INTRODUCTION

The lumen of all blood vessels and heart is covered by endothelial cells which play pivotal roles for the integrity of the cardiovascular structure as well as for physiological functions. In contrast to the physiological and clinical importance, developmental and molecular mechanisms underlying endothelial cell differentiation are only poorly understood. We report the first vascular endothelial cell lineage-specific (including angioblastic precursor cells) 1.2 kb promoter in transgenic mice. Moreover, deletion analysis of this promoter region in transgenic embryos revealed multiple elements that are required for the maximum endothelial cell lineage-specific expression. This is a powerful molecular tool that will enable us to identify factors and cellular signals essential for the establishment of vascular endothelial cell lineage. It will also allow us to deliver genes specifically into this cell type in vivo to test specifically molecules that have been implicated in cardiovascular development. Furthermore, we have established embryonic stem (ES) cells from the blastocysts of the transgenic mouse that carry the 1.2 kb promoter-LacZ reporter transgene. These ES cells were able to differentiate in vitro to form cystic embryoid bodies (CEB) that contain endothelial cells determined by PECAM immunohistochemistry. However, these in vitro differentiated endothelial cells did not express the LacZ reporter gene. This indicates the lack of factors and/or cellular interactions which are required to induce the expression of the reporter gene mediated by this 1.2 kb promoter in this in vitro differentiation system. Thus this system will allow us to screen for the putative inducers that exist in vivo but not in vitro. These putative inducers are presumably important for in vivo differentiation of vascular endothelial cells.

Key words: endothelial cells, visceral mesenchyme, angioblasts, transgenic mouse, mouse development, promoter

SUMMARY

Vascular endothelial cells play essential roles in the function and development of the cardiovascular system. However, due to the lack of lineage-specific markers suitable for molecular and biochemical analyses, very little is known about the molecular mechanisms that regulate endothelial cell differentiation. With the use of such promoters and enhancers in transgenic mice, it will be possible to introduce gene expression specifically in vascular endothelial cells during development. This will provide a useful animal model system to study basic mechanisms for the development and function of the cardiovascular system in vivo. Such promoter and enhancer elements can serve as biochemical and molecular means to decipher the cascades of molecular events that regulate the differentiation of endothelial cells from their presumptive progenitor cells, angioblasts.

Recently, a family of receptor tyrosine kinase genes specifically expressed in mammalian endothelial cell lineage was cloned and characterized (Dumont et al., 1992, 1993; Iwama et al., 1992; Quinn et al., 1993; Sato et al., 1993; Schnurch and Risau, 1993; Ziegler et al., 1993; Yamaguchi et al., 1993; Ziegler et

Vascular endothelial cell lineage-specific promoter in transgenic mice

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al., 1993). Among those endothelial receptor tyrosine kinases, Tie2 (also called Tek) was unequivocally shown to be expressed specifically in endothelial cell lineage including their precursor cells, angioblasts (Dumont et al., 1992; Schurch and Risau, 1993). Thus, we analysed the 5′-flanking region of the Tie2 gene in transgenic mice for its ability to drive the reporter gene, E. coli lacZ (LacZ), expression specifically in endothelial cell lineage.

MATERIALS AND METHODS

Cloning, transgene construction and DNA purification

The genomic DNA clones encoding 5′-flanking region of the Tie2 gene was isolated from the mouse 129/sv genomic library (Stratagene) using the cDNA (Sato et al., 1993). The 5′ flanking genomic fragments were subcloned into the LacZ reporter gene vector, p46D (Desmarais et al., 1992) (Figs 1, 6). For Tie2(5PH)gal, the 5′-flanking 2.0 kb HindIII-HindIII fragment was subcloned into the HindIII site of the p46D vector plasmid and the PstI-PstI fragment was excised for injections. For Tie2(5BH)gal, the insert was excised by digesting the plasmid with BamHI. The 5′-flanking EcoRI-HindIII, SacI-HindIII, SphI-HindIII fragments were subcloned into the Smal and HindIII sites of the p46D plasmid after blunting the 5′-restriction sites by T4 DNA polymerase to construct T2(5RH)gal, T2(Sac)lacZ and T2(SpH)lacZ, respectively. The T2(5RH)gal was digested with SylI and HindIII, and both ends were blunted by T4 DNA polymerase and then plasmid DNA was religated to make T2(5RH)gal. The 5′-flanking sequence of the promoter and promoter-LacZ junction regions of all the constructs were completely sequenced to confirm no mutations had occurred during the subcloning steps. All the promoter-LacZ insert DNAs were excised out by digestion of the construct DNA with XhoI. The restriction enzyme-digested DNA was separated by low melting temperature agarose gel electrophoresis in 1× TAE and the DNA bands were excised. The DNA was purified from the excised gel bands using GELase (Epigennt Enterprises Technologies, Madison, WI) according to the manufacturer’s “High activity” protocol except phenol/chlorophorm extraction steps were included. The DNA was further purified by using Elutip-d columns (Schleicher & Schuell, Keene, NH) and the final DNA was dissolved in 10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA for microinjection. The concentration of the purified DNA was spectrophotometrically determined and the integrity of DNA was analyzed by agarose gel electrophoresis. The final concentration of 2.5 ng/µl DNA was stored at −20°C in small aliquot until microinjection.

Transgenic mice

Microinjection and other surgical procedures were performed as described (Hogan et al., 1986) using either fertilized FVB or B6CBAF1/J (C57BL/6jxCBA/J) oocytes. The established transgenic lines were screened by Southern blot using HybondN (Amersham, Arlington Heights, IL) and the injected DNA fragment as a probe and PCR. The final wash for the Southern blot was in 0.1× SSC, 0.1× SDS at 67°C. For embryos, the yolk sac was lysed in the PCR proteinase-K digestion buffer (McMahon and Bradley, 1990) at 55°C and the heat-inactivated digest was subjected to the PCR reaction. PCR primers are Tie2-T5: 5′-GGGAATCGAAGTGGTGTGATTGT-3′ and GAL-3.1: 5′-AAAGCATCGATGGCTTGGAGAAGT-3′ (Fig. 1). This pair of primers amplifies a Tie2 promoter-LacZ-specific 190bp product. For transgenic embryos carrying T2(5RH)gal construct, the 5′-primer was replaced by T2P11: 5′-TCCAGGTATAGTTTCCT-3′ (Tie2 promoter-LacZ-specific PCR primer). This pair of primers amplifies a 500 bp product. All transgenic lines were maintained on FVB background. For ES cell isolation, the transgenic mice were backcrossed to the mice of mixed strain of 129/sv and C57BL/6j.

LacZ staining

The mid-day of the plug observation was counted as E0.5. The embryos were dissected out in ice-cold PBS and fixed in ice-cold 2% paraformaldehyde, 2 mM MgCl2, 2 mM EGTA, 0.1 M Pipes buffer, pH 6.9 for 15 minutes. The embryos were rinsed with PBS three times for 5 minutes each. The LacZ expression was detected by incubating the embryos at 30°C overnight in 0.1% X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 1 M magnesium chloride, 0.002% NP-40, 0.01% sodium deoxycholate, PBS, pH 7.0. After the staining, embryos were rinsed in PBS and postfixed at 4°C overnight in 2% paraformaldehyde, 0.1% glutaraldehyde, PBS, pH 7.0. For whole-mount photography, the postfixed embryos were rinsed in PBS and equilibrated in 50% glycerol and then in 70% glycerol. For staining embryos older than E13.5 or postnatal animal tissues, the animals or embryos were first perfused with the fixation solution and

**Fig. 1.** Diagram of the 5′-flanking region of the mouse Tie2 gene and the transgenes. The restriction map of the 5′-flanking region of the Tie2 gene is shown at the top. exon I (E1) is indicated as a filled box. ATG translation start site is also indicated. Exon 1 contains approximately 400 bp 5′ untranslated region and 52 bp translated region which encode the complete signal peptide sequence. The PCR primers, Tie2-T5 and GAL-3.1, are also indicated by arrowheads. The first construct, Tie2(5PH)gal, contains upstream PstI-HindIII fragment (2.2 kb upstream from the transcription start site and the 0.3 kb 5′ non-coding sequence) fused to E. coli lacZ followed by SV40 polyadenylation signal sequence. The second construct contains BamHI-HindIII fragment (the 0.2 kb upstream from the transcription start site and the 0.3 kb 5′ non-coding sequence) fused to E. coli lacZ followed by SV40 polyadenylation signal sequence. The third construct contains PstI-HindIII fragment (the 0.2 kb upstream from the transcription start site and the 0.3 kb 5′ non-coding sequence) fused to E. coli lacZ followed by SV40 polyadenylation signal sequence.
the cryosections were prepared before staining. The LacZ staining was performed by incubating the sections on the slides with the same staining solutions as described above.

Whole-mount immunohistochemistry

For whole-mount immunohistochemistry with monoclonal antibody, MEC13.3, to mouse PECAM (Schurch and Risau, 1993; Vecchi et al., 1994), embryos were fixed in 4% paraformaldehyde, PBS at 4°C overnight. The fixed embryos were subsequently rinsed in PBS at room temperature and dehydrated in the series of methanol. The dehydrated embryos were bleached in 5% hydrogen peroxide in methanol for 4–5 hours at room temperature and then rinsed a couple of times with methanol. The bleached embryos were rehydrated and blocked in PBS (3% instant skim milk, 0.1% Triton X-100, PBS) for 1 hour twice. The embryos were then incubated with 1:10 diluted MEC13.3 hybridoma supernatant (provided by Dr Annunziata Vecchi) in PBS at 4°C overnight. On the next day, the embryos were washed with PBS at 4°C five times (1 hour each) and then incubated with horseradish peroxidase-conjugated goat anti-rat IgG (Kirkegaard and Perry Lab., Gaithersburg, MD) in PBS (1:100 dilution) at 4°C overnight. On the third day, the embryos were rinsed in PBS at 4°C five times (1 hour each) and finally in PBT (0.2% BSA, 0.1% Triton X-100, PBS) for 20 minutes at room temperature. The peroxidase staining was performed by incubating embryos in 0.3 mg/ml DAB (Sigma), 0.5% NiCl2 in PBT for 20 minutes followed by the addition of H2O2 to the final concentration of 0.03%. The best signal-to-background ratio was typically achieved by 10 minutes incubation. The staining reaction was stopped by rinsing in PBT and then PBS. The stained embryos were postfixed in 2% paraformaldehyde, 0.1% glutaraldehyde in PBS at 4°C overnight. The detailed protocol for the whole-mount immunohistochemistry is available from the corresponding author upon request.

In situ hybridization

The in situ hybridization was performed essentially as described (Wilkinson et al., 1987) except the probe concentration for hybridization was 12–14 ng/ml. The GALpBSKII(+) was constructed by subcloning the HindIII-EcoRI fragment (3286 bp) of LacZ from pCH110 (Pharmacia) into the pBlueScript SKII (+) (Stratagene). The GALpBSKII(+) was linearized by HindIII digestion and used as a template to synthesize LacZ antisense riboprobe. The nt-301-#101 of the 5′-flanking region of Tie2 (Matthews et al., 1991) was amplified using mouse lung poly(A)+RNA by RT-PCR and subcloned into pBlueScript ISK(+) to make pflk-1(1/2). The pflk-1(1/2) was linearized by NotI digestion for antisense Flk1 riboprobe synthesis. The riboprobe for Tie2 was described elsewhere (Sato et al., 1993). Emulsion-coated slides with LacZ and Flk1 probes were exposed for 7–8 days and the slides hybridized with the Tie2 probe were exposed for 2–4 weeks.

ES cell lines

ES cell lines were established from the blastocysts isolated from the transgenic mice of the mixed strain (FVB/129/sv/C57BL/6J) carrying the T2(5PH)gal transgene. ES cell isolation and cloning was described (Robertson, 1987) and three ES clones carrying the transgene and one wild-type ES cell clone were established. Undifferentiated ES cell lines were maintained in high glucose Dulbecco’s modified Eagle’s media (DMEM) supplemented with 10,000 units/ml purified recombinant mouse leukemia inhibitory factor (ESGRO, Gibco/BRL), 15% selected lots of fetal bovine serum (Hyclone), 2 mM L-glutamine, 0.1 mM non-essential amino acid, 10 mM 2-mercaptoethanol and 300 mM nucleosides mix on confluent feeder layers of mitomycin C-treated primary mouse embryonic fibroblasts. Karyotypes of all the four established ES cell clones were determined (the detail protocol for karyotyping ES cells can be obtained from the corresponding author). Two of the transgenic and the one wild-type ES cell clones were found to have normal karyotype. The ES cells were induced to differentiate in vitro essentially as described (Wang et al., 1992). The dispase-treated ES cell cultures were transferred to the Fisher brand 10 cm bacteriological Petri dishes and cultured in CEB/FLATS media (high-glucose DMEM, 20% selected lots of fetal bovine serum, 2 mM monothioglyserol, 1× gentamycin) for in vitro differentiation. Embryoid bodies and cystic embryoid bodies (CEBs) were processed for whole-mount LacZ staining and immunohistochemistry as described above.

RESULTS

The 1.2 kb 5′-flanking region is sufficient to confer endothelial cell lineage-specific expression in transgenic embryos

The 1.2 kb 5′-flanking region was sufficient to drive expression of LacZ specifically in endothelial cell lineage during embryonic development in all four LacZ-expressing transgensics (Fig. 2). None of the four transgenic lines with the 0.2 kb 5′-flanking region (Fig. 1) expressed LacZ (data not shown). LacZ expression was first detected in subsets of visceral mesenchyme at the late primitive streak stage (embryonic day 7.25–7.50 [E7.25–E7.50]) (Figs 2A, 3A). Interestingly, all the LacZ-positive mesenchyme cells were always close in contact with underlying visceral endoderm (Fig. 3A). At this stage, no morphologically distinguishable hematopoietic cells were found. These LacZ-positive mesenchymal cells were also known to express endogenous Tie2 and another known early endothelial cell lineage-specific receptor tyrosine kinase, Flk1 (Dumon et al., 1992; Millauer et al., 1993; Schurch and Risau, 1993; Yamaguchi et al., 1993). This indicates that LacZ expression driven by the 1.2 kb promoter is a landmark for one of the earliest endothelial cell lineage establishments. During the subsequent somitogenesis, E8.5, LacZ-positive cells were identified in endothelial cells of embryonic proper such as at the pair of dorsal aorta, intersomatic arteries and endocardium as well as in the endothelial cells of extraembryonic yolk sac blood island (Figs 2B-C, 3B-E). As the embryo developed further, expression of LacZ was found to be more restricted to the specific vascular system. At E9.5 (Fig. 2D), LacZ-positive cells were identified in dorsal aorta, aortic arches (I, II and III) and aortic sac. Only weak and patchy LacZ staining was observed in endocardium and intersomitic arteries. Intense LacZ staining was detected in vessels in liver bud. At this stage, abundant LacZ expression was still detected in endothelial cells of the larger vessels in the yolk sac. However, only patchy staining was observed in small capillaries interconnecting large vessels (Fig. 2E). At E10.5 (Fig. 3F), LacZ staining in intersomitic arteries was barely detected. At E11.5 (Fig. 3G), LacZ-positive cells were identified in endothelial cells of embryonic proper such as at the pair of dorsal aorta, ectodermal and endocardium as well as in the endothelial cells of extraembryonic yolk sac blood island (Figs 2F, 3H). As the embryo developed further, expression of LacZ was found to be more restricted to the specific vascular system. At E12.5 (Fig. 3I), LacZ-positive cells were identified in dorsal aorta and third aortic arch. Liver vessels were strongly positive for LacZ staining. Only weak LacZ staining was detected in endocardium. By E12.5 stage, fetal liver sinusoids and aortic arteries were the major LacZ-positive vascular system (Fig. 4A). LacZ expression in liver sinusoidal endothelial cells persisted during the early postnatal period (P1–P2) (Fig. 4P1). However, no LacZ expression was detected elsewhere. In adult (8–10 weeks old) there was no evidence of LacZ expression in any vascular endothelial cells (data not shown). These LacZ stainings in later embryonic and early postnatal stages were not due to the accumulation of LacZ protein in the endothelial cells but rather to the existence of the LacZ transgene mRNA in...
these cells. This was confirmed by the presence of \textit{lacZ} mRNA in these cells (Fig. 4Bb,Bc). Throughout embryonic development, there was no evidence of LacZ expression in hematopoietic lineage (Figs 3, 5). All LacZ-positive cells were found to
co-localize with another endothelial cell-specific marker, PECAM (Fig. 5). These analyses confirmed the endothelial cell lineage-specific expression of the transgene throughout the vascular system development. Furthermore, comparison of the staining patterns of the LacZ and PECAM revealed the heterogeneous expression patterns of the transgene during the vascular system development.

LacZ expression does not correlate with angiogenesis

It was previously shown that Tie2 expression is upregulated in endothelial cells of adult maternal decidua vessels during pregnancy that are undergoing active angiogenesis (Sato et al., 1993; Schnurch and Risau, 1993). However, LacZ expression was not detected in these vessel endothelial cells, although
abundant expression of the endogenous Tie2 and Flk1 transcripts was detected (data not shown).

Multiple elements within the 5’-flanking region are required for the endothelial cell-specific expression

In order to facilitate biochemical analyses of putative transcription factors required for the endothelial cell lineage-specific expression through the Tie2 promoter, further deletion analysis was performed in transgenic embryos (Fig. 6). Each construct DNA was injected into the pronucleus of fertilized eggs and generation 0 embryos were stained for β-galactosidase at 8.5 dpc to 9.5 dpc. The deletion of another 550 bp (down to the 5’-SacI site) did not affect the reporter gene expression pattern. However, deletion of further 220 bp (down to the 5’-SphI site) dramatically affected the reporter gene expression. Only faint and patchy LacZ expression was detectable in the posterior part of the cardinal vein and middle part of the dorsal aorta endothelial cells. No LacZ expression was detected in any other endothelial cells. Furthermore, deletion of the 250 bp SstI-HindIII fragment within the 5’-untranslated region (i.e. within the first exon) completely abolished the reporter gene expression. These results clearly show that multiple elements within the 5’-flanking region of the Tie2 gene are essential for the maximum endothelial cell lineage-specific expression.
The in vitro differentiation of ES cells derived from the transgenic blastocysts

The in vitro system where putative cascades of signals required for this promoter-mediated LacZ expression could be identified was investigated. ES cells were cloned from the heterozygous transgenic blastocysts and their lacZ expression during the in vitro differentiation (Doetschman et al., 1993; Risau et al., 1988; Wang et al., 1992) was studied. Although it is very low level, endogenous Tie2 gene expression was reported during the in vitro differentiation of ES cells (Yamaguchi et al., 1993). The rationale behind this strategy was (1) if the in vitrodifferentiating endothelial cells express LacZ, devising the way to inhibit expression, such as by using inhibitors for growth factor ligand-receptor signalling cascades, second messengers, etc., would provide clues as to how this gene expression might be regulated, and (2) if the in vitro differentiating endothelial cells do not express LacZ, finding the way to induce LacZ expression by modifying the culture conditions, such as by adding exogenous factors, would provide clues for the mechanism of this gene expression. All the ES cell clones that had normal karyotypes were tested for their ability to give rise to vascular endothelial cells. Approximately 64% of the embryoid bodies formed CEBs within 16 days after the induction of in vitro differentiation. About 75% of the CEBs contained the clearly visible PECAM-positive endothelial-like cells which formed the vascular cords (Fig. 7). However, none of these PECAM-positive endothelial-like cells were stained for LacZ (data not shown). The CEBs were also systematically studied from 4 to 16 days of differentiation for PECAM and LacZ expression. Although PECAM-positive blood island-like structures were visible during the early stage of CEB formation, no LacZ-positive cells were detected (data not shown) at any stages. This indicates that there are factors and/or cellular interactions lacking that are required for the
LacZ expression during the normal embryonic differentiation of the vascular endothelial cells. It is now possible using these ES cells to test various reagents and culture conditions to induce the expression of the LacZ transgene.

**DISCUSSION**

This report describes the first endothelial cell lineage-specific promoter in vivo. Not only differentiated endothelial cells, but also subsets of visceral mesenchymal cells were also LacZ positive. These LacZ-positive cells in visceral mesenchyme at this embryonic stage were previously described to express Tie2 and Flk1 (Dumont et al., 1992; Millauer et al., 1993; Schnurch et al., 1993). This indicates that this subpopulation of mesenchyme cells is most likely to be of endothelial cell lineage. It is unknown whether these mesenchyme cells represent hemangioblasts, putative bipotential stem cells for both endothelial and hematopoietic cell lineages (Sabin, 1917; Murray, 1932) or angioblasts, stem cells for endothelial cell-specific lineage (McClure, 1921; Sabin, 1917). Lineage-specific cell ablation in vivo (Evans, 1989) using this promoter may give some clues to distinguish these two possibilities. The direct cellular interaction of the LacZ-positive mesenchyme cells with underlying visceral endoderm (Fig. 3A) may indicate that induction of LacZ in these precursor cells requires direct visceral mesenchyme-endoderm interaction.

The restricted expression of the reporter gene only in certain vascular system endothelial cells may reflect the existence of the tissue-specific inducer for the LacZ expression through this Tie2 promoter in vivo. In order to eliminate the possibility of instability of LacZ protein or accessibility of x-gal or other experimental artifacts that might account for the LacZ-negative endothelial cells, all the sections were also analyzed by in situ hybridization with lacZ riboprobe (data not shown). This in situ hybridization experiment confirmed the LacZ-staining results, which strongly argues against the possibility of the experimental artifacts. The LacZ reporter gene expression is rather more likely mediated by the specific signals localized at these embryonic regions, which include visceral mesenchyme,

![Image](image-url)

**Fig. 6.** Deletion analysis of the Tie2 promoter in transgenic embryos. The restriction sites of the upstream region of the Tie2 gene is indicated at the top and the first exon is indicated as a filled box. The bars indicate the upstream promoter fragments that were fused to the LacZ SV40 poly(A) cassettes and their construct nomenclatures are indicated on the right. Embryos (8.5-9.5 dpc) derived from the injection of both T2(5RH)gal and T2(ScH)LacZ constructs had identical LacZ expression patterns as described with embryos injected with Tie2(5PH)gal. All three LacZ-expressing transgenic embryos (indicated by *) derived from T2(5RH)gal and T2(ScH)LacZ construct exhibited faint and patchy LacZ staining only in a part of the posterosial cardinal veins and in a middle part of the dorsal aorta. No LacZ staining was detected from the 12 transgenic embryos derived from the T2(5RHi5)gal construct injection.

**Fig. 7.** In vitro differentiation of the transgenic ES cells. The whole-mount CEB stained for PECAM (A) and subsequent paraffin-sections (B) are shown. The arrowheads indicate the PECAM-positive endothelial-like cells. None of these PECAM-stained endothelial cells were LacZ positive (data not shown). Erythroblastic cells were also visible within the vessel cord cavity. These erythroblastic cells were stained with benzidine (data not shown). Bar, 25 µm.
extraembryonic blood islands, aortic vascular system and fetal liver. Identification of these signals may shed light on the molecular details of vascular endothelial cell differentiation. For this purpose, it is essential to identify other regulatory elements in the Tie2 gene which are required for the expression of LacZ in the vascular system where the 1.2 kb promoter was unable to confer the expression, such as brain blood vessels. We have tested up to 7.2 kb upstream and approximately 6 kb of the second intron for their ability to confer the reporter gene expression in vascular endothelial cells more ubiquitously. Neither of them showed the endothelial cell-specific enhancer activity in transgenic embryos (data not shown).

Our deletion analysis of the Tie2 promoter identified two small regions (200-250 bp) within the 5'-flanking sequences. One region (SacI-SphI) upstream from the transcription start site did not completely abolish the endothelial cell-specific expression. Its deletion affected the level of reporter gene expression but did not abolish expression completely. However, the other region (Styl-HindIII) within the first exon completely eliminated the endothelial cell expression of the reporter gene. The nucleotide sequence of this 250 bp 5'-untranslated region was found to be extremely conserved between murine and bovine (data not shown). This species conservation may also indicate the importance of this region for the lineage-specific expression. These DNA fragments are small enough to be biochemically analyzed to identify putative transcription factors involved in the endothelial cell lineage-specific gene expression. It is noteworthy that endothelial-cell-specific positive regulation in vitro of another endothelial gene, von Willebrand factor gene, is also mediated by elements in 5'-untranslated region of the gene (Jahroudi and Lynch, 1994).

The in vitro differentiation of ES cells dissociated the morphological differentiation and endothelial cell marker, PECAM, expression from the LacZ expression. This indicates that these endothelial cell differentiation programs are separable. This in vitro system will also allow us systematically to screen putative cellular factors and signals required for this promoter-mediated LacZ expression in endothelial cells.

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