, contractile

phenotype to a secretory, proliferative phenotype, during vascular inflammation or injury. KLFs appear to regulate phenotypic switch, proliferation, migration, apoptosis, and inflammation in VSMCs. Accordingly, studies indicate that KLFs in VSMCs are involved in vascular diseases such as restenosis, atherosclerosis, and aneurysm (Figure 2).

KLF4 and VSMCs

KLF4 and VSMC phenotypic switch. KLF4 deficiency in smooth and cardiac muscles in mice results in postnatal death and growth restriction, underscoring its importance in cardiovascular development (Yoshida et al., 2010). KLF4 is upregulated by platelet-derived growth factor BB (PDGF-BB) via transcription factor Sp1, a potent inhibitor of VSMC differentiation (Deaton et al., 2009). In turn, KLF4 overexpression inhibits the expression of VSMC contractile markers, including myocardin (Turner et al., 2013), and mediates the elongation of long-chain fatty acid family member 6 (Elovl6)-induced VSMC phenotypic switch (Sunaga et al., 2016). Therefore, KLF4 is a critical driver of VSMC phenotypic switch from a contractile to a secretory phenotype. In contrast, TGF-β, a positive regulator of VSMC differentiation from secretory to contractile phenotype, reduces KLF4 expression through miR-143/145 in VSMCs (Davis-Dusenbery et al., 2011). Furthermore, bone morphogenetic proteins (BMP) 2, 4, 6 and TGF-β share KLF4 as a common downstream molecule in maintaining the VSMC contractile phenotype (King et al., 2003). All-trans retinoic acid (ATRA) induces VSMC differentiation and inhibits proliferation via downregulation of KLF4 (Wang et al., 2008; Yu et al., 2011). Mechanistically, KLF4 inhibits myocardin, a co-activator of serum response factor (SRF) essential to maintain VSMC differentiation and contractile phenotype (Owens et al., 2004; Turner et al., 2013).

Additional mechanisms could mediate KLF4 regulation of VSMC phenotypic switch. KLF4 cooperates with ELK-1 (a co-repressor of SRF) and HDAC2 to suppress VSMC differentiation markers *in vitro* and *in vivo* (Salmon et al., 2012). Some evidence indicates that KLF4 promotes VSMC differentiation by Smad and p38 MAPK pathway (Li et al., 2010). Additionally, high phosphate induces VSMC switch to an osteogenic phenotype via upregulation of KLF4 (Yoshida et al., 2012), suggesting a positive and complex effect of KLF4 on VSMC dedifferentiation and osteogenic differentiation.

KLF4 and vascular injury. KLF4 is barely expressed in normal, contractile VSMCs *in vivo*, but is induced upon vascular injury (Liu et al., 2005). KLF4 inhibits VSMC proliferation and neointimal formation (Zheng et al., 2009; Wang et al., 2012b). Conditional knockout of *Klf4* delays VSMC dedifferentiation, but increases VSMC proliferation by removing KLF4-dependent upregulation of p21, resulting in exacerbated neointimal formation after vascular injury in mice (Yoshida et al., 2008). A recent study demonstrated that SMC-specific KLF4 deficiency reduces the numbers of SMC-derived adventitial progenitors, which have the potential to differentiate into multiple lineages, including mature SMCs, resident macrophages, and endothelial-like cells, potentially contributing to intimal lesions *in vivo* (Majesky et al., 2017).

KLF4 and atherosclerosis. Many studies have shown that VSMC plasticity contributes to the development of atherosclerosis (Gomez and Owens, 2012). SMC-specific knockout of Klf4 results in reduced numbers of SMC-derived macrophage and mesenchymal stem cell-like cells, a marked reduction in atherosclerosis and an increase in plaque stability, compared to wild-type controls, reinforcing the contribution of SMCs to atherosclerotic plaques (Shankman et al., 2015).

KLF4 and aneurysm. At the histological level, inflammation, VSMC apoptosis, extracellular matrix degradation, and oxidative stress have been recognized as visible hallmarks of aortic aneurysm pathogenesis (Kuivaniemi et al., 2015). KLF4 expression is progressively increased in the vessel wall after aortic elastase perfusion in a mouse model of aneurysm. Conditional KLF4 deletion attenuates abdominal aortic aneurysm in both elastase perfusion and angiotensin II infusion-induced mouse models of aneurysm (Salmon et al., 2013). KLF4 knockdown also attenuates the TNFα-induced phenotypic switch from contractile phenotype to secretory phenotype in cerebral SMCs (Ali et al., 2013). These findings underscore the importance of KLF4 in the mechanisms behind intracranial and aortic aneurysms.

KLF5 and VSMCs

Like KLF4, KLF5 is also induced in VSMCs after vascular injury (Watanabe et al., 1999). However, unlike KLF4, KLF5 promotes VSMC proliferation and aggravates neointimal formation after carotid balloon injury in rat (Suzuki et al., 2009; Shi et al., 2012). KLF5 heterozygous deficient mice ($KLF5^{+/-}$) exhibited reduced vascular injury response, vascular remodeling, angiogenesis, and cardiac hypertrophy and fibrosis (Shindo et al., 2002). Angiotensin II increases KLF5 in a PKC, p38 MAPK, and NADH/NADPH oxidase-dependent pathway (Gao et al., 2006). In turn, KLF5 mediates the pro-proliferative effect of angiotensin II (AngII) via interaction with c-Jun in VSMCs (Liu et al., 2010), thus creating a positive feedback loop to regulate VSMC response to AngII. KLF5 increases proliferation and decreases apoptosis in pulmonary artery SMCs, of relevance to development of PAH (Courboulin et al., 2011). Indeed, periostin-positive cells (both VSMC and fibroblast)-specific KLF5 deficiency attenuates vascular remodeling in deoxycorticosterone-acetate (DOCA) salt-induced mouse hypertension model (Zempo et al., 2016). Short-hairpin RNA (shRNA)-mediated KLF5 knockdown attenuates pulmonary artery remodeling in a rat PAH model (Li et al., 2016b). Beyond those roles, clinical evidence demonstrates that KLF5 is highly expressed in large and giant unruptured cerebral aneurysms in human samples, although the specific role of KLF5 and the mechanism underlying its potential role in cerebral aneurysms remain unclear (Nakajima et al., 2012).

KLF15 and VSMCs

KLF15 is expressed in quiescent VSMCs and decreased upon PDGF-BB *in vitro* or after vascular injury *in vivo*. $Klf15^{-/-}$ mice exhibited increased neointimal formation after vascular injury

and *Klf15* deficiency enhanced VSMC proliferation and migration (Lu et al., 2010). Furthermore, KLF15 is downregulated in human atherosclerotic lesion and SMC-specific KLF15 deficiency aggravates atherosclerosis development in *ApoE*$^{-/-}$ mice through increased proinflammatory activation of VSMCs (Lu et al., 2013), suggesting that KLF15 has a protective effect on vascular inflammatory diseases.

The regulation of KLFs in the vascular system

The transcriptional and posttranscriptional regulation of KLFs is a critical way for cells to adapt to changes under both physiological and pathophysiological conditions (McConnell and Yang, 2010). Further understanding of KLFs regulation will provide new avenues to modulate their activity according to their roles in the context of cellular process. Here, we briefly discuss key emerging aspects of the regulation of KLFs at both transcriptional and posttranscriptional levels in the vascular system.

DNA methylation is the most stable epigenetic hallmark that confers persistent changes in gene expression and plays a key role in maintaining endothelial cell homeostasis and participates in vascular disease development. Methylation in the KLFs promoters changes the transcription level of KLFs. Disturbed oscillatory blood flow upregulates expression of DNA methyltransferases (DNMTs) both *in vitro* and *in vivo*, which alters genome-wide DNA methylation and global gene expression (Dunn et al., 2015). Upon disturbed hemodynamics, CpG islands within the KLF4 promoter are hypermethylated in a DNMT-dependent fashion in ECs (Dunn et al., 2015), which significantly represses KLF4 transcription, while DNMT inhibitors and knockdown of DNMT3A rescue such epigenetic silencing (Jiang et al., 2014). Low-density lipoprotein (LDL) cholesterol induces endothelial dysfunction and is a major risk factor for coronary heart disease. Pharmacological inhibition or genetic inactivation of DNMTs prevents LDL-induced downregulation of KLF2 in ECs (Kumar et al., 2013). Altogether, these data suggest that DNA methylation may directly affect KLF expression in ECs in a context-dependent manner.

Acetylation is important for KLF4-mediated transactivation. ATRA increases KLF4 acetylation by inducing HDAC2 phosphorylation and further promotes VSMC secretory phenotype (Meng et al., 2009). Endogenous KLF4 is acetylated by p300/CBP and mutagenesis of the acetylated lysines in KLF4 protein results in a decreased ability of KLF4 to activate target genes (Evans et al., 2007). The deacetylase HDAC1 can negatively regulate KLF5 activity through direct interaction with the first zinc finger of KLF5, inhibiting the binding of p300 to the same region of KLF5 (Matsumura et al., 2005). Additionally, SUMOylation can alter protein activity and stability. In the presence of PDGF-BB, KLF4 SUMOylation leads to release of the p300 co-activator required for p21 expression, recruitment of co-suppressors, and downregulation of p21 expression with the ensuing increase of VSMC proliferation (Nie et al., 2016).

Interaction with an extensive network of co-regulators modulates the transcriptional activities of KLFs, which cannot be covered in depth here. Briefly, besides CBP and p300, some KLFs (e.g. KLF3, 8, and 12) bind to transcriptional regulator C-terminal-binding protein 1 (CtBP) through a consensus-binding element in their N-terminal regions to recruit histone deacetylases and histone methyltransferases to transcriptional complexes, thus affecting chromatin remodeling of the target genes (Turner and Crossley, 1998). Furthermore, KLF9, 10, 11, 13, 14, and 16 recruit the transcriptional repressor Sin3A, which binds to HDAC1 and HDAC2 to modify chromatin conformation (McConnell and Yang, 2010). Heterochromatin protein 1 (HP1) interacts with KLF11 to compact chromatin and silence gene expression (Lomberk et al., 2012). Additionally, KLFs can interact with nuclear receptors to further modulate their functions. Upon agonist stimulation of PPARδ, KLF5 is deSUMOylated and binds to transcriptional activation complexes containing both ligand-bound PPARδ and CBP to cooperatively regulate transcriptional pathways of lipid metabolism in C2C12 cells, an immortalized mouse myoblast cell line (Oishi et al., 2008). Through a genome-wide and high-throughput co-activation screening, KLF11 was demonstrated to interact with PPARγ to attenuate mouse cerebral vascular EC dysfunction (Yin et al., 2013).

Hemodynamic flow and KLFs

Hemodynamic flow-induced mechanotransduction regulates vascular cell homeostasis, including inflammation, proliferation, survival, metabolism, and cytoskeletal reorganization. Steady laminar flow (atheroprotective) maintains EC homeostasis, while disturbed flow (athero-prone) induces an activated and proinflammatory phenotype in ECs (Chiu and Chien, 2011). KLF2 is well-recognized as upregulated by laminar shear stress (LSS), exhibiting anti-inflammatory, anti-proliferative, and anti-thrombotic effects in vascular ECs (Dekker et al., 2002). Transcriptome analysis reveals that KLF2 and Nrf2 upregulation accounts for most of the LSS-induced gene expression (Fledderus et al., 2007, 2008). Besides driving epigenetic control of KLF2 transcription, LSS further increases KLF2 mRNA abundance though multiple signaling pathways, including enhanced MEK5/ERK5/myocyte enhancing factor-2A (MEF2) activity (Parmar et al., 2006). Additionally, LSS stimulates HDAC5 phosphorylation and nuclear translocation, further enhancing MEF2 transcriptional activity, and hence inducing the expression of KLF2 (Wang et al., 2010; Kwon et al., 2014). Under LSS, several transcription factors, including heterogeneous nuclear ribonucleoprotein D (hnRNP-D) and nucleolin, have been identified to induce histone H3 and H4 acetylation-related chromatin remodeling in the KLF2 promoter in a PI3K-dependent manner (Huddleson et al., 2005, 2006). TGFβ/activin receptor-like kinase 5 (Alk5) signaling (Walshe et al., 2013) and AMP-activated protein kinase (Young et al., 2009) are required for the LSS induction of KLF2 in ECs. Beside this complex transcriptional regulation, prolonged LSS stabilizes KLF2 mRNA, also in a PI3K-dependent fashion (van Thienen et al., 2006). Conversely, disturbed oscillatory shear stress (OSS) decreases KLF2 expression through multiple pathways. OSS inhibits KLF2 transcription through MEF2 deacetylation mediated by class I HDAC (HDAC3/5/7) (Lee et al., 2012). Non-receptor tyrosine kinase Src (Wang et al., 2006) and thioredoxin-interacting protein (TXNIP) (Wang et al., 2012a) also mediate the inhibitory effect of OSS on KLF2 expression. Furthermore, KLF2 mRNA is directly downregulated by miR-92a, which, in

turn, is decreased by atheroprotective laminar flow in ECs (Wu et al., 2011; Fang and Davies, 2012). The pathways that modulate KLF2, KLF4, and KLF11 activity and expression levels in ECs are summarized in Figure 3. Unlike KLF2, which is suppressed by proinflammatory cytokines such as TNFα and IL1β in ECs (Cunningham and Gotlieb, 2004; SenBanerjee et al., 2004), basal expression of KLF4 is low, but KLF4 can be induced by TNFα, IL-1β, and interferon γ (Hamik et al., 2007). Therefore, KLF2 may be constitutive in basal conditions, while KLF4 is adaptive in ECs under different stimuli. KLF4 is upregulated by LSS in human ECs (McCormick et al., 2001; Methe et al., 2007), whereas OSS induces DNA methylation of the KLF4 promoter, resulting in decreased KLF4 transcription (Jiang et al., 2014). Like KLF2, KLF4 is also downregulated by miR-92a (Fang and Davies, 2012). In addition, KLF4 protein stability is increased by PPARγ via activation of Akt signaling and reduction of KLF4 ubiquitination (Sun et al., 2014). The basal expression level of KLF11 in human coronary artery ECs (HCAECs) is as low as that of KLF4, but RNA-sequencing analysis shows that KLF11 is upregulated by LSS (Qiao et al., 2016) and increased by TNFα in a dose-dependent manner in ECs (Fan et al., 2012). As a whole, these findings indicate that KLFs are key downstream effectors of mechanotransduction in vascular cells. Further understanding of the specific roles of individual KLFs and their complex network of interactions will likely accelerate finding interventions to address the multifaceted and deleterious effects of hemodynamic flow changes leading to CVDs.

Clinical perspective: modulation of KLFs activity in CVDs?

Recent advances in the understanding of KLFs biology and the increasingly recognized importance of KLFs to CVD, cancer, and digestive disease have stimulated researchers' fervor towards development of drugs targeting KLFs, in an increased effort to modulate KLF activity (upregulation or downregulation) on the basis of their roles in the context of specific diseases (Bialkowska et al., 2009; Guo et al., 2015; Khedkar et al., 2015; Ruiz de Sabando et al., 2016). KLFs regulate various cellular functions such as growth and differentiation, activation, and development, and may control a whole cluster of functionally related genes in response to physiological and pathological conditions. Therefore, therapeutic targeting of a selective KLF might achieve desirable biological effects in specific diseases. Unfortunately, there is high sequence and structural conservation in the KLF family and possible functional redundancy among some KLFs. Furthermore, unlike nuclear receptors, KLFs lack clear molecule 'pockets', thus thwarting efforts to find small therapeutic molecules. However, alternative strategies have been employed to achieve this goal.

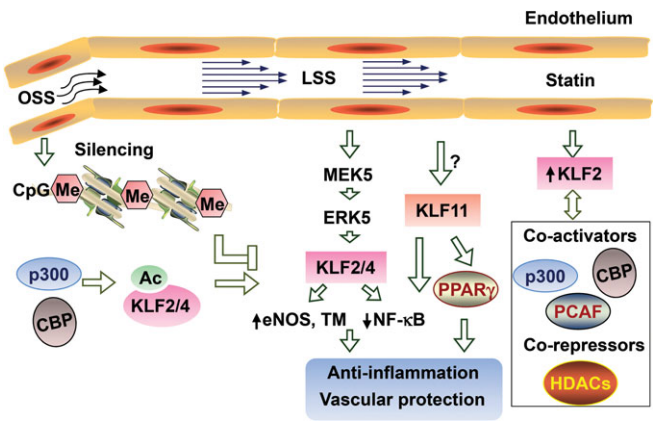

Figure 3 The regulation of KLFs in ECs. In ECs, laminar shear stress (LSS) upregulates, while oscillatory shear stress (OSS) downregulates KLF2 and KLF4. Epigenetic regulation (DNA methylation or histone acetylation) and MEK5–ERK5 signaling mediate the shear stress-dependent regulation of KLF2 and KLF4. KLF2, KLF4, and KLF11 potently inhibit inflammation through suppression of the NF-κB pathway. KLF11 facilitates PPARγ protective effect on ECs, while KLF2 dynamically interacts with co-activators or co-repressors to modulate cell function. Statins upregulate KLF2 in ECs.

Drugs that regulate KLF expression

Some drugs or chemical compounds have been identified to regulate KLF expression. Statins, the most widely used drugs for lipid-lowering and prevention of CVDs, effectively reduce clinical cardiovascular events in a variety of patients, not only in those with established CVD but also those who are at risk for CVD (Heart Protection Study Collaborative, 2002). Several studies demonstrated that statins increase KLF2 transcription in ECs (Parmar et al., 2005; Sen-Banerjee et al., 2005), while siRNA-mediated KLF2 knockdown abolishes the regulatory effect of statins on eNOS, TM, and prostaglandin D2 synthase (Parmar et al., 2005; Sen-Banerjee et al., 2005), indicating a critical role of KLF2 in mediating the atheroprotective role of statins in ECs. Resveratrol, an NAD-dependent deacetylase sirtuin-1 (SIRT1) activator, induces the expression of KLF2 and KLF2-dependent atheroprotective genes in ECs (Gracia-Sancho et al., 2010). Furthermore, KLF4 is upregulated by simvastatin and resveratrol in ECs (Villarreal et al., 2010), and increased by rapamycin in VSMCs (Wang et al., 2012b).

A third-generation small molecule compound, ML264, derived from ultrahigh-throughput screenings, potently inhibited KLF5 expression in colorectal cancer models (Ruiz de Sabando et al., 2016) but remains to be tested in the context of CVD. Recently, through a high-throughput compound screening based on KLF14 promoter-reporter assays, our lab demonstrated that perhexiline, an FDA-approved drug for chronic heart failure and refractory angina, transcriptionally upregulated KLF14 expression and further increased ApoA1 level. Perhexiline treatment, consequently and significantly, inhibits atherosclerosis in *ApoE*$^{-/-}$ mice (Guo et al., 2015).

Small molecule compounds that disrupt DNA-binding activity of KLFs

A large, 'shallow' pocket was identified within the middle zinc finger region of KLF10 leading to identification of small molecule compounds that bind in this pocket and inhibit the KLF10–DNA interaction interface, using computer-aided drug design screening of chemical libraries (Khedkar et al., 2015). This study provides optimism regarding the feasibility to identify small molecules that directly target KLFs. However, due to the conserved protein

structure of the zinc finger region among KLF members, the selectivity of these compounds remains unclear.

Nucleic acid-based gene regulation of KLFs

Because of its specificity and efficacy, RNA interference (RNAi)-mediated gene silencing using siRNA or short hairpin RNA (shRNA) is a particularly promising approach to decrease expression of target genes, especially genes whose products are not considered to be practical drug targets (e.g. transcription factors). Liposome–siRNA complexes or polymer-based RNA complexes have been often used in preclinical studies for cardiovascular disorders. MicroRNAs (miRNAs) are critical to the regulation of vascular function through posttranscriptional modification or translational repression of target genes. It has been demonstrated that miR-143/145 targets KLF4, leading to decreased expression of KLF4 in vascular smooth muscle cells (Cordes et al., 2009; Davis-Dusenbery et al., 2011), while miR-92a decreases the expression of KLF2 and KLF4 in ECs (Wu et al., 2011; Fang and Davies, 2012). miRNA mimics and antagonists such as antisense oligonucleotides, anti-miRNA oligonucleotides, and aptamers targeting specific KLFs may provide specific tools for treatment of CVD (Deshpande et al., 2016). The rapidly developing gene editing technologies, such as transcription activator-like effector nucleases (TALEN), Zinc Finger Nuclease (ZNF), and CRISPR/Cas9, make it possible to regulate gene expression and could be eventually applied to treat human diseases (Hsu et al., 2014).

Conclusions and future perspectives

Although the KLF family shares a similar protein structure with conserved zinc finger domain, members have distinct tissue distribution patterns and depict different biological functions in various CVDs. The differential expression of KLF members is dynamically changed in response to specific pathophysiological processes through transcriptional regulation and posttranscriptional modifications and dynamic interactions with co-regulators.

Essential roles of KLFs in vascular biology have been extensively demonstrated, especially in the vascular wall cells: ECs and VSMCs. Nonetheless, key aspects of their involvement and mechanisms of action still remain obscure. The plethora of findings on relative alterations in KLF gene expression in CVDs opens the question on whether there will be a compensatory effects among KLFs when enhancing or decreasing a given KLF member by specific targeting. It also remains to be investigated whether there is a cooperative or independent effect among KLFs in the same cell/tissue and disease manifestation. For instance, vascular integrity is broken and gene expression of VEGF receptor 2, eNOS, and occludin is reduced in E9.5 *Klf2*$^{-/-}$/*Klf4*$^{-/-}$ double knockout (EC-DKO) compared to *Klf2*$^{-/-}$ embryos (Chiplunkar et al., 2013). Moreover, a recent study, using inducible endothelial-specific deletion of *Klf2* and/or *Klf4* mouse models, revealed that *Klf2* and *Klf4* EC-DKO leads to acute death from myocardial infarction, heart failure, and stroke. EC-DKO mice also exhibit impaired vascular integrity and coagulation (Sangwung et al., 2017). Collectively, these studies establish a requirement for both KLF2 and KLF4 for maintenance of vascular integrity in both embryo development and the adult animal. However, whether KLF2 and KLF4 regulate EC functions synergistically through common mechanisms or individually through distinct mechanisms, with the ensuing implications for therapeutic targeting, is a question for future studies.

The newly found genetic associations of KLFs with CVD are a new and promising area of research. Recent genetic studies have revealed the associations of KLFs with human CVDs. Missense mutations in the KLF10 gene were identified to be positively associated with hypertrophic cardiomyopathy (Bos et al., 2012). In addition, a single nucleotide polymorphism located at −1282 bp within the KLF5 locus is associated with an increased risk of hypertension (Oishi et al., 2010). Recently, a novel missense mutation (p.H288Y) located in the zinc finger domain of KLF2, which is a recurrent somatic mutation in B-cell lymphoma, was found to likely disrupt gene function and lead to heritable pulmonary arterial hypertension (HPAH) (Eichstaedt et al., 2017). Considering the multiple homeostatic roles of this extensive family, it is likely that, as further targeted genetic studies are conducted, novel variants in KLF genes or their regulatory regions that affect KLF expression/activity in the cardiovascular system may be linked to CVD development. In spite of its essential involvement in CVD, a genetic association between KLF4 with human CVDs is yet to be found. Conversely, human genetic studies revealed an association between KLF11 and diabetes (Neve et al., 2005), but it is still unknown whether KLF11 plays an important role in diabetes-associated CVD. The answers to these important questions will facilitate our understanding of the roles of KLFs in human CVDs and inform new therapeutic strategies.

Strategies to utilize chemical compounds and nucleotides as therapeutic agents require efficient delivery systems. Since the KLFs have distinct and complicated functions in different tissues and are involved in various diseases, cell or tissue-specific delivery of therapeutic agents and optimized dosage are a prerequisite to avoid side effects. Drug delivery systems (DDS) are always critical to enhance the efficacy and safety of therapeutic agents and overcome their limitations, such as poor organ specificity, toxicity, low water solubility, and low bioavailability. Nanoparticle-mediated DDS (nano-DDS) modify the *in vivo* kinetics of therapeutic agents and are superior in that drug targeting can leverage physiologic and pathophysiological properties specific to certain disease conditions. Two polymers, polylactide (PLA) and poly (lactide-co-glycolide) (PLGA), are been extensively used for the synthesis of polymeric biodegradable nano-DDS (Matoba and Egashira, 2014). For ECs, specific cell surface molecular determinants, such as membrane receptors and adhesion molecules, can be targets for the delivery of a variety of agents-loaded pharmaceutical carriers (Koren and Torchilin, 2011). Therapeutic agents targeting specific KLF combined with optimal advanced nano-DDS may represent a promising approach to treat or prevent vascular diseases.

Although the high similarity of protein structure among the large number of KLF members, along with the scarcity of drug targetable pockets largely hindering the development of KLFs as therapeutic agents, the critical functions of KLFs have provided

new insights for novel pharmacological perspectives. Further elucidation of the association of KLFs with human diseases, the KLF biological functions, molecular mechanisms and structure analysis, and drug development will be essential for the ascension of KLFs to the clinical arena in cardiovascular medicine.

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