Biochemical and Biophysical Research Communications 391 (2010) 984–989

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Defining the regulation of KLF4 expression and its downstream transcriptional targets in vascular endothelial cells

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ARTICLE INFO

Article history:
Received 2 December 2009
Available online 5 December 2009

Keywords:
Endothelial cells
KLF4
MEK5
KLF2
Shear stress
Resveratrol

ABSTRACT

The Kruppel-like factor 2 (KLF2) and Kruppel-like factor 4 (KLF4) transcription factors have recently been shown to act as critical regulators of endothelial homeostasis. While several insights have been made into the signaling mechanisms orchestrating endothelial KLF2 expression, those governing the expression of KLF4 in the vascular endothelium remain largely unknown. Here, we show that diverse vasoprotective stimuli including an atheroprotective shear stress waveform, simvastatin, and resveratrol induce the expression of KLF4 in cultured human endothelial cells. We further demonstrate that the induction of KLF4 by resveratrol and atheroprotective shear stress occurs via a MEK5/MEF2-dependent signaling pathway. Since MEK5 activation is also critical for the expression of KLF2, we assessed the individual contribution of KLF4 and KLF2 to the global transcriptional activity triggered by MEK5 activation. Genome-wide transcriptional profiling of endothelial cells overexpressing KLF4, KLF2, or constitutively active MEK5 revealed that 59.2% of the genes regulated by the activation of MEK5 were similarly controlled by either KLF2 or KLF4. Collectively, our data identify a significant degree of mechanistic and functional conservation between KLF2 and KLF4, and importantly, provide further insights into the complex regulatory networks governing endothelial vasoprotection.

Introduction

The vascular endothelium, which forms the inner most lining of blood vessels, plays a critical role in the transduction and regulation of biological responses to various types of physiological stimuli, including biomechanical and humoral signals [1–3]. The inability of the endothelium to modulate these responses and maintain a normal homeostatic state, a process known as endothelial dysfunction, is a hallmark in the development of several cardiovascular diseases including atherosclerosis [4].

The Kruppel-like family of transcription factors, in particular Kruppel-like factor 2 (KLF2) and Kruppel-like factor 4 (KLF4), are key regulators of endothelial function. Specifically, these transcription factors have been shown to coordinate transcriptional programs important for the establishment of an anti-inflammatory, vasodilatory, and anti-thrombotic vascular endothelial phenotype [5–9]. Recent work by our laboratory and others has identified KLF2 as a common critical mediator of the vascular protective effects conferred by distinct physiological and pharmacological stimuli, namely atheroprotective shear stress, statins, and resveratrol [5,10–12]. In the context of these three distinct stimuli, KLF2 is necessary for the induction of key anti-inflammatory, anti-thrombotic, and vasodilatory factors such as thrombomodulin (TM), endothelial nitric oxide synthase (eNOS), and c-type natriuretic peptide (CNP). Phylogenetic studies indicate that KLF4 is most closely related to KLF2 amongst the other members of the KLF family [13]. Indeed, these transcription factors have recently been shown to play functionally similar roles both in the regulation of self-renewal of embryonic stem cells [14] as well as the induction of key vasoprotective genes such as eNOS, TM, and CNP in the vascular endothelium [6].

While several insights have been made regarding the stimuli and molecular mechanisms governing the expression of KLF2 in endothelial cells, those mediating KLF4 expression remain largely unknown. Given the functional similarity between KLF2 and KLF4, we hypothesized that KLF4 is similarly induced by known physiological and pharmacological stimuli of KLF2, and furthermore, that some of the signaling pathways mediating the expression of these two transcription factors in endothelial cells may be conserved.

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doi:10.1016/j.bbrc.2009.12.002

Materials and methods

Endothelial cell culture, dynamic flow system, RNA isolation, and real-time Taqman PCR. Primary human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described [11]. For shear stress experiments, cells were exposed to an atheroprotective waveform using a dynamic flow system as described by Dai et al. [15]. Following the completion of each respective experiment, cells were lysed, RNA isolated, and real-time Taqman PCR performed as described previously by our laboratory [11].

Adenoviral-mediated infection. Endothelial cells at 85–90% confluency were infected with either Ad-MEK5-DN or Ad-GFP (MOI = 20) for 12 h, washed with media, and incubated for an additional 12 h after media exchange. Cells were then treated for 8 h with either 100 μM resveratrol (Sigma) or ethanol vehicle. For Ad-MEF2ASA and Ad-GFP (MOI = 50) experiments, endothelial cells at 85–90% confluency were infected for 24 h, then washed with media and incubated for an additional 16 h after media exchange. Cells were then treated for 8 h with either 100 μM resveratrol or ethanol vehicle. For Ad-MEK5-CA and Ad-GFP (MOI = 20) experiments, cells were infected for 18 h, washed with media, and incubated an additional 24 h. For Ad-hKLF4-V5 and Ad-NC-V5 control (MOI = 10) experiments, cells were infected and lysed 24 h later. Samples were then processed for microarray analysis as previously described [5].

siRNA experiments. Transfections were conducted as previously described [11] with minor modifications. Briefly, endothelial cells were transfected with siERK5 (Invitrogen Stealth siRNA HSS183373; 100 nM) or siControl (Invitrogen Stealth siRNA LO GC negative control; 100 nM) at a confluency of 30–40% using Oligofectamine (Invitrogen). At 24 h post-transfection, cells were replated at 90–100% confluency. 45 h post-transfection, cells were incubated for an additional 8 h with either 100 μM resveratrol or ethanol vehicle. ERK5 siRNA results were validated using an additional siRNA (Ambion; s11149) targeting a different region of the transcript.

Transcriptional profiling. Total genome oligonucleotide microarrays from Applied Biosystems containing approximately 30,096 features representing 28,790 human genes were used. Labeling, hybridization, spot normalization, and analyses were performed as previously described [5]. Three independent experiments were run for each condition. Ad-hKLF4-V5 vs. Ad-NC-V5 control and Ad-MEK5-CA vs. Ad-GFP microarray excel data are located in the Supplemental material. Microarray data for Ad-KLF2 vs. Ad-GFP used for the multiple comparisons presented here were previously reported [5].

Western blotting. HUVEC at 100% confluency were treated for 8 h with 100 μM resveratrol, 1.0 μM simvastatin, or ethanol vehicle. Following lysis, SDS–PAGE and immunoblotting were performed as previously described [5]. ERK5 polyclonal antibody (Cell Signaling; #3372) and alpha-tubulin monoclonal antibody (Santa Cruz Biotechnology Inc.; sc-14262) were both used at a dilution of 1:1000.

Statistics. Statistical significance was determined using Student's *t*-test or one-way ANOVA followed by Tukey's HSD Post-hoc test when appropriate. Differences were considered significant at $p < 0.05$. For microarray data, gene regulation differences of $p < 0.001$ were considered significant as determined using Z-pool statistical methodology as described previously [16].

Results

KLF4 expression in endothelial cells is induced by distinct vasoprotective stimuli

To assess our hypothesis that KLF4 expression is similarly increased by known physiological and pharmacological inducers of KLF2, human endothelial cells (EC) were exposed to an atheroprotective shear stress waveform, simvastatin, and resveratrol. As shown in Fig. 1A, EC cultured under an atheroprotective shear stress waveform for 24 h displayed a significant upregulation of KLF4 mRNA expression. Likewise, EC incubated with increasing concentrations of simvastatin or resveratrol exhibited a significant induction in the expression of KLF4 (Fig. 1B and C). Since the endothelial vasoprotective effects conferred by treatment with 1.0 μM simvastatin or 100 μM resveratrol are well-characterized [10–12,17–20], we next conducted a time course analysis of KLF4 upregulation using these selected concentrations. As seen in Fig. 1D and E, simvastatin and resveratrol displayed differing kinetics in their induction of KLF4. While the resveratrol-mediated upregulation in KLF4 expression seen at 8 h was steadily maintained through later time points, the expression of KLF4 in simvastatin-treated cells followed a statistically significant reduction after 12 h.

Resveratrol induces endothelial KLF4 expression via a MEK5/MEF2-dependent, ERK5-independent signaling pathway

Given the similar response of KLF4 to well-characterized KLF2 stimuli, in addition to their known overlapping functional roles in endothelial cells, we sought to assess if the mechanisms governing KLF4 and KLF2 upregulation were conserved. Since MEK5 has been shown to be critical for the resveratrol-mediated induction of KLF2 [12], we first investigated the involvement of this kinase in the induction of KLF4 by resveratrol. To this end, EC were infected with either a dominant negative MEK5 adenovirus (Ad-MEK5-DN) or control GFP virus (Ad-GFP) and subsequently treated with resveratrol. As shown in Fig. 2A, MEK5-DN significantly blocked the upregulation of KLF4 by resveratrol. To determine whether MEK5 was sufficient for KLF4 induction, EC were infected with either a constitutively active form of MEK5 (Ad-MEK5-CA) or control GFP virus (Ad-GFP). As seen in Fig. 2B, MEK5-CA led to a significant increase in KLF4 mRNA expression as compared to control infected cells. Together, these data establish MEK5 as a critical component for the regulation of endothelial KLF4.

One of the well-characterized targets of MEK5 is the extracellular signal related kinase 5 (ERK5) [21,22]. ERK5 has been shown to be activated by and required for the upregulation of KLF2 by atheroprotective shear stress [5,23,24]. Previously, our laboratory demonstrated that MEK5 is both necessary and sufficient for the ERK5 phosphorylation by atheroprotective shear stress in EC [5]. To determine the importance of ERK5 for the resveratrol-mediated induction of KLF4, we first assessed its activation by resveratrol. As shown in Fig. 2C, resveratrol did not induce ERK5 phosphorylation. In contrast, treatment with simvastatin, a known ERK5 activator [12], led to the phosphorylation of ERK5. These data suggest that ERK5 activation is not necessary for the upregulation of KLF4 by resveratrol. To further define the role of ERK5 in the induction of KLF4, we muted its expression using a specific siRNA (Fig. 2D). Using this siRNA, we demonstrate that while ERK5 silencing modestly reduced KLF4 expression under basal conditions, its silencing had no significant effect on the KLF4 upregulation by resveratrol (Fig. 2D). Thus, these observations demonstrate that ERK5 is not necessary for the resveratrol-mediated induction of KLF4 in endothelial cells.

Previous studies have identified MEK5 as a critical activator of the myocyte enhancer factor 2 (MEF2) family of transcription factors [22,25,26]. Indeed, recent work by our group and others has shown that the MEF2 family functions as a critical regulator of endothelial KLF2 expression [5,27]. Additionally, analysis of the human KLF4 promoter revealed several putative MEF2 binding sites. Therefore, we next elucidated the role of MEF2 in the resveratrol-mediated upregulation of KLF4 by infecting EC with either a

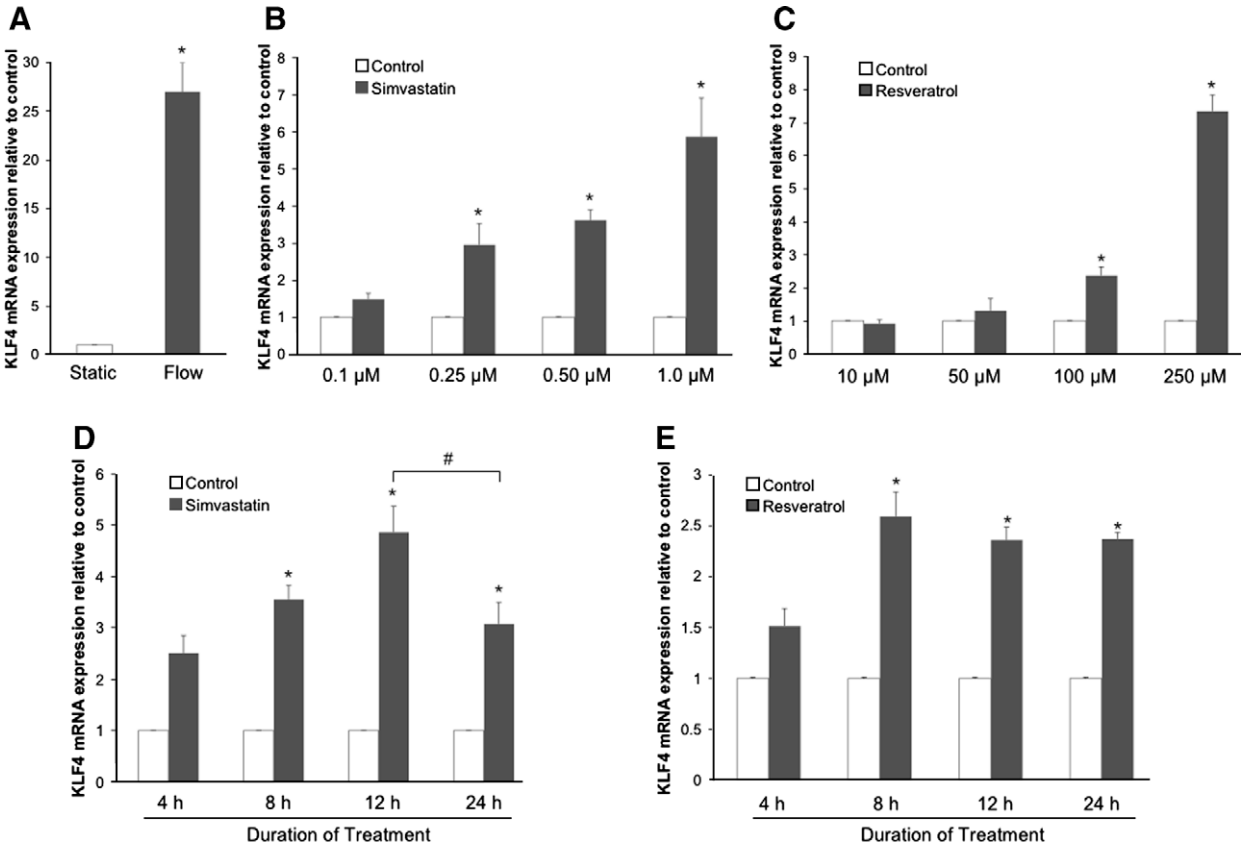

Fig. 1. Distinct physiological and pharmacological vasoprotective stimuli induce the expression of endothelial KLF4. (A) KLF4 mRNA expression in HUVEC exposed to either static (no flow) or an atheroprotective shear stress waveform for 24 h. (B) KLF4 mRNA expression in HUVEC following 12 h treatment with 0.1, 0.25, 0.5, and 1.0 μM simvastatin or DMSO control. (C) mRNA expression of KLF4 in HUVEC incubated for 8 h with 10, 50, 100, and 250 μM resveratrol or ethanol control. (D) KLF4 mRNA expression following incubation with 1.0 μM simvastatin or DMSO control for the indicated periods of time. (E) mRNA levels of KLF4 in HUVEC cultured with 100 μM resveratrol or ethanol control for the indicated periods of time. All data are expressed as means ± SEM from three or four independent experiments ($^{*}p < 0.05$ vs. respective control; $^{\#}p < 0.05$).

dominant negative MEF2 mutant adenovirus (Ad-MEF2ASA) or control GFP virus (Ad-GFP). As seen in Fig. 2E, the upregulation of KLF4 by resveratrol was abolished in MEF2ASA infected EC, demonstrating that the MEF2 family is necessary for the induction of KLF4 by resveratrol. Importantly, we also documented that the MEK5/MEF2 pathway plays a critical role in the upregulation of endothelial KLF4 by the atheroprotective shear stress waveform (Supplemental Fig. 1), suggesting that MEK5/MEF2 may function as a common pathway in the activation of KLF4 by different vasoprotective stimuli.

KLF4 expression controls multiple endothelial vasoprotective transcriptional programs

To begin to comprehensively assess the role of KLF4 in global endothelial cell gene expression, genome-wide transcriptional profiling was performed in EC overexpressing either KLF4 or a control adenoviral vector. Statistical analysis of these transcriptional profiling data identified 1899 genes differentially regulated by the expression of KLF4 in EC. Among these genes, several were found to mediate important endothelial functions including inflammation, thrombosis, vasomotor tone, blood vessel development, and oxidative stress (Table 1). While some of these genes such as endothelial nitric oxide synthase (eNOS), thrombomodulin (THBD), interleukin 6 (IL-6), and monocyte chemoattractant protein-1 (CCL2) are established targets of both KLF4 [6] and KLF2 [5], potentially novel KLF4-specific targets including forkhead box O1 (FOXO1), vascular endothelial growth factor (VEGF), and kelch-like ECH-associated protein 1 (KEAP1) were identified. These genes, which play important roles in angiogenesis and oxidative stress, displayed no overlap with previously published endothelial KLF2 targets derived from overexpression and silencing microarray datasets obtained by our laboratory and others [5,7]. Importantly, collective analysis of these gene expression data strongly suggests that KLF4 confers an anti-inflammatory, anti-thrombotic, and vasodilatory endothelial phenotype similar to that previously defined for KLF2.

MEK5 activation evokes endothelial gene expression programs similar to those triggered by the expression of KLF2 or KLF4 in endothelial cells

Since we have established MEK5 as a critical inducer of both KLF2 [5] and KLF4 expression in endothelial cells, we next sought to assess the global transcriptional activity evoked by MEK5 activation and compare it with that triggered by the expression of KLF2 or KLF4. To this end, we conducted a genome-wide transcriptional profiling screen of EC infected with either Ad-MEK5-CA or Ad-GFP as a control. Comparison of the downstream targets regulated by MEK5 and either KLF2 or KLF4 revealed a substantial overlap (Fig. 3A). Specifically, 383 (59.2%) of the MEK5-regulated genes were similarly modulated (up and down) by either KLF2 or KLF4, suggesting that these Kruppel factors function as critical transcriptional regulators for the activation/repression of genes downstream of MEK5 activation. Among the genes identified to be commonly regulated by MEK5, KLF2, and KLF4 (Fig. 3B) were TEK tyrosine kinase (Tie-2), argininosuccinate synthetase 1 (ASS1),

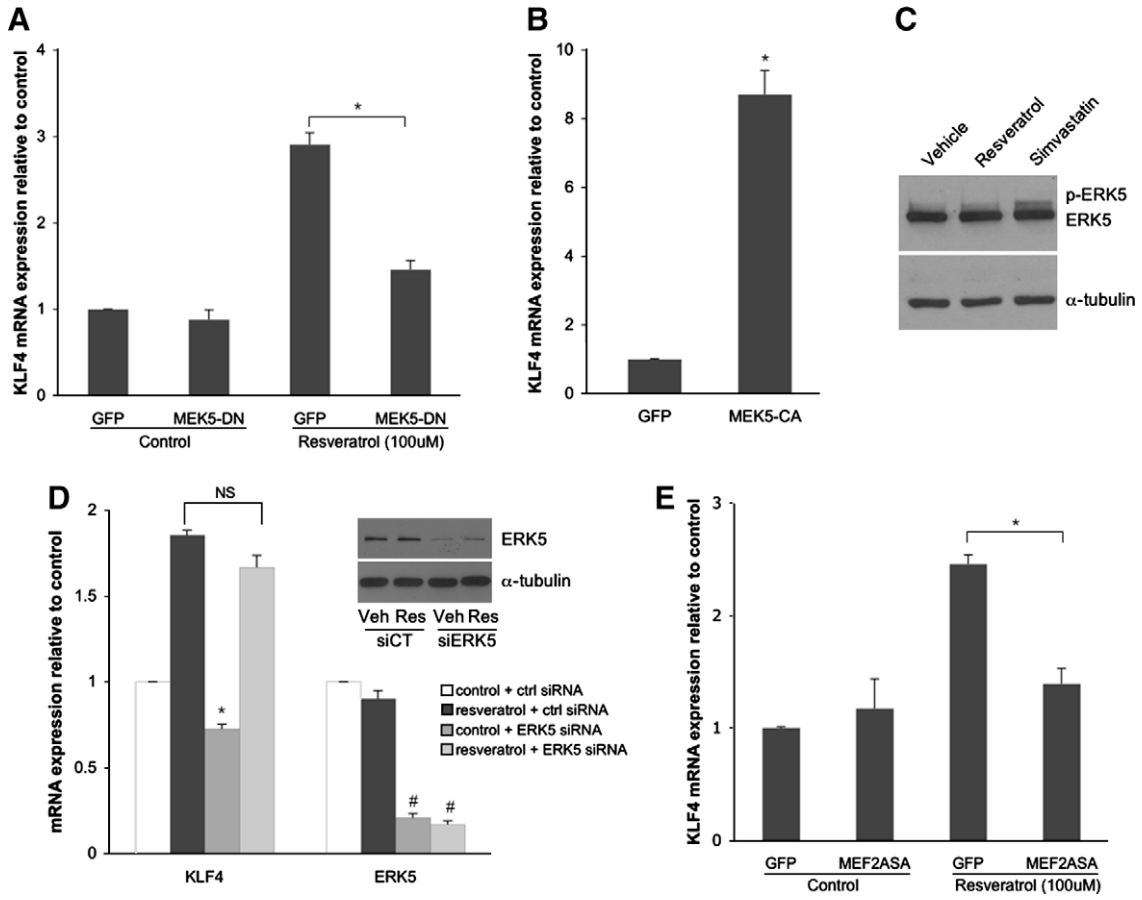

Fig. 2. Resveratrol induces endothelial KLF4 expression via a MEK5/MEF2-dependent, ERK5-independent pathway. (A) KLF4 mRNA expression in HUVEC infected with either control GFP or MEK5-DN adenovirus followed by incubation with 100 μM resveratrol or its vehicle. (B) KLF4 mRNA expression from HUVEC infected with either control GFP or MEK5-CA adenovirus. (C) Representative western blot showing the effect on ERK5 phosphorylation from HUVEC stimulated for 8 h with either vehicle, resveratrol (100 μM), or simvastatin (1.0 μM). (D) mRNA expression of KLF4 and ERK5 from HUVEC transfected with either ERK5 siRNA or control siRNA followed by incubation with 100 μM resveratrol or its vehicle (*$p < 0.01$ vs. control + ctrl siRNA; #$p < 0.001$ vs. respective ctrl siRNA group). Insert shows representative western blot demonstrating the silencing efficiency of ERK5. (E) Effect of MEF2ASA or GFP adenovirus on the induction of KLF4 mRNA expression by 100 μM resveratrol or its vehicle. All data are expressed as means ± SEM from three independent experiments (*$p < 0.01$).

Table 1
Select endothelial genes identified by transcriptional profiling to be differentially regulated by Ad-hKLF4-V5 vs. Ad-NC-V5 Control infected cells.

RefSeq	Gene Name	Ratio	Function
Upregulated			
NM_000050	Argininosuccinate synthetase 1	25.1	Vessel tone
NM_000641	Interleukin 11	18.6	Vasoprotection
NM_002514	Nephroblastoma overexpressed gene	13.9	Cell communication
NM_000501	Elastin	7.0	ECM structural protein
NM_000903	NAD(P)H dehydrogenase, quinone 1	5.8	Oxidative stress
NM_001025366	Vascular endothelial growth factor[a]	4.1	Angiogenesis
NM_000361	Thrombomodulin	3.8	Thrombosis/hemostasis
NM_002133	Heme oxygenase 1	3.2	Inflammation/oxidative stress
NM_000603	Nitric oxide synthase 3	2.6	Vessel tone
NM_000459	TEK tyrosine kinase	1.6	Vascular development
Downregulated			
NM_203500	Kelch-like ECH-associated protein 1[a]	−1.4	Oxidative stress
NM_002015	Forkhead box O1[a]	−1.7	Cell proliferation/oxidative stress
NM_001200	Bone morphogenetic protein 2	−1.8	Inflammation
NM_016270	Kruppel-like factor 2	−2.7	Vasoprotection
NM_001147	Angiopoietin 2	−2.9	Angiogenesis
NM_000584	Interleukin 8	−2.9	Inflammation
NM_001955	Endothelin 1	−3.0	Vessel Tone
NM_000600	Interleukin 6	−3.1	Inflammation
NM_003246	Thrombospondin 1	−3.5	Angiogenesis/platelet aggregation
NM_002982	Chemokine (C–C motif) ligand 2	−7.3	Inflammation

[a] Gene not a published transcriptional target of endothelial KLF2.

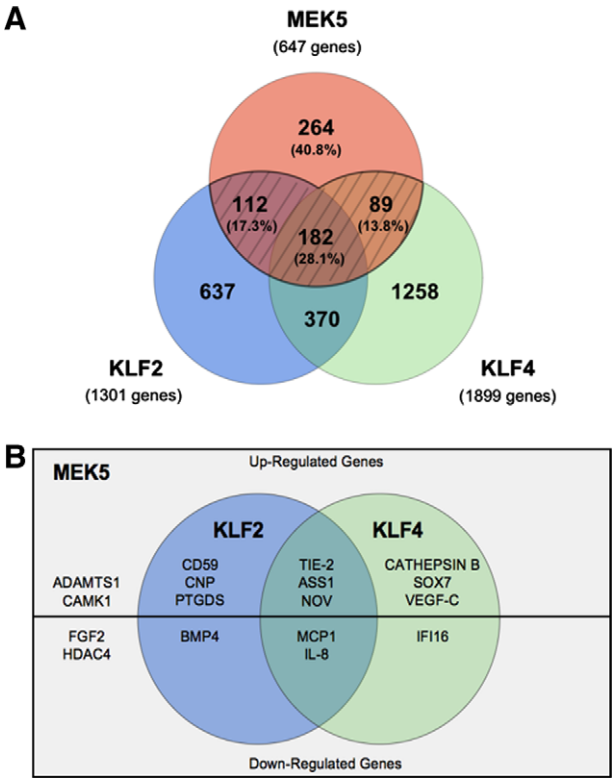

Fig. 3. Comparative analysis of MEK5, KLF2, and KLF4 global gene regulation. (A) Venn diagram demonstrating the quantitative overlap between endothelial MEK5, KLF2, and KLF4-regulated genes. Percentages represent the proportion of all MEK5-controlled genes present in that particular sector. (B) MEK5-regulatory gene domain highlighting specific KLF2 and KLF4 targets contained within this domain.

nephroblastoma overexpressed gene (NOV), and interleukin 8 (IL-8), all of which mediate diverse endothelial functions including vasomotor tone, inflammation, and blood vessel development. Given the strong similarity between the previously published downstream transcriptional targets of KLF2, and those described here for KLF4, we next determined the degree of global overlap between KLF2 and KLF4-regulated genes. Analyses of these datasets demonstrated that 552 (42.4%) of the genes regulated by KLF2 were similarly controlled by KLF4 (Fig. 3A), suggesting a significant degree of transcriptional redundancy between these two closely related KLF members.

Discussion

The vascular endothelium plays a fundamental role in the health and disease of the cardiovascular system [4]. The Kruppel-like transcription factors, KLF2 and KLF4, have previously been shown to control important aspects of endothelial function leading to the establishment of a vasoprotective phenotype [5–9]. Despite the advances that have been made in our understanding of the stimuli and molecular mechanisms governing KLF2 expression in endothelial cells, little is known regarding the regulation of KLF4 and the degree of functional convergence between these two transcription factors in the vascular endothelium.

In the present study, we demonstrate that distinct, well-characterized physiological and pharmacological stimuli of KLF2, including atheroprotective shear stress, simvastatin, and resveratrol also induce the expression of endothelial KLF4. Using resveratrol stimulation as our specific experimental tool, we show that the signaling mechanisms governing KLF4 upregulation occur via a MEK5/MEF2-dependent, ERK5-independent pathway. These mechanisms are the same as those recently described by our group to mediate the induction of KLF2 by resveratrol in EC [12]. In addition, we demonstrate that MEK5/MEF2 are also important for KLF4 upregulation by atheroprotective shear stress, suggesting that this signaling pathway for KLF4 induction may be shared by multiple vasoprotective stimuli. Taken together, our data reveal that at least part of the regulatory machinery governing the induction of KLF2 and KLF4 by vasoprotective stimuli in endothelial cells is conserved. It remains to be defined, however, if the previously documented dependencies of KLF2 induction on geranylgeranyl pyrophosphate (statins) and SIRT1 (resveratrol) are also important for KLF4 upregulation by these two respective stimuli.

A critical aspect of KLF4 biology that remains unexplored is the extent to which KLF4 orchestrates endothelial transcriptional programs. To begin addressing this issue, we conducted a genome-wide transcriptional profiling of cells overexpressing KLF4. These experiments revealed that 1899 genes are differentially regulated by KLF4 expression. Among the KLF4-regulated transcripts found were a number of vasoprotective genes commonly induced by atheroprotective shear stress, simvastatin, and resveratrol. These genes included eNOS, thrombomodulin, and CNP, key mediators of the endothelial functional phenotype. Since several of the genes identified to be regulated by KLF4 in this study are also regulated by KLF2 [5], we compared the genome-wide transcriptional profiles of endothelial cells overexpressing either KLF2 or KLF4. This analysis revealed that 42.4% of the genes regulated by KLF2 were similarly controlled by KLF4, suggesting a significant degree of shared downstream transcriptional targets. Because KLF2 and KLF4 share a common canonical CACCC transcriptional binding site, at the present time we cannot rule out the possibility of non-physiologically relevant gene activation/suppression resulting from this type of *in vitro* overexpression experiment. Importantly, however, Jiang et al. have demonstrated that 89% of KLF2 *in vivo* binding sites are shared by KLF4 in embryonic stem cells [14]. In this same study only 26% of KLF4 *in vivo* binding sites were shared by KLF2. This result is similar to our analysis in endothelial cells whereby 29.1% of KLF4 targets were shared by KLF2, suggesting that KLF4 may play a more extensive transcriptional regulatory role than KLF2 in endothelial cells. Notably however, KLF4 null mice do not display any reported vascular abnormalities [28], while mice lacking KLF2 show defects in vessel wall maturation, stability, and hemodynamic adaptation, and are embryonic lethal at approximately day 12.5–14.5 [29–31]. These data, together with *in vitro* KLF2 silencing experiments [5,11,12], suggest that KLF2 and KLF4 have important non-compensatory transcriptional regulatory functions. The specific roles of endothelial KLF2 and KLF4, and their functional overlap in the context of adult pathophysiological states remains to be defined.

In this study, we also demonstrate that MEK5 activation, which has been shown to be necessary for the establishment of an endothelial vasoprotective phenotype [5,12], functions as a critical upstream regulator of endothelial KLF4 expression. Comparisons of genome-wide expression profiles from cells overexpressing KLF2, KLF4, or a constitutively active MEK5 showed that 59.2% of the MEK5-regulated genes are controlled in a similar manner by either KLF2 or KLF4. These data indicate that these two KLF members serve as important transcriptional integrators of MEK5 activation, and importantly, identify MEK5 as a critical regulatory node for endothelial vasoprotective stimuli.

The studies presented here provide novel insights into the regulatory mechanisms controlling KLF4 expression in vascular endothelial cells. Furthermore, they identify a significant degree of mechanistic and functional conservation between KLF2 and KLF4, and indicate that this two transcription factors are critical for the downstream transcriptional programs triggered by MEK5 activation. Collectively, these observations reveal important molecular

interactions involved in the complex regulatory processes mediating endothelial vasoprotection.

Acknowledgments

This work was supported by the National Institutes of Health [HL-076686 and HL-090856 to G.G.-C.]; the Howard Hughes Medical Institute Research Training Fellowship for Medical Students [G.V.]; the American Federation for Aging Research/National Institute on Aging [T35 AG026781 to G.V.]; the Department of Innovation, Universities and Enterprise, Government of Catalonia, Spain, and the Spanish Association for the Study of the Liver [J.G.-S.].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.002.

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