Development 136, 1115-1125 (2009) doi:10.1242/dev.029538

Krüppel-like factor 2 cooperates with the ETS family protein ERG to activate *Flk1* expression during vascular development

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The VEGF receptor, FLK1, is essential for differentiation of the endothelial lineage and for embryonic vascular development. Using comparative genomics, we have identified conserved ETS and Krüppel-like factor (KLF) binding sites within the *Flk1* enhancer. In transgenic studies, mutation of either site results in dramatic reduction of *Flk1* reporter expression. Overexpression of KLF2 or the ETS transcription factor ERG is sufficient to induce ectopic *Flk1* expression in the *Xenopus* embryo. Inhibition of KLF2 function in the *Xenopus* embryo results in a dramatic reduction in *Flk1* transcript levels. Furthermore, we show that KLF2 and ERG associate in a physical complex and that the two proteins synergistically activate transcription of *Flk1*. Since the ETS and KLF protein families have independently been recognized as important regulators of endothelial gene expression, cooperation between the two families has broad implications for gene regulation during development, normal physiology and vascular disease.

KEY WORDS: *Tie2*, VE-cadherin, VEGFR2, *Xenopus*, Endothelial lineage, Vascular development, KLF2

INTRODUCTION

Vascular endothelial growth factor (VEGF) and its high-affinity receptor, fetal liver kinase 1 (FLK1, also called VEGFR2 or KDR), are essential for formation of the vasculature. Mice lacking a single *Vegf* allele die at E10.5 owing to defects in the differentiation of endothelial cells and severe disorganization of the vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Similarly, homozygous *Flk1*-null embryos die at E8.5-9.5 with severe reduction in endothelial cell number and complete disruption of subsequent vascular development (Shalaby et al., 1995). Since FLK1 is amongst the earliest markers of the endothelial lineage, characterization of transcription mechanisms regulating *Flk1* gene expression will help to advance our understanding of the regulatory pathways controlling vascular development.

Mouse transgenic studies have shown that endothelial-specific expression of *Flk1* is regulated by an enhancer in the first intron, which contains binding elements for the transcription factors TAL1 (SCL) and members of the GATA and ETS families (Kappel et al., 1999). Mutation of the GATA or ETS motifs abolishes reporter expression in endothelial cells of transgenic mice, whereas mutation of the TAL1 site results in reduced expression levels (Kappel et al., 2000). Additional ETS motifs are located in the promoter region of the mouse *Flk1* gene and these have been shown to function together with HIF-2α (EPAS1 – Mouse Genome Informatics) to regulate *Flk1* transcription (Elvert et al., 2003). The requirement for ETS binding sites in *Flk1* regulatory regions is consistent with the established function of ETS transcription factors in the regulation of vascular development (Dejana et al., 2007). Approximately 30 ETS factors are known in mammals and all members share a conserved DNA-binding domain that recognizes the core recognition sequence GGA(A/T) (Lelievre et al., 2001; Sharrocks, 2001). ETS proteins frequently interact with partners to influence tissue-specific gene regulation (Lelievre et al., 2001; Sharrocks, 2001; Oikawa and Yamada, 2003), but very little is known about possible ETS partners in endothelial cells. Transgenic analysis has demonstrated the importance of ETS motifs for expression of several vascular genes in addition to *Flk1*, including *Tie2* (Schlaeger et al., 1997) and VE-cadherin (cadherin 5) (Gory et al., 1999). Gain-of-function experiments have shown that ETS factors can upregulate endothelial gene expression in cultured cells (Birdsey et al., 2008; Hasegawa et al., 2004; Schwachtgen et al., 1997; Wakiya et al., 1996). Overexpression of the ETS factor ERG in *Xenopus* embryos is sufficient to activate ectopic transcription of the vascular marker *X-msr* (Baltzinger et al., 1999).

At least four ETS genes, *Ets1*, *Erg*, *Fli1* and *Er71* (*Etv2*), are expressed in mouse embryonic endothelial cells (Lelievre et al., 2001; Lee et al., 2008). Owing to functional redundancy between family members, most loss-of-function studies of individual ETS factors have not revealed early vascular phenotypes. A striking exception is the knockout of the mouse *Er71* gene, which shows greatly reduced angioblast cell numbers and severe disruption of vascular development (Lee et al., 2008). Zebrafish studies have shown that knockdown of four vascular ETS genes results in a near complete loss of endothelial cells, whereas single knockdowns of individual genes exhibit less severe phenotypes (Pham et al., 2007).

The Krüppel-like factor (KLF) family of transcription regulators is also involved in the regulation of vascular gene expression (Atkins and Jain, 2007). KLFs bind a consensus recognition sequence of CACCC (Bieker, 2001; Dang et al., 2001), and three of the 17 family members, KLF2, KLF4 and KLF6, are expressed in the mouse embryonic vasculature (Kuo et al., 1997; Yet et al., 1998; Kojima et al., 2000; Botella et al., 2002; Lee et al., 2006). KLF proteins can act as either transcriptional activators or repressors and domain mapping of KLF2 has identified transactivating and transrepression domains within the protein (Conkright et al., 2001). Numerous endothelial genes have KLF binding sites in their promoter regions and cell culture studies have shown that KLF2 activates the expression of vascular genes including thrombomodulin (Lin et al., 2005) and *eNOS* (*Nos3*) (Parmar et al., 2006; Dekker et al., 2005), but inhibits expression of other vascular genes including endothelin and adrenomedullin (Dekker et al., 2006). Mice lacking either KLF2 or KLF4 activity are not viable; however, early vascular development in both knockouts is normal (Kuo et al., 1997; Lee et al., 2006; Segre et al., 1999). Mice lacking KLF6 function exhibit

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Accepted 2 February 2009

vascular assembly defects in the yolk sac, but endothelial gene expression and development of blood vessels in the embryo itself are apparently normal (Matsumoto et al., 2006). It is interesting to note that embryonic stem (ES) cells lacking KLF6 activity show reduced levels of FLK1 protein after differentiation into embryoid bodies (Matsumoto et al., 2006), suggesting that KLF6 might function as an activator of *Flk1* expression. Like the ETS proteins, it is possible that redundant expression of different KLF family members can exert a rescuing function and, indeed, redundancy has been demonstrated in studies using ES cells in which any one of three specific KLFs (KLF2, KLF4 or KLF5) could substitute for the other two in maintaining ES cells in an undifferentiated state (Jiang et al., 2008). Of particular relevance to the study of *Flk1* transcriptional regulation, both cell culture and microarray studies using adult endothelial cells have suggested that KLF2 functions as a repressor of *Flk1* expression (Bhattacharya et al., 2005; Dekker et al., 2006).

Our investigation into the transcriptional regulation of *Flk1* strongly suggests a positive role for KLF proteins during embryonic vascular development. We show that KLF2 is sufficient to activate *Flk1* expression in the *Xenopus* embryo and that inhibition of KLF2 function results in the disruption of normal vascular development. Furthermore, we show that ETS and KLF proteins physically interact and synergistically activate embryonic expression of the *Flk1* gene.

MATERIALS AND METHODS

Preparation of in situ probes and mRNAs

The insert from a full-length *Xenopus laevis Klf2* clone (BC043732) was isolated using *Not*I and *Sal*I and inserted into pBluescript SK(+). For in situ probe synthesis, *Klf2*-pBluescript was linearized with *Sal*I and transcribed with T3 RNA polymerase (Megascript Kit, Ambion). *Xenopus tropicalis VE-cadherin* sequences were inserted into pGEM T-easy, linearized with *Sal*I and transcribed with T7 RNA polymerase. Synthesis of *Flk1*, *Erg* and *X-msr* in situ hybridization probes has been described previously (Cleaver et al., 1997; Baltzinger et al., 1999; Devic et al., 1996). The KLF2 coding region was PCR amplified from BC043732 with Pfu polymerase, subcloned into pT7TS and the sequence verified. For synthesis of *Klf2* mRNA, *Klf2*-pT7TS was linearized with *Xba*I and transcribed with T7 RNA polymerase (Message Machine Kit, Ambion). A dominant-repressor form of KLF2 (DR-KLF2) lacking the first 87 amino acids that make up the transactivation domain was generated by inverse PCR from *Klf2*-pT7TS template using Pfu polymerase and the sequence verified. The ERG coding sequence (AJ224126) was PCR amplified using Pfu polymerase, cloned into pT7TS, sequence verified, linearized with *Xba*I and mRNA synthesized using T7 RNA polymerase.

Microinjection and embryological manipulation

For embryonic expression experiments, mRNAs encoding ERG, KLF2, DR-KLF2 and EGFP were injected into a single vegetal blastomere of a four-cell stage embryo in 0.4× MMR containing 6% Ficoll and cultured thereafter in 0.2× MMR until assay (stages 34-36). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Whole-mount in situ hybridization was carried out using digoxigenin-labeled probes and standard conditions (Harland, 1991). Preparation and microinjection of morpholino oligomers (MOs) was performed as described previously (Garriock et al., 2005). *Klf2* antisense MO (*Klf2* MO, 5′-ATCCGAATCAGATTGTCA-GCAAAAC-3′) was targeted to the 5′ untranslated region (UTR) of *Klf2* transcripts. *Klf2* MO effectively blocked translation of *Klf2* test transcripts containing the 5′ UTR plus a portion of the coding region sequences of *Klf2* fused to the coding region of EGFP (see Fig. 3D,E). For in vivo experiments, 12.5, 25 or 50 ng of *Klf2* MO or control antisense MO (5′-GGT-AGTAATAGATGCTGTGATCTAT-3′) was microinjected into the mediolateral region of one cell of two-cell staged embryos and later assayed at stage 34 for *Flk1* transcripts by whole-mount in situ hybridization. For measuring *Flk1* transcript levels, *Klf2* or control MO was injected at the one-cell stage.

Xenopus transgenics and transient assays

A *Flk1* genomic fragment comprising ~2.5 kb of sequence from upstream of the transcriptional start site to within exon 2 was isolated. For transgenic analysis, the EGFP coding region was inserted into exon 1 of a *Flk1* construction containing 2.5 kb of 5′ flanking sequences plus 1.5 kb of first intron sequences. Transgenic mutant constructs for the ETS and KLF sites were generated by inverse PCR using Pfu polymerase and the sequence verified. The ETS site 5′-GGAT-3′ was mutated to 5′-GTAT-3′ and the KLF site 5′-CACCCT-3′ was mutated to 5′-CGGTCG-3′. *Xenopus* transgenic embryos were generated as described (Kroll and Amaya, 1996; Sparrow et al., 2000).

For luciferase assays, mouse bEnd.3 endothelial cells (ATCC# CRL-2299) were transfected with 0.5 μg of reporter plasmid using Fugene 6 (Roche). *Flk1* reporter constructions (wild-type, ETS mutant and KLF mutant) were identical to transgenic constructions except the luciferase coding region was substituted in place of EGFP. For assay of the ETS/KLF module alone, three copies of the ETS/KLF sequence were inserted in tandem upstream of the minimal SV40 promoter in the luciferase reporter construction pGL3-Promoter vector (Promega). For both sets of experiments, cells were co-transfected with 0.1 μg of plasmid containing a CMV promoter driving β-galactosidase for normalization of transfection efficiency. Transfected cells were incubated in a 12-well plate for 20 hours and luciferase and β-galactosidase activity was measured using a luminometer. Experimental transfections were performed in quadruplicate.

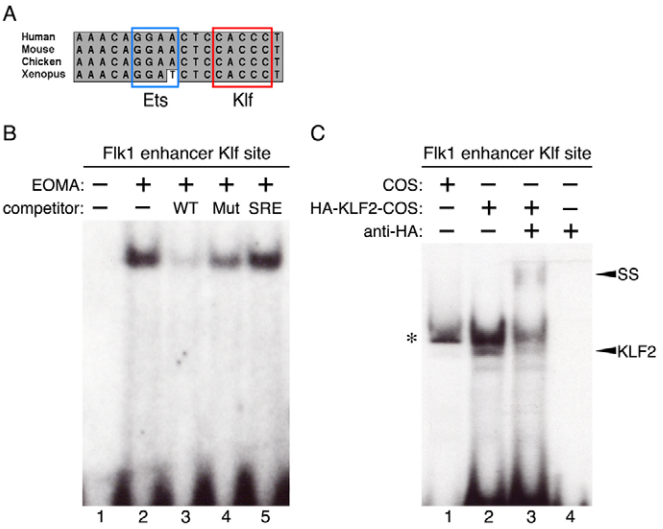

Fig. 1. The *Flk1* enhancer contains conserved ETS and KLF binding sites. (**A**) Alignment of sequences within the *Flk1* (*Kdr*) first intron from human, mouse, chicken and *Xenopus* reveals the presence of conserved tandem ETS and KLF binding sites. (**B**) Nuclear protein extracts from a mouse endothelial cell line (EOMA) were used in electophoretic mobility shift assays with probe corresponding to a consensus KLF site (WT). The shifted band (lane 2) was competed by cold wild-type probe (WT) but not by cold probe for a mutated KLF site (Mut) or serum-response element (SRE). (**C**) KLF2 binds to the KLF sequence located in the *Flk1* enhancer. Extracts from COS-7 cells produced a shifted band (asterisk in lane 1). Extracts from COS-7 cells expressing HA-KLF2 show the presence of a specific complex (arrowhead labeled KLF2 in lane 2). The shifted band formed in the presence of HA-KLF2 is supershifted following treatment with anti-HA antibody (arrowhead labeled SS in lane 3).

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts from mouse EOMA (ATCC# CRL-2586) and L cell fibroblast (CCL-1) cell lines were isolated according to standard procedures. HA-tagged mouse KLF2 was generated by transfection of a HA-KLF2 expression plasmid into COS-7 cells using Fugene 6, and protein extracts were isolated in 15 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, 120 mM NaCl, 25 mM KCl and protease inhibitors. Radiolabeled probes included: WT used in Fig. 2A (5′-GTACTCTCCACCCTGGTGC-3′) and WT used in Fig. 2B (5′-TAAGACTCCACCCTGGCC-3′). Competition oligonucleotides included a mutated KLF site (Mut, 5′-GTACTTT-AGATGCAGGTGC-3′) and a serum-response element (SRE, 5′-CTA-GGTTTCAGGGTCCTGCCATAAAAG-3′). EMSA was performed using standard techniques.

PCR analysis

Quantitative RT-PCR analysis was performed using a Rotor-gene 6000 (Corbett). Ventral posterior tissue from 15 embryos (stage 34) injected with 150 pg of *Klf2* mRNA, 25 pg of *Erg* mRNA and 500 pg *GFP* mRNA alone or in combination were processed for RNA isolation, the RNA converted into cDNA and used for real-time PCR analysis. The following primers were used (F, forward; R, reverse): *Flk1* F, 5′-ACATTCCTGTAGAGCCTGTGGT-3′ and R, 5′-GGACTGGTAGT-CGCTAGTTTGG-3′; *Tie2* F, 5′-TGAGAAGCCTCTGAACTGTG-3′ and R, 5′-TTCAGCAGAACAGTCAATCC-3′; *PECAM* F, 5′-ATG-AGTGTGACGTGTTCTGTG-3′ and R, 5′-GACAGCCATGCAATGT-CTATG-3′; *ornithine decarboxylase* (*ODC*) was used to normalize samples (Bouwmeester et al., 1996).

Co-immunoprecipitation experiments

Co-immunoprecipitation experiments were conducted as described (Meadows et al., 2008). Briefly, HA-tagged mouse KLF2 was generated by transfection into COS-7 cells and verified by protein blotting with detection using chemiluminescent solution (Supersignal, West Dura). ERG and EGFP proteins were radiolabeled with ^{35}S methionine by translation in the Wheat Germ Cell-Free Translation System (Promega). COS-7 cell extracts containing HA-KLF2 were mixed with in vitro translated products and immunoprecipitation was carried out using standard protocols and anti-HA antibody (Roche). Bound proteins were fractionated on a 10% SDS-PAGE gel and visualized by autoradiography. Human ERG was modified with a FLAG epitope, inserted into the expression vector pcDNA3.1+ (Promega) and co-transfected into COS-7 cells with HA-KLF2. Immunoprecipitation was carried out using anti-FLAG monoclonal antibody (Sigma) and proteins detected as described above.

RESULTS

Identification of a conserved KLF binding site in the *Flk1* enhancer

In order to explore regulatory pathways that may be involved in development of the vascular endothelial lineage, we investigated the transcriptional control of the gene encoding FLK1. Alignment of sequences within the first intron of the *Flk1* gene from different species identified a ~120 bp segment that is highly conserved from human to frog. This conserved element is contained within a region of the mouse *Flk1* intron that has previously been demonstrated to possess enhancer activity (Kappel et al., 1999; Kappel et al., 2000). As shown in Fig. 1A, a module within the larger conserved region contains consensus binding sites for ETS and KLF proteins. This ETS binding site in the mouse *Flk1* gene is known to be essential for expression in transgenic animals (Kappel et al., 2000), but the KLF element has not previously been reported. The conservation of the putative KLF binding site in evolutionarily distant organisms suggests that it might also be important for regulation of *Flk1* expression.

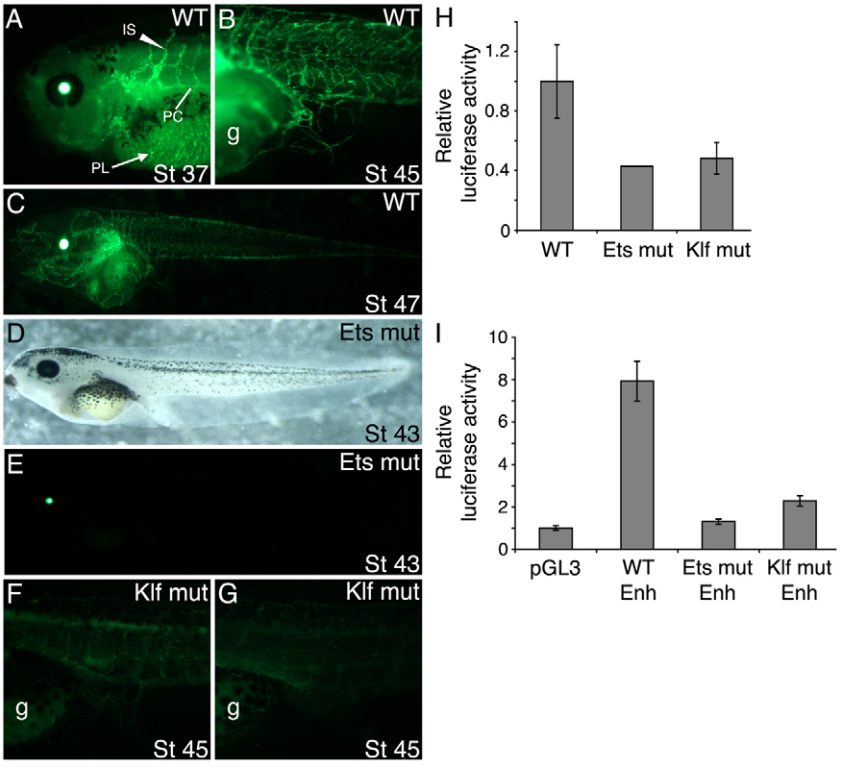

Fig. 2. The conserved KLF binding site is required for *Flk1* reporter expression in transgenic *Xenopus* embryos. (**A**) Fluorescent image of a transgenic embryo (stage 37) showing GFP reporter expression driven by the *Flk1* wild-type (WT) construction. Strong GFP expression was observed in developing blood vessels, including the posterior cardinal vein (PC), intersomitic vessels (IS) and vascular plexus (PL). GFP fluorescence in the eye was driven by the γ-crystallin reporter construction that serves as a marker for transgenesis. (**B**) Fluorescent image of the trunk region of a typical transgenic embryo (stage 45) showing GFP expression in minor blood vessels. The gut (g) is indicated for orientation. (**C**) Fluorescent image of entire transgenic embryo (stage 47) showing GFP expression throughout the vasculature. (**D**) Bright-field view of embryo transgenic for the ETS mutant construction. (**E**) Fluorescent image of embryo shown in D. Mutation of the conserved ETS site dramatically reduces detectable GFP fluorescence in the vasculature. (**F**,**G**) Fluorescent images of embryos transgenic for the KLF mutant construction, showing major reduction in GFP reporter expression. To allow direct comparison, images in B,F,G were collected at the same magnification and exposure. (**H**) Mutation of the KLF binding site reduces *Flk1* reporter expression in an endothelial cell line. The luciferase coding region was substituted for GFP in the *Flk1* reporter construction and transfected into a mouse endothelial cell line (bEnd.3). Relative luciferase activity of wild-type and mutant constructions is indicated. Assays were carried out in quadruplicate. (**I**) Three copies of the ETS/KLF binding sequence, or equivalent ETS and KLF mutated sequences, were inserted upstream of a minimal promoter in a luciferase reporter construction and the resulting plasmids transfected into bEnd.3 cells. Mutations of either the ETS or KLF binding sites result in a reduction in relative luciferase activity. Assays were carried out in quadruplicate.

KLF2 binds to the consensus KLF element in the *Flk1* enhancer

To determine the possible functional relevance of the KLF site, we used the electrophoretic mobility shift assay (EMSA) to determine whether nuclear proteins from endothelial cells associate with the KLF element. Nuclear extracts were prepared from the mouse EOMA endothelial cell line, which expresses the *Flk1* gene (data not shown). A single EMSA band was observed when protein extracts were incubated with an oligonucleotide containing the KLF binding sequence found in the enhancer (Fig. 1B, lane 2). This shift could be competed with wild-type KLF sequences (Fig. 1B, lane 3), but not with a mutated KLF sequence or with other binding site sequences (Fig. 1B, lanes 4 and 5). Nuclear proteins from a fibroblast cell line (CCL-1) failed to form a complex with the KLF binding sequence (data not shown). These experiments indicate that a binding activity specific for the KLF site in the *Flk1* enhancer is present in endothelial cell nuclear extracts.

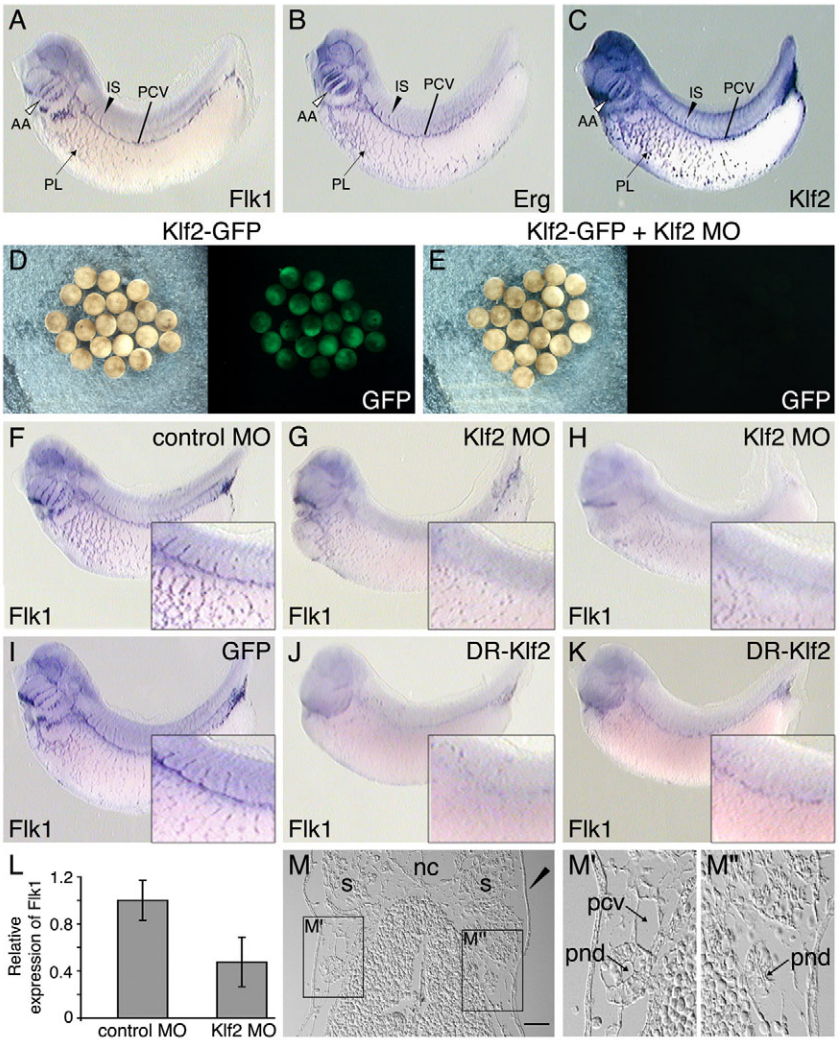

Fig. 3. Inhibition of KLF2 function results in reduced *Flk1* expression in the *Xenopus* embryo. (**A-C**) Whole-mount in situ hybridization analysis of *Flk1*, *Erg* and *Klf2* expression in *Xenopus* embryos (stage 34, lateral view). For each gene, expression is observed in the endothelial cells of the major developing vessels, including the posterior cardinal vein (PCV), intersomitic vessels (IS), aortic arches (AA) and in the forming plexus on the flank of the embryo (PL). (**D**,**E**) *Klf2* MO effectively blocks translation of a control *Klf2* transcript. (D) Bright-field and fluorescent images of embryos injected with a control transcript in which the 5′ UTR of *Klf2* was fused to the coding sequences of GFP (*Klf2-GFP*). (E) Bright-field and fluorescent images of embryos injected with *Klf2-GFP* transcript plus *Klf2* MO (25 ng). Note that GFP reporter fluorescence is greatly inhibited by *Klf2* MO treatment. (**F**) Embryo injected with 50 ng of a control MO and assayed for expression of *Flk1* transcripts. The inset is a higher magnification view, centered on the developing posterior cardinal vein. (**G**,**H**) Two different embryos injected with 50 ng of *Klf2* MO and assayed for expression of *Flk1*. *Klf2* MO-injected embryos show a dramatic reduction of *Flk1* expression. (**I**) Embryo injected with 500 pg of *GFP* mRNA and assayed for *Flk1* transcripts. (**J**,**K**) Two different embryos injected with 250 pg of mRNA encoding a dominant-repressor form of KLF2 (DR-KLF2) and assayed for *Flk1* transcripts. Embryos expressing the DR-KLF2 construction show a dramatic reduction of *Flk1* transcripts. (**L**) qRT-PCR analysis reveals significant reduction in *Flk1* transcript levels in *Klf2* MO-treated embryos. Results shown are the average of three separate embryos for each MO treatment. (**M-M″**) *Klf2* MO treatment eliminates vascular tubes. Histological section through a stage 42 embryo injected with *Klf2* MO. Somites (s) and notochord (nc) are indicated. Scale bar: 100 μm. Injected side is to the right (arrowhead). M′ and M″ show enlargements of the region of the posterior cardinal vein on the untreated and treated sides, respectively. Although the pronephric duct (pnd) is visible on both sides, no tube corresponding to the posterior cardinal vein (pcv) is visible on the treated side.

To determine whether KLF proteins interact with the conserved element, gel shift assays were performed using protein extracts from COS-7 cells expressing an epitope-tagged KLF2 protein, as this family member is known to be expressed in endothelial cells (Kuo et al., 1997). Extracts from non-transfected COS-7 cells yielded a shifted band (Fig. 1C, lane 1). Since KLF proteins are expressed in a wide range of cell types (Kaczynski et al., 2003), we cannot exclude the possibility that this band results from the binding of one or more KLF proteins to the probe sequence. However, an additional, prominent complex was detected when nuclear extracts expressing HA-tagged KLF2 were incubated with the probe (Fig. 1C, lane 2). This band could be supershifted using an antibody directed against the HA-tag on the KLF2 protein (Fig. 1C, lane 3). These experiments demonstrate that KLF2, a representative endothelially expressed member of the KLF protein family, specifically binds to the KLF motif located in the *Flk1* enhancer.

Conserved ETS and KLF binding sites are required for embryonic expression of *Flk1*

To address the function of the ETS and KLF sites for transcriptional control of *Flk1*, we utilized the *Xenopus* transgenesis system (Kroll and Amaya, 1996; Sparrow et al., 2000). A construction containing 2.5 kb of *Xenopus Flk1* 5′ flanking sequences plus first intron sequences is sufficient to drive GFP expression throughout the blood vessels of transgenic embryos (Fig. 2A-C) (Warkman et al., 2004; Doherty et al., 2007). Expression is evident in major vessels including the posterior cardinal veins, intersomitic vessels and the vascular plexus, and is equivalent to endogenous *Flk1* expression at a comparable stage (compare Fig. 2A with Fig. 3A). Reporter expression persists during subsequent development, when GFP fluorescence is observed throughout the smallest vessels of the trunk and tail (Fig. 2B,C) (Doherty et al., 2007).

To determine whether the conserved ETS and KLF binding sites were required for embryonic expression of the *Flk1* gene, the individual sites were mutated in the context of the GFP reporter construction. In these experiments, the γ-crystallin promoter driving GFP expression in the eye was used as a marker for transgenesis. As expected from previous mouse studies (Kappel et al., 2000), mutation of the core ETS sequence reduced transgene expression to almost undetectable levels (n=28) (Fig. 2D,E). To assess the role of the KLF binding site, the consensus sequence in the *Flk1*-GFP reporter construction was altered from CACCC to CGGTC and transgenic embryos were generated. Transgenic embryos showed a dramatic reduction in GFP reporter expression compared with the wild-type construction (n=31) (compare Fig. 2F,G with 2B), but any detectable expression remained endothelial-specific. We conclude from these experiments that the KLF site is required for efficient expression of the *Flk1* gene during embryonic development and that the normal function of the site is to activate transcription.

Previous studies have suggested that KLF2 acts as an inhibitor of *Flk1* expression (Bhattacharya et al., 2005; Dekker et al., 2006). Therefore, we wished to confirm that the KLF site in the enhancer indeed functioned as a positive regulatory element. First, the coding region of luciferase was substituted for GFP in the wild-type and ETS and KLF mutant *Flk*1 reporter constructions. These constructions were transfected into a mouse endothelial cell line (bEnd.3) and luciferase activity was measured. The results indicated that mutation of either the ETS or KLF elements results in a significant reduction in transcriptional activity compared with the wild-type construction (Fig. 2H). Second, in order to investigate the activity of the ETS and KLF binding sites independent of other potential regulatory sequences in the *Flk1* gene, we inserted three tandem copies of the ETS/KLF module upstream of a minimal promoter driving a luciferase reporter. Reporter expression was then assayed in transfected bEnd.3 cells. As shown in Fig. 2I, mutation of either the ETS or the KLF binding site resulted in a reduction in reporter expression in endothelial cells. Taken together, the transgenesis and cell culture studies strongly suggest that the ETS and KLF sites in the *Flk1* enhancer function as positive regulators of *Flk1* transcription.

Table 1. Inhibition of KLF2 function reduces *Flk1* expression

Treatment	Gene expression*		Total number of embryos
	Normal	Reduced	
Control MO (50 ng)	46 (96)	2 (4)	48
Control MO (25 ng)	26 (100)	0 (0)	26
Control MO (12 ng)	21 (100)	0 (0)	21
Klf2 MO (50 ng)	12 (36)	21 (64)	33
Klf2 MO (25 ng)	54 (84)	10 (16)	64
Klf2 MO (12 ng)	14 (93)	1 (7)	15
GFP (500 pg)	51 (98)	1 (2)	52
DR-KLF2 (250 pg)	5 (13)	33 (87)	38
DR-KLF2 (150 pg)	15 (52)	14 (48)	29
DR-KLF2 (75 pg)	22 (69)	10 (31)	32
DR-KLF2 (37 pg)	27 (82)	6 (18)	33

*The number of embryos is shown, with the percentage in parentheses.

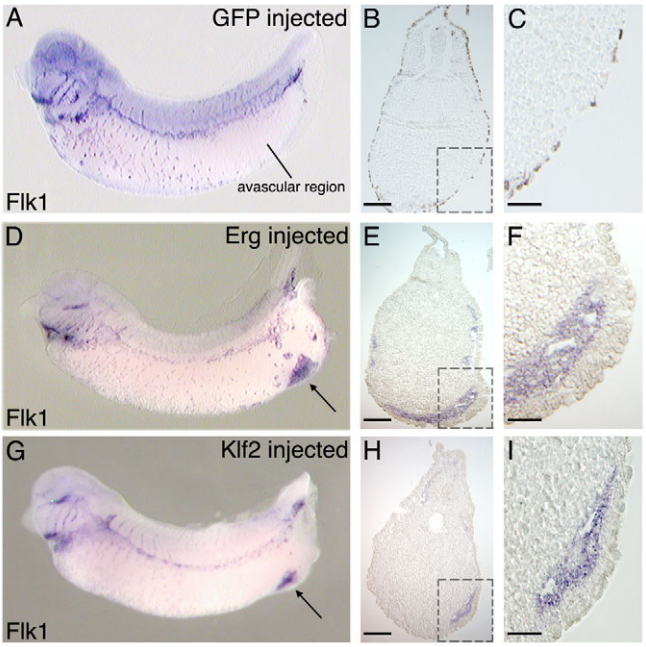

Fig. 4. KLF2 and ERG activate ectopic expression of *Flk1* in *Xenopus* embryos. (**A**) Embryo injected with 500 pg of mRNA encoding GFP and assayed for *Flk1* transcripts by in situ hybridization at stage 34. Injected mRNA was targeted to the posterior avascular region of the embryo. (**B**,**C**) Low- and high-magnification views of a transverse section through the embryo in A. (**D**) Embryo injected with 500 pg of *Erg* mRNA displays ectopic expression of *Flk1* (arrow). (**E**,**F**) Low- and high-magnification views of a transverse section through the embryo in D showing robust ectopic expression of *Flk1*. (**G**) Embryo injected with 500 pg of *Klf2* mRNA shows ectopic expression of *Flk1* (arrow). (**H**,**I**) Low- and high-magnification views of a transverse section through the embryo in G showing strong ectopic expression of *Flk1*. Scale bars: 100 μm in B,E,H; 40 μm in C,F,I.

Klf2 is expressed in endothelial cells in the *Xenopus* embryo

Several ETS family genes, including *Ets1*, *Fli* and *Erg*, are known to be expressed in *Xenopus* embryonic endothelial cells (Stiegler et al., 1990; Stiegler et al., 1993; Meyer et al., 1993; Meyer et al., 1995; Meyer et al., 1997; Baltzinger et al., 1999). For example, whole-mount in situ hybridization for *Flk1* and *Erg* transcripts (Fig. 3A,B) show very similar expression patterns, with transcripts detected in endothelial cells of the posterior cardinal veins, intersomitic vessels, aortic arches and the ventral vascular plexus. Previous reports have shown that *Klf2* is expressed in the developing vasculature of mouse and zebrafish embryos (Kuo et al., 1997; Oates et al., 2001; Lee et al., 2006), but expression of *Klf2* in *Xenopus* has not been reported. As shown in Fig. 3C, *Klf2* is expressed in all major developing blood vessels of the *Xenopus* embryo, in a pattern very similar to *Flk1* and *Erg*. The expression of *Erg* and *Klf2* in early endothelial structures indicates that these factors are present in the correct place at the correct time to regulate embryonic transcription of *Flk1*.

Inhibition of KLF2 function results in reduced transcription of *Flk1* in vivo

To address the function of KLF2 in *Flk1* expression during development, we used an antisense morpholino oligomer (MO) to specifically inhibit the translation of KLF2 in the embryo. The MO effectively blocked translation of a test mRNA transcript in control experiments (Fig. 3D,E). The MO was injected into one cell of the two-cell embryo so that the uninjected side functioned as a time-matched control. *Klf2* MO-treated *Xenopus* embryos were assayed for *Flk1* expression at the tailbud stage by whole-mount in situ hybridization. MO treatment resulted in a major reduction of *Flk1* expression in the vascular plexus, aortic arches, posterior cardinal vein and intersomitic vessels when compared with embryos injected with control MO (compare Fig. 3G,H with 3F). The effects on *Flk1* expression were dose-dependent, with 64% of embryos showing a significant inhibition of transcript levels when 50 ng of *Klf2* MO was injected (Table 1). Quantitation of the reduction by PCR indicated that *Flk1* transcript levels were significantly reduced (Fig. 3L), but were not eliminated, consistent with the results of the transgenic studies and in situ detection of *Flk1* expression (Fig. 2F,G and Fig. 3G,H, respectively).

To complement and extend the MO studies, we used a dominant-repressor form of the KLF2 protein (DR-KLF2) that lacks the transactivation domain but retains the repressor domain (Conkright et al., 2001). Since at least three KLF proteins are expressed in mouse embryonic endothelial cells, we reasoned that a dominant-repressor form of KLF2 might inhibit the function of possibly as yet uncharacterized KLF proteins that are also expressed in the frog vasculature. *Xenopus* embryos were injected with mRNA encoding DR-KLF2 and assayed at the tailbud stage for *Flk1* expression. Similar to the results obtained with *Klf2* MO, DR-KLF2-expressing embryos displayed a significant reduction in *Flk1* transcripts throughout the embryonic vasculature (compare Fig. 3J,K with 3G,H). Again, the effect was dose-dependent, and at the highest levels of DR-KLF2 transcript injected (250 pg), 87% of embryos exhibited a reduction in *Flk1* expression (Table 1). The observation that general inhibition of KLF function using DR-KLF2 was slightly more inhibitory than with *Klf2* MO, raises the possibility that additional KLF proteins might also be involved in the regulation of *Flk1* expression in the frog embryo. Finally, we examined whether knockdown of KLF2 activity inhibited formation of vascular structures in the embryo, as would be expected when FLK1 receptor function is reduced. In histological sections, vascular tubes were absent on the injected side of a *Klf2* MO-treated embryo (Fig. 3M-M″). Taken together, these inhibition studies support a role for KLF proteins in the activation of *Flk1* expression during embryonic development.

Table 2. ERG and KLF2 induce ectopic expression of *Flk1* transcripts

mRNA	Gene expression*		Total number of embryos
	Normal	Ectopic	
GFP (500 pg)	65 (96)	3 (4)	68
Erg (1 ng)	14 (25)	42 (75)	56
Erg (500 pg)	20 (35)	38 (65)	58
Erg (250 pg)	29 (50)	47 (50)	58
Klf2 (1 ng)	6 (21)	23 (79)	29
Klf2 (500 pg)	41 (45)	50 (55)	91
Klf2 (250 pg)	20 (65)	11 (35)	31

*The number of embryos is shown, with the percentage in parentheses.

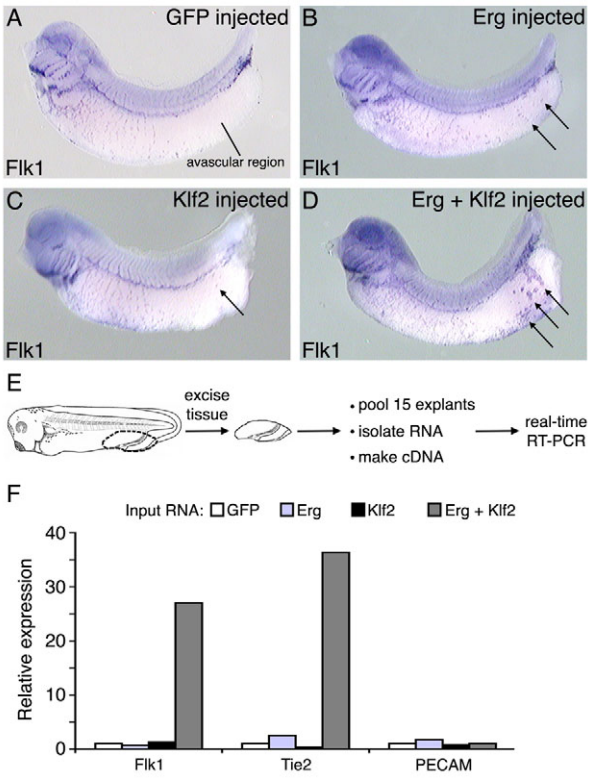

Fig. 5. KLF2 and ERG cooperate to activate expression of *Flk1*. Embryos were assayed for expression of *Flk1* by whole-mount in situ hybridization. (**A**) Embryo injected with *GFP* control mRNA (500 pg). The region showing minimal expression of *Flk1* is labeled as the avascular region. (**B**) Embryo injected with *Erg* mRNA (25 pg) showing very low ectopic expression of *Flk* (arrows). (**C**) Embryo injected with *Klf2* mRNA (150 pg) showing very low levels of ectopic *Flk1* expression (arrow). (**D**) Co-expression of *Klf2* (150 pg) and *Erg* (25 pg) mRNAs resulted in dramatic expansion of ectopic *Flk1* expression (arrows). (**E**) Outline of procedure for dissection of tissue and qRT-PCR analysis of ectopic *Flk1* expression. (**F**) qRT-PCR analysis of *Flk1*, *Tie2* and *PECAM* transcript levels after mRNA injection. Each sample contained tissue from 15 dissections. Samples were normalized to *ornithine decarboxylase* transcript levels. Co-expression of low levels of KLF2 and ERG resulted in synergistic activation of *Flk1* and *Tie2* expression, but *PECAM* was not upregulated.

Table 3. ERG and KLF2 synergize to activate ectopic *Flk1* expression

mRNA	Gene expression*				Total number of embryos
	Normal	Weak, ectopic	Moderate, ectopic	Strong, ectopic	
GFP (500 pg)	65 (96)	3 (4)	0 (0)	0 (0)	68
Klf2 (150 pg)	18 (64)	6 (21)	3 (11)	1 (4)	28
Erg (25 pg)	16 (55)	11 (38)	2 (7)	0 (0)	29
Erg (25 pg) + *Klf2* (150 pg)	6 (22)	6 (22)	6 (22)	9 (33)	27

*The number of embryos is shown, with the percentage in parentheses.

KLF2 and ERG activate *Flk1* expression in vivo

Previous studies have shown that expression of the ETS factor ERG in the *Xenopus* embryo is sufficient to induce ectopic expression of the endothelial marker *X-msr* (Baltzinger et al., 1999). This activation of an endothelial marker is a remarkable observation and has not been replicated with any other class of transcription factor. In order to determine whether expression of ERG is also sufficient to activate ectopic expression of *Flk1*, we injected *Erg* mRNA into the frog embryo. Injections were targeted to ventral blastomeres that contribute to posterior ventral regions of the embryo, which are largely free of endothelial cells. In all mRNA injection experiments, the test mRNA was co-injected with *EGFP* mRNA, which served as a tracer. Injection of *Erg* mRNA resulted in strong, ectopic expression of *Flk1* in a dose-dependent manner (Fig. 4D-F, Table 2), whereas embryos injected with *EGFP* mRNA alone showed no ectopic expression (Fig. 4A-C, Table 2). Significantly, injection of *Klf2* mRNA was also sufficient to activate ectopic expression of the *Flk1* gene (Fig. 4G-I). At the highest doses of *Klf2* examined (1 ng of mRNA), 79% of embryos showed the presence of ectopic *Flk1* transcripts (Table 2). Ectopic expression was observed in both mesodermal and endodermal tissue but apparently not in the ectoderm. These experiments demonstrate that ERG and KLF2 are independently sufficient to activate ectopic *Flk1* transcription in the frog embryo.

KLF2 and ERG cooperate to promote *Flk1* gene expression

ETS proteins are known to interact with many other transcription factors to modulate expression of target genes (Sharrocks, 2001; Oikawa and Yamada, 2003). However, much less is known concerning possible regulatory partners for the KLF family proteins (Atkins and Jain, 2007; Turner and Crossley, 1999). Since our results (Fig. 4) show that ERG and KLF2 are independently capable of activating *Flk1* transcription, and because the ETS and KLF sites are adjacent in the *Flk1* enhancer region (Fig. 1A), we considered the possibility that ETS and KLF proteins might cooperate to activate transcription of *Flk1*. To test this, we first titrated the individual doses of *Erg* and *Kfl2* mRNA to amounts that resulted in little to no ectopic activation of *Flk1* (Fig. 5B,C, Table 3). However, when these limiting amounts of *Klf2* and *Erg* mRNA were co-expressed, robust ectopic expression of *Flk1* was observed in a large proportion of embryos (Fig. 5D, Table 3). The large increase in expression observed when both *Klf2* and *Erg* sequences were present suggests possible synergistic activation of *Flk1* transcription.

To better characterize the effect of *Klf2* and *Erg* co-expression on *Flk1* transcript levels, we carried out quantitative (q) RT-PCR analysis. Posterior ventral regions were dissected from *Xenopus* embryos expressing either GFP control, low doses of KLF2 or ERG alone, or a combination of KLF2 and ERG. These samples were then assayed for *Flk1* transcripts by qRT-PCR (Fig. 5F). Samples expressing *GFP* mRNA showed very low levels of *Flk1* transcripts, probably owing to the presence of a few endogenous endothelial cells in the dissected tissue. As expected, samples expressing KLF2 or ERG alone also showed extremely low levels of *Flk1* transcripts, essentially equivalent to background levels. However, co-expression of KLF2 and ERG resulted in strong, synergistic activation of *Flk1* expression to ~25-fold above background levels. Strong activation was also observed for the vascular-specific angiopoietin receptor gene *Tie2*, but not for the *PECAM* gene. These experiments demonstrate that KLF2 and ERG synergize to activate embryonic expression of *Flk1*. Although cooperation was also observed for *Tie2* expression, failure to activate *PECAM* indicates that not all endothelial genes respond to KLF/ERG co-regulation.

ERG and KLF2 physically associate

The functional cooperation between KLF2 and ERG in the regulation of *Flk1* raised the possibility that the two proteins might physically associate. To test this, we carried out co-immunoprecipitation (Co-IP) experiments using HA-tagged KLF2 protein (HA-KLF2) produced in COS-7 cells, and radiolabeled in vitro translated ERG protein (Fig. 6A, lanes 1 and 2). ERG co-

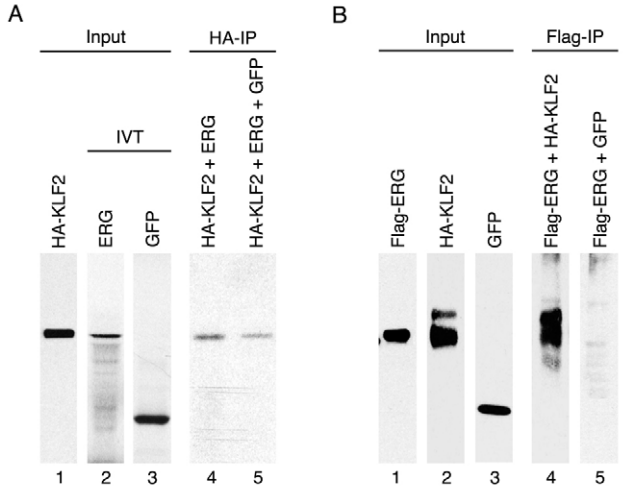

Fig. 6. Physical interactions between KLF2 and ERG proteins. (**A**) HA-tagged KLF2 was produced in COS-7 cells and detected by western blot using an anti-HA antibody. Radiolabeled ERG and GFP were produced by in vitro translation and detected by autoradiography. HA-KLF2 cell extracts were incubated with in vitro translation products and immunoprecipitated with anti-HA antibody. Co-precipitated proteins (HA-IP) were detected by autoradiography. Input for lane 4 contained ERG only and input for lane 5 contained ERG and GFP. (**B**) FLAG-ERG plus HA-KLF2 or GFP were co-expressed in COS-7 cells. Lanes 1-3 show input proteins detected by western blotting. Following immunoprecipitation with anti-FLAG antibody, co-precipitated proteins were detected by western blotting. Lane 4 shows HA-KLF2 recovered following the immunoprecipitation reaction. Lane 5 shows that no GFP is recovered under identical immunoprecipitation conditions.

precipitated with HA-KLF2 (Fig. 6A, lanes 4 and 5), whereas control GFP protein (Fig. 6A, lane 3) failed to associate with HA-KLF2 (Fig. 6A, lane 5). In reciprocal experiments, FLAG-tagged ERG and HA-tagged KLF2 proteins were co-expressed in COS-7 cells. Using anti-FLAG antibody, KLF2 co-precipitated with ERG protein, but no significant precipitation of GFP control protein was observed under the same conditions (Fig. 6B, lanes 4 and 5). These studies indicate that ERG and KLF2 are components of a single physical complex and very likely associate through direct protein-protein interactions.

KLF2 activates additional vascular marker genes

Our studies suggest that KLF2 binds to a site within the *Flk1* enhancer and directly activates transcription of *Flk1*. It would be interesting to determine whether KLF2 also regulates the expression of other vascular marker genes. Using overexpression and MO knockdown approaches, we determined that KLF2 is required for normal expression of both the apelin receptor *X-msr* and the vascular cell-adhesion molecule *VE-cadherin* (Fig. 7, Table 4). These results raise the possibility that KLF2 is involved in the regulation of a considerable number of vascular genes in the embryo, in addition to *Flk1*. It remains to be determined, however, whether this activation is direct, through specific binding of KLF2 to regulatory elements in the *X-msr* and *VE-cadherin* genes, or whether activation is indirect, occurring via other transcription factors.

DISCUSSION

ETS and KLF sites within the *Flk1* enhancer are necessary for expression in vivo

Examination of sequences within the first intron of the *Flk1* gene revealed the presence of paired ETS and KLF binding sites that are conserved in all species examined, from frog to human (Fig. 1A). Previous studies have shown that the ETS site is essential for expression of a *Flk1* reporter in transgenic mice (Kappel et al., 2000), but the KLF site has not previously been recognized. Using a *Flk1* transgenic reporter construction in *Xenopus*, we confirmed the importance of the ETS binding site (Fig. 2D,E) and also demonstrated that the KLF site is essential for efficient *Flk1* expression (Fig. 2F,G). These results demonstrate that both the conserved ETS and KLF motifs in the intronic enhancer play an important role in the developmental regulation of the *Flk1* gene.

KLF2 function is required for embryonic expression of *Flk1*

Numerous studies have demonstrated the importance of ETS proteins for developmental regulation of vascular endothelial gene expression (Dejana et al., 2007; Lelievre et al., 2001; Pham et al., 2007). However, less attention has been directed towards the role of KLF family proteins during early vascular development. At least three KLF genes (*Klf2*, *Klf4* and *Klf6*) are expressed in the endothelium during mouse development (Kuo et al., 1997; Yet et al., 1998; Kojima et al., 2000; Botella et al., 2002; Lee et al., 2006). Similarly, a *Klf2* gene (*klf2a*) is expressed in endothelial cells of the zebrafish embryo (Lee et al., 2006; Oates et al., 2001). Mouse embryos lacking KLF2 or KLF6 function die as embryos owing to defects in the ability to recruit smooth muscle cells to blood vessels and disorganization of the yolk sac vasculature, respectively (Kuo et al., 1997; Matsumoto et al., 2006), but early formation of the embryonic endothelium is normal in these animals. Knockout of *Klf4*, which is also endothelially expressed, results in perinatal death owing to loss of skin barrier function (Segre et al., 1999), but vascular development is again normal. The presence of multiple

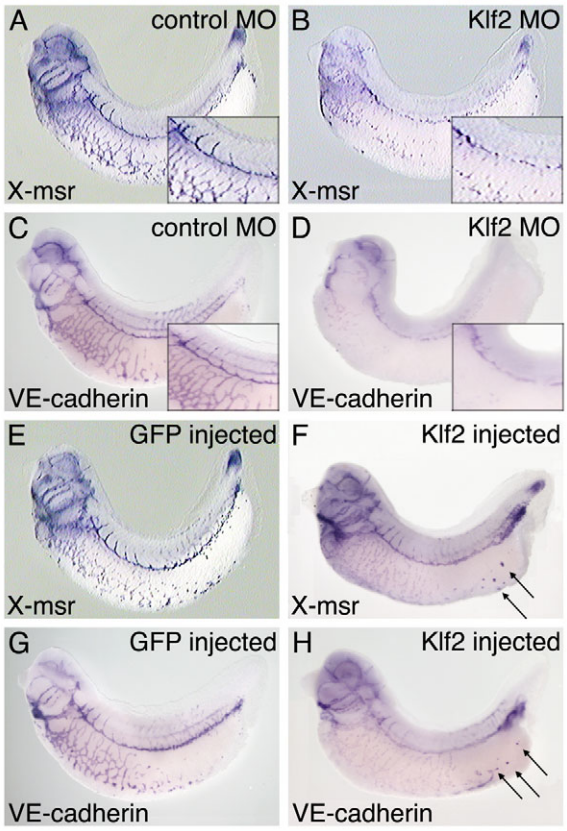

Fig. 7. KLF2 is necessary and sufficient for expression of other vascular marker genes. In situ hybridization analysis of *X-msr* and *VE-cadherin* expression in injected *Xenopus* embryos (stage 34, lateral view). (**A-D**) *Klf2* MO blocks expression of the vascular markers *X-msr* and *VE-cadherin*, as compared with control MO-injected embryos. The insets are higher magnification views, centered on the developing posterior cardinal vein. (**E-H**) Injection of *Klf2* mRNA (500 pg) induces ectopic expression of *X-msr* and *VE-cadherin* (arrows).

KLF proteins in the endothelium raises the possibility of functional redundancy. An argument for functional equivalence of at least some KLF proteins is supported by studies carried out using ES cells, which suggested that any one of three specific KLF proteins (KLF2, KLF4 or KLF5) could substitute for the other two in maintaining ES cells in an undifferentiated state (Jiang et al., 2008). In agreement with observations from other species, we determined that *Klf2* is expressed in early endothelial cells of the *Xenopus* embryo (Fig. 3C). Inhibition of KLF2 function, using either a sequence-specific MO (Fig. 3F-H, Table 1) or more general interference with KLF function using a dominant-repressor construction (Fig. 3I-K, Table 1), results in significant downregulation of *Flk1* expression in the *Xenopus* embryo. We note, however, that inhibition of KLF function never resulted in complete loss of *Flk1* expression. This is consistent with the results of the transgenic studies and cell transfection experiments (Fig. 2F-I), in which mutation of the KLF site resulted in a reduction, but not elimination, of reporter gene expression. Taken together, these results indicate that KLF2 is required for normal regulation of *Flk1* expression in the frog embryo. We cannot exclude the possibility that other KLF genes are expressed in frog embryonic endothelial cells, but the KLF2-specific knockdown studies indicate that any such proteins are insufficient to completely rescue the loss of KLF2 function.

Table 4. KLF2 is necessary and sufficient for expression of *X-msr* and *VE-cadherin*

Marker	Treatment	Gene expression* Normal	Reduced	Total number of embryos
X-msr	Control MO (50 ng)	33 (92)	3 (8)	36
	Klf2 MO (50 ng)	4 (10)	35 (90)	39
	GFP (500 pg)	35 (97)	1 (3)	36
	DR-KLF2 (250 pg)	21 (20)	82 (80)	103
VE-cadherin	Control MO (50 ng)	39 (97)	1 (3)	40
	Klf2 MO (50 ng)	7 (14)	44 (87)	51
	GFP (500 pg)	52 (91)	5 (9)	57
	DR-KLF2 (250 pg)	20 (22)	73 (78)	93
Marker	mRNA	Gene expression* Normal	Ectopic	Total number of embryos
X-msr	*GFP* (500 pg)	34 (94)	2 (6)	36
	Klf2 (500 pg)	5 (13)	33 (87)	38
VE-cadherin	*GFP* (500 pg)	53 (93)	4 (7)	57
	Klf2 (500 pg)	7 (21)	27 (79)	34

*The number of embryos is shown, with the percentage in parentheses.

KLF2 is an activator of *Flk1* expression during vascular development

Our studies consistently indicate that KLF proteins function as activators of *Flk1* expression. First, we have shown that mutation of the KLF site within the first intron of the *Flk1-EGFP* reporter construction results in a major reduction of reporter expression in transgenic embryos (Fig. 2). Second, expression of KLF2 in the frog embryo has the remarkable ability to activate ectopic transcription of *Flk1* (Fig. 4G-I). Third, inhibition of KLF2 activity using MOs or a dominant-repressor protein reduces embryonic expression of the endogenous *Flk1* gene (Fig. 3, Table 1). These observations stand in apparent contradiction to previous studies showing that KLF2 may act as an inhibitor of *Flk1* expression (Bhattacharya et al., 2005; Dekker et al., 2006). When KLF2 was introduced into human primary endothelial cells using an adenoviral vector, levels of both *FLK1* mRNA and protein were reduced (Bhattacharya et al., 2005). Using a reporter construction containing the human *FLK1* promoter, but not the conserved first intron sequences, KLF2 inhibitory activity was found to be mediated through SP1 binding sites close to the transcription start site. Another study suggesting KLF2 inhibition involved microarrays, where *FLK1* was included in a population of genes downregulated following expression of KLF2 in HUVECs (Dekker et al., 2006). In these experiments, gene expression was analyzed after 7 days of forced KLF2 expression, raising the possibility that the effect on *FLK1* transcript levels was indirect. Our results, however, are in broad agreement with studies of mouse KLF6 which indicated that FLK1 expression was reduced in embryoid bodies derived from *Klf6*$^{-/-}$ ES cells (Matsumoto et al., 2006). The most likely explanation for these contrasting observations is that the studies were carried out over different time periods and using different sources of endothelial cells. KLF proteins exhibit both activator and repressor properties (Conkright et al., 2001; Yet et al., 1998) and different regulatory activities are likely to be revealed in distinct cellular contexts, probably through interactions with different partner proteins.

KLF2 and ERG cooperate to activate expression of *Flk1*: a general vascular mechanism?

ETS family proteins frequently interact with partner proteins to regulate gene expression (Lelievre et al., 2001; Sharrocks, 2001; Oikawa and Yamada, 2003). These binding partners act to modulate ETS DNA-binding affinity and transcriptional activity (Li et al., 2000). However, despite many documented interacting proteins, no partner that functions specifically in endothelial cells has been characterized. Our studies suggest that endothelially expressed KLF and ETS family members might function together to regulate transcription of *Flk1*. First, both ERG and KLF2 are expressed in embryonic angioblasts during early vascular development (Fig. 3A-C) and are therefore in the right place at the right time to activate or maintain *Flk1* transcription. Second, we have shown that ERG and KLF2 regulate *Flk1* expression synergistically. When low amounts of *Klf2* and *Erg* mRNA were expressed in the frog embryo, we never observed high levels of ectopic *Flk1* expression (Fig. 5B,C, Table 3). However, when the same low doses were co-expressed, approximately one-third of embryos showed strong ectopic *Flk1* expression (Fig. 5D, Table 3). Quantitation of equivalent experiments using real-time PCR showed that co-expression of ERG and KLF2 resulted in a ~25-fold increase in *Flk1* transcript levels over either ERG or KLF2 alone, strongly suggesting synergistic activation of *Flk1* expression. Finally, we demonstrated that ERG and KLF2 are able to physically associate with each other (Fig. 6A,B). Our experiments do not exclude the possibility that other proteins are present in the complex, and do not demonstrate direct physical interaction between ERG and KLF2, but they do place the two proteins in the same complex. Additional studies will be required to determine whether the ability to form a physical complex is specific for ERG and KLF2 or whether this property is shared by additional family members.

Our studies have focused largely on transcriptional regulation of *Flk1* because this represents one of the earliest and most important steps in the endothelial differentiation program. However, the observed cooperation between ERG and KLF2 might have implications for regulation of additional vascular endothelial genes. ETS binding sites are important for regulation of many endothelial genes (Dejana et al., 2007; Schlaeger et al., 1997; Gory et al., 1999) and recent experiments have shown that a large number of endothelial genes are potentially responsive to KLF2 regulation (Dekker et al., 2006). Although preliminary, we have obtained results showing that KLF2 is required for the expression of other embryonic vascular markers including *X-msr* and *VE-cadherin* and is capable of activating ectopic transcription of these genes (Fig. 7, Table 4). However, until functional studies are carried out, we do not

know whether KLF2 is a direct transcriptional regulator of these genes. In summary, our studies suggest that cooperation between ETS and KLF proteins is important for activation of the vascular program in the embryo. Cooperation might also play a role in the maintenance of normal vascular function and in vascular disease.

We thank Jianzhu Chen for the *HA-Klf2* construction, Ke Xu and Ondine Cleaver for the *Xenopus* VE-cadherin plasmid, and Brad Davidson for comments on the manuscript. P.A.K. is the Allan C. Hudson and Helen Lovaas Endowed Professor of the Sarver Heart Center at the University of Arizona College of Medicine and is supported by the Sarver Heart Center and by the NHLBI of the NIH (grant #HL74184). Deposited in PMC for release after 12 months.

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