The Down syndrome critical region gene 1 short variant promoters direct vascular bed–specific gene expression during inflammation in mice

Takashi Minami, … , William C. Aird, Tatsuhiro Kodama

*J Clin Invest.* 2009;119(8):2257-2270. [https://doi.org/10.1172/JCI35738](https://doi.org/10.1172/JCI35738).

Down syndrome critical region gene 1 (DSCR-1) short variant (DSCR-1s) is an inhibitor of calcineurin/NFAT signaling encoded by exons 4–7 of *DSCR1*. We previously reported that VEGF induces DSCR-1s expression in endothelial cells, which in turn negatively feeds back to attenuate endothelial cell activation. Here, in order to characterize the role of the promoter that drives DSCR-1s expression in mediating inducible expression in vivo and to determine the functional relevance of DSCR-1s in inflammation, we targeted a DNA construct containing 1.7 kb of the human *DSCR1s* promoter coupled to the *lacZ* reporter to the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) locus of mice. We determined that *lacZ* was uniformly expressed in the endothelium of transgenic embryos but was markedly downregulated postnatally. Systemic administration of VEGF or LPS in adult mice resulted in cyclosporine A–sensitive reactivation of the *DSCR1s* promoter and endogenous gene expression in a subset of organs, including the heart and brain. The *DSCR1s* promoter was similarly induced in the endothelium of tumor xenografts. In a mouse model of endotoxemia, DSCR-1s–deficient mice demonstrated increased sepsis mortality, whereas adenovirus-mediated DSCR-1s overexpression protected against LPS-induced lethality. Collectively, these data suggest that the *DSCR1s* promoter directs vascular bed–specific expression in activated endothelium and that DSCR-1s serves to dampen the host response to infection.

Find the latest version:

http://jci.me/35738-pdf
The Down syndrome critical region gene 1 short variant promoters direct vascular bed–specific gene expression during inflammation in mice

Takashi Minami,1,2 Kiichiro Yano,3 Mai Miura,1 Mika Kobayashi,1 Jun-ichi Suehiro,1 Patrick C. Reid,4 Takao Hamakubo,1 Sandra Ryeom,5 William C. Aird,3 and Tatsuhiko Kodama1,2

1Research Center for Advanced Science and Technology and 2Translational Systems Biology and Medicine Initiative (TBSMI), University of Tokyo, Tokyo, Japan. 3Department of Medicine, Center for Vascular Biology Research, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA. 4PeptiDream Inc., Tokyo, Japan. 5Vascular Biology Program, Department of Surgery, Children’s Hospital Boston, Boston, Massachusetts, USA.

Down syndrome critical region gene 1 (DSCR-1) short variant (DSCR-1s) is an inhibitor of calcineurin/NFAT signaling encoded by exons 4–7 of DSCR1. We previously reported that VEGF induces DSCR-1s expression in endothelial cells, which in turn negatively feeds back to attenuate endothelial cell activation. Here, in order to characterize the role of the promoter that drives DSCR-1s expression in mediating inducible expression in vivo and to determine the functional relevance of DSCR-1s in inflammation, we targeted a DNA construct containing 1.7 kb of the human DSCR1s promoter coupled to the lacZ reporter to the hypoxanthine guanine phosphoribosyl transferase (Hprt) locus of mice. We determined that lacZ was uniformly expressed in the endothelium of transgenic embryos but was markedly downregulated postnatally. Systemic administration of VEGF or LPS in adult mice resulted in cyclosporine A–sensitive reactivation of the DSCR1s promoter and endogenous gene expression in a subset of organs, including the heart and brain. The DSCR1s promoter was similarly induced in the endothelium of tumor xenographs. In a mouse model of endotoxemia, DSCR-1s–deficient mice demonstrated increased sepsis mortality, whereas adenovirus-mediated DSCR-1s overexpression protected against LPS-induced lethality. Collectively, these data suggest that the DSCR1s promoter directs vascular bed–specific expression in activated endothelium and that DSCR-1s serves to dampen the host response to infection.

Introduction
Endothelial cell phenotypes vary in structure and function, in space and time, and in health and disease (reviewed in refs. 1, 2). Endothelial cells are adapted to meet the diverse needs of the underlying tissue. Phenotypic heterogeneity is governed by a combination of extracellular signals and epigenetic forces (reviewed in ref. 3). Under normal conditions, signals from the extracellular environment are integrated at the transcriptional and posttranscriptional levels, resulting in tight control of hemostatic balance, leukocyte trafficking, barrier function, migration, and proliferation. When signaling is excessive, oversustained and/or spatially and temporally misplaced, pathology may ensue. Among the important regulatory mechanisms in the endothelium is negative feedback inhibition of transcription networks.

The Down syndrome critical region gene 1 (DSCR1) (also known as MCIP-1, calcipressin-1, Adapt 78, and RCAN), so designated because it resides within the Down syndrome critical region of human chromosome 21, encodes a protein that binds to and inhibits the catalytic subunit of calcineurin (4–6). The DSCR1 gene includes 7 exons and 6 introns and encodes 4 different isoforms. The two major isoforms are referred to as DSCR-1 long variant (DSCR-1L) and DSCR-1 short variant (DSCR-1s). DSCR-1L contains exons 1, 5, 6, and 7, whereas DSCR-1s contains exons 4, 5, 6, and 7. The two isoforms are differentially regulated; the s’ promoter drives Notch/Hes-1–inhibitable expression of DSCR-1L (7), while an intergenic promoter located between exons 3 and 4 mediates calcineurin/NFAT–dependent expression of DSCR-1s (8).

We recently reported that VEGF and thrombin result in dramatic and rapid upregulation of DSCR-1s (8). VEGF-mediated induction of DSCR-1s was shown to involve the cooperative binding of NFATc and GATA2/3 to neighboring consensus motifs in the upstream promoter. DSCR-1s, in turn, feeds back to inhibit calcium/calcineurin/NFAT signaling, resulting in attenuation of inflammation and angiogenesis (8).

Here, we wished to extend our studies to the in vivo setting. We asked whether the DSCR1s promoter, as delineated in our cell culture studies, contains information for inducible expression in vivo. Moreover, we tested the hypothesis that DSCR-1–mediated attenuation of inflammation influences the host innate immune response. Here, we show that the DSCR1s promoter directs widespread basal expression of lacZ throughout the vascular tree in the developing embryo, while reporter gene activity is significantly downregulated in the postnatal period. However, in the adult, the DSCR1s promoter is upregulated in different vascular beds in the...
response to VEGF and LPS and as a consequence of tumor growth. In addition, mice with a targeted deletion of both DSCR-1 isoforms demonstrated increased sepsis mortality, while overexpression of DSCR-1s protected against LPS-induced lethality. Taken together, the results provide new insights into vascular bed–specific expression of DSCR-1s in vivo and point to DSCR-1s as a potential therapeutic target in vascular inflammation.

Results

Hprt-targeted DSCR-1 intergenic promoter directs age-dependent expression in the intact endothelium. In a previous study, we demonstrated that VEGF and thrombin induced the expression of DSCR-1s in HUVECs, resulting in secondary attenuation of calcium/NFAT signaling (8). A region of the DSCR1s promoter between −1,664 and +83 was shown to contain information for VEGF and thrombin responsiveness (8). To determine whether the same promoter region directed inducible expression in vivo, we coupled the −1,664/+83 DSCR1s promoter to the lacZ reporter gene and targeted the resulting transgenic cassette (DSCR-1–lacZ) to the Hprt locus of mice using homologous recombination (Figure 1A). Four independent high-percentage male chimeras, derived from 2 recombinant ES cell clones, were bred with wild-type mice to achieve germline transmission. F1 agouti females were mated with DSCR-1–lacZ-Hprt hemizygous males and harvested for embryos at various gestational ages. At E11, whole-mount lacZ staining revealed widespread expression of the transgene in the vasculature (Figure 1B). In cryosections, strong staining was observed in the dorsal aorta, intersomatic vessels, carotid arteries, caudal veins, the primary head vein branch, and the endocardium (Figure 1C shows dorsal aorta and intersomatic vessels). lacZ colocalized with endothelialPECAM-1 (data not shown). At E14, lacZ expression was markedly downregulated (data not shown). At E19, reporter gene activity was further reduced, with detectable expression limited to the endocardium, umbilical artery, and umbilical vein (data not shown). In adult mice, DSCR-1–lacZ activity was detected in only a subset of endothelial cells in the brain, heart, lung, and kidney (Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI35738DS1). Expression was also observed in occasional neurons, vascular smooth muscle cells, cardiomyocytes, and renal epithelial cells. In contrast, DSCR-1s–lacZ activity was undetectable in the liver, spleen, thigh skeletal muscle, and thymus (Supplemental Figure 1, E and F, shows liver and skeletal muscle, respectively). These findings suggest that the DSCR1s promoter, though widely expressed in the endothelium of E11 embryos, is downregulated in later stages of development and in adults.

Hprt-targeted DSCR-1 intergenic promoter directs vascular bed–specific expression in response to VEGF and LPS. Next, we wished to determine whether the DSCR1s promoter confers response to inflammatory stimuli in vivo. To that end, DSCR-1s–lacZ mice were systemically administered VEGF (500 μg/kg, i.v.) or LPS (10 mg/kg, i.p.). Organs were harvested 6 hours later and stained for lacZ. In whole-mount preparations, the X-gal reaction product was detectable in the brain and heart of untreated mice and was further upregulated by VEGF and LPS (Figure 2A and B). In contrast, lacZ staining was not observed in skeletal muscle under any condition (Figure 2C shows control and LPS treatment). By comparison, mice in which the Flt1 promoter was targeted to the Hprt locus (Flt-1–lacZ) demonstrated β-galactosidase activity in skeletal muscle vasculature (Figure 2C). Flt1 promoter activity was not altered by treatment with LPS or VEGF, suggesting that the stimulatory effect of these agonists is specific to the DSCR1s promoter.

Tissue sections from the DSCR-1s–lacZ mice revealed inducible expression of the promoter in the aorta, brain, lung, and kidney but not in the spleen, liver, or thymus (Supplemental Figure 2, A–D, and data not shown). VEGF-mediated induction of the DSCR-1 transgene in the aorta was restricted to the endothelial lining, as evidenced by colocalization of lacZ and PECAM-1 (Supplemental Figure 2A). In contrast, LPS-mediated induction of the transgene occurred in both the endothelial and smooth muscle cell layers of the aorta (Supplemental Figure 2A). Both VEGF and LPS significantly enhanced reporter gene expression in the microvascular endothelium of the brain (Supplemental Figure 2B). In the lung,
systemic administration of VEGF and LPS resulted in increased transgene expression in both micro- and macrovascular endothelium (Supplemental Figure 2C). In the kidney, LPS administration increased lacZ expression in PECAM-1–negative cells of the glomerulus and proximal tubule, while VEGF administration induced expression mainly in glomerular endothelial cells (Supplemental Figure 2D). To determine whether expression of the transgene correlated with that of the endogenous gene, immunostaining was performed using a mouse monoclonal anti-DSCR-1 antibody. VEGF stimulated DSCR-1 expression in the endothelial lining of multiple organs in a pattern that correlated with that of lacZ (Supplemental Figure 3 shows heart and kidney). Together, these data suggest that inflammatory mediators result in vascular bed–specific induction of DSCR1s promoter activity.

Real-time PCR analysis was used to quantify changes in transgene expression. Under basal conditions, lacZ mRNA expression was highest in the heart, followed by the brain, lung, and kidney (Figure 3A). Expression in skeletal muscle, spleen, and liver was below the level of detection. VEGF and LPS resulted in significant induction of lacZ transcripts in the heart (4.5-fold and 8.1-fold, respectively), brain (2.9-fold and 4.0-fold, respectively), lung (2.7-fold and 4.2-fold, respectively), and kidney (3.1-fold and 6.2-fold, respectively), but not in spleen, liver, or skeletal muscle (Figure 3A). LPS-mediated induction of the endogenous Dscr1s gene was similarly restricted to the heart (25.3-fold), brain (7.0-fold), lung (10.3-fold), and kidney (9.3-fold) (Figure 3B). We have previously shown that i.v. administration of VEGF results in phosphorylation of Flk-1 in multiple organs, including those in which DSCR-1s is not induced (e.g., liver and skeletal muscle) (9). To confirm that this was the case in the current study, we carried out immunoprecipitation and Western blot analysis for phospho–Flk-1 in the lung, heart, skeletal muscle, liver, and spleen of control and VEGF-treated mice. Ten minutes after i.v. administration of VEGF, phosphorylation of Flk-1 was induced in the lung, heart, skeletal muscle, and liver, with comparable ratios of phospho–Flk-1 versus total Flk-1 (Supplemental Figure 4A). These results argue against organ-specific differences in Flk-1 signaling in mediating differential induction of DSCR1s. Moreover, both VEGF and LPS induced expression of Egr-1 in all organs except the brain, indicating that these agonists result in widespread activation, even in DSCR-1s–negative tissues (Supplemental Figure 4B). Finally, agonist treatment failed to alter DSCR-1L transcript levels (Figure 3B). Thus, VEGF and LPS promote vascular bed–specific expression of both the DSCR1s promoter and the endogenous Dscr1s gene.

Hprr-targeted DSCR-1 intergenic promoter directs expression in tumor endothelium. Solid tumors produce a variety of proangiogenic molecules and inflammatory cytokines, which have important paracrine effects on surrounding endothelial cells. Thus, we investigated whether the DSCR-1s transgene is activated in tumor blood vessels. To that end, B16-F1 melanoma and Lewis lung carcinoma (LLC) cells were implanted s.c. into the flank of DSCR-1s–human prolactin (Hprt)–targeted DSCR-1 intergenic promoter-directed mice. (A and B) Comparative whole-mount lacZ stains of thigh skeletal muscle from Flt-1-lacZ-Hprt or DSCR-1s-lacZ-Hprt mice treated with LPS (right panel) or without (left panel) or with VEGF (right panel).

Figure 2
Systemic administration of VEGF and LPS results in organ-specific increases in DSCR1s promoter activity. (A and B) Comparative whole-mount lacZ stains of brain (A) and heart (B) taken from untreated (control), LPS-treated, or VEGF-treated mice. (C) Whole-mount lacZ stains of thigh skeletal muscle from Flt-1-lacZ-Hprt or DSCR-1s-lacZ-Hprt mice treated without (left panel) or with LPS (right panel).
The Journal of Clinical Investigation

Volume 119 Number 8 August 2009

in endothelial cells. To that end, human microvascular endothelial cells (HMVECs) were transiently transfected with a plasmid containing 1.7 kb DSCR1s promoter coupled to luciferase (DSCR-1s–luc). The transfected cells were treated in the absence or presence of various agonists. Consistent with our data in HUVECs, VEGF and thrombin stimulation of microvascular endothelial cells resulted in 11- and 8.2-fold induction of promoter activity, respectively, while TNF-α had only a modest effect on promoter expression (1.5-fold induction) (Figure 5A). In contrast, LPS failed to alter luciferase activity (Figure 5A). A similar pattern of response was observed for the endogenous Dscr1s gene (Figure 5B). These data suggest that the effect of LPS on DSCR-1s expression in vivo is mediated by secondary signals.

We reported that VEGF- and thrombin-mediated induction of DSCR-1 involves the cooperative binding of NFATc and GATA2 to neighboring consensus motifs in the upstream promoter (located at positions −220 and −254, respectively) (4). GATA2, NFATc1, NFATc2, and/or NFATc3 are expressed in HMVECs (data not shown). To delineate which of these factors are responsible for mediating inducible expression of DSCR-1s, we employed siRNA to knock down their expression in HMVECs. The various siRNAs resulted in more than 75% downregulation of their respective target genes (Supplemental Figure 5). GATA2 knockdown significantly inhibited (63%) VEGF-mediated induction of DSCR-1s mRNA, whereas single knockdown of NFATc1, NFATc2, or NFATc3 had no such effect (Figure 5C). In contrast, the VEGF

Figure 3
Systemic administration of VEGF and LPS results in organ-specific increases in lacZ and DSCR-1s mRNA expression. (A) Real-time PCR quantitation of lacZ expression levels in various organs from control mice, VEGF-treated mice, or LPS-treated mice. Data are expressed as mean ± standard deviation; n = 5 in each treatment. *P < 0.04, **P < 0.01 compared with mock treatment in each organ. (B) Real-time PCR quantitation of DSCR-1L and DSCR-1s mRNA expression levels in various organs from LPS-treated mice. The results are the mean and standard deviation of expression levels relative to cyclophilin A, derived from at least 4 independent mice. †P < 0.001 compared with DSCR-1s expression levels without LPS treatment.
with conditioned medium from LLC and B16-F1 melanoma cells
luciferase activity. As shown in Figure 6A, incubation of HMVECs
and assayed the cells 2 hours later for
mediating inducible DSCR-1s expression. GATA2 (Figure 5C). Together, these data support an essential
resulted in a 7.2-fold and a 5.5-fold in
of solid tumors are exposed to a relatively hypoxic environment.

To investigate whether tumor-secreted factors induce DSCR1s
promoter activity, we incubated DSCR1s-luc-transfected
HMVECs were incubated in the absence or presence of tumor
cell–conditioned medium and assayed the cells 2 hours later for
luciferase activity. As shown in Figure 6A, incubation of HMVECs
with conditioned medium from LLC and B16-F1 melanoma cells
resulted in a 7.2-fold and a 5.5-fold in DSCR1s promoter activation,
respectively, while the medium from normal skin fibroblasts failed
to upregulate the DSCR1s promoter. Cells residing in the inner core
of solid tumors are exposed to a relatively hypoxic environment.

Thus, we asked whether hypoxia induced DSCR1s promoter activity
in transiently transfected HMVECs. As shown in Figure 6A, luciferase activity was not upregulated in cells grown in 2% oxygen for
24 hours. Promoter induction by tumor cell–conditioned medium was significantly inhibited by neutralizing anti–Flk-1 antibodies
(Figure 6A). These data are consistent with a role for tumor-derived VEGF in mediating DSCR1s expression. However, the inhibitory
effect was not complete, suggesting a role for additional tumor-
derived mediators in this process. Finally, combined treatment
with siGATA2 and cyclosporine A (CsA) completely blocked the
effect of LLC-conditioned medium on DSCR1s promoter activation
(Figure 6A). Thus, tumor cell–mediated induction of DSCR1s involves GATA2- and NFATc-dependent pathways.

To confirm that endogenous DSCR1s protein levels are similarly
regulated by GATA2 and NFATc activity, we carried out Western blot analyses. As shown in Figure 6B, VEGF- or thrombin-mediated induction of DSCR1s protein expression was profoundly inhibited by CsA. In addition, the VEGF effect was significantly attenuated by siRNA against GATA2 (Figure 6C). Similar results were observed for tumor cell–conditioned medium (Figure 6D).
Together with the real-time PCR findings, these findings support
an essential role for GATA2 and NFATc1, -c2, and -c3 transcription factors in mediating inducible DSCR1s expression.

NFATc and GATA2 regulate inducible expression of DSCR1s in vivo. To
determine the role of NFAT in mediating DSCR1s expression in vivo, we pretreated DSCR1s-lacZ-Hprt mice with CsA (1 mg/kg i.p.
every second day for 2 weeks) prior to VEGF administration. CsA significantly inhibited VEGF induction of lacZ (Figure 7, A and B,
shows aorta and lung; Supplemental Figure 6, A and B, shows diaphragm and chest wall). In B16-F1 melanoma xenografts, injection
with 1 mg/kg CsA every other day for 20 days resulted in a profound reduction in lacZ staining (Figure 7C). To elucidate the role of GATA2 in mediating inducible expression of DSCR1s in vivo, we employed a microRNA against GATA2 (miGATA2). Treatment of cultured mouse endothelial cells (MS-1 cells) with miGATA2, but not miControl, significantly (89%) inhibited expression of
GATA2 (Supplemental Figure 7A). Systemically administrated miGATA2 resulted in marked downregulation of GATA2 protein
expression in aortic endothelium (Supplemental Figure 7B) and GATA2 mRNA expression in aorta and lung (Supplemental Figure 7C). Importantly, miRNA against GATA2 significantly reduced VEGF-mediated induction of lacZ expression in the diaphragm, aorta, chest wall, and lung (Figure 7, D and E, shows aorta and lung; Supplemental Figure 6, C and D, shows diaphragm and chest wall). To determine the effect of miGATA2 on DSCR1s promoter activity in tumor endothelium, B16 melanoma cells were implanted subcutaneously in mice in the presence of adenovirus expressing either miGATA2 or miControl (Ad-miGATA2 or

Figure 4
GATA- and NFATc-dependent DSCR1s promoter and protein expres-
sion is upregulated in tumor vasculature. (A) Upper panels: lacZ stain-
ngs were performed with 10-μm cryosections of LLC and B16-F1 melano-
ma xenografts. Lower panels: Merged images of immunofluorescence
staining with antibodies against lacZ (green) or PECAM-1 (red) or with
DAPI (blue). lacZ/PECAM-1 double-positive cells are yellow/orange.
Scale bars: 50 μm. (B) Cryosections from LLC and B16-F1 melanoma
xenografts were stained with anti–DSCR1-1 antibody (green), anti–
PECAM-1 antibody (red), and DAPI (blue). Merged images are shown
in the lower panels. Scale bars: 50 μm.

response was inhibited 32% by double knockdown of NFATc1 and NFATc2; 52% by combined knockdown of NFATc1, NFATc2, and NFATc3; and 94% by siRNA against NFATc1, NFATc2, and GATA2 (Figure 5C). Together, these data support an essential role for GATA2 and NFATc1, -c2, and -c3 transcription factors in mediating inducible DSCR1s expression.
Ad-miControl). After 2 weeks, the mice received intratumor injection of $1 \times 10^{10}$ PFU of Ad-miGATA2 or miControl. Three days later, xenografts were harvested, sectioned, and stained for lacZ. These studies show that DSCR1s promoter activity in tumor vessels was abolished in the presence of miRNA against GATA2 (Figure 7F).

Together, these data strongly suggest a role for NFATc and GATA2 in the induction of DSCR-1s expression in the endothelium.

DSCR-1s attenuates endothelial cell activation in vivo. We previously demonstrated that DSCR1s attenuates VEGF-mediated activation of cultured endothelial cells (10). Together with our present find-
ings, these data led us to hypothesize that VEGF- and LPS-inducible expression of DSCR-1s in mice may serve as a negative feedback inhibitor of endothelial activation in vivo. To test this hypothesis, we examined the effect of DSCR-1 deficiency or overexpression on endotoxemia phenotype. The generation of Dscr1–/– mice, which carry a targeted deletion of both DSCR-1s and DSCR-1L, has been previously described (11). To overexpress DSCR-1s, we employed an adenoviral delivery system in which the endothelium-specific Flt1 promoter is coupled to DSCR-1s followed by an IRES-EGFP cassette (Ad-Flt1-DSCR-1s). As evidence of its cell type specificity, Ad-Flt1-DSCR-1s resulted in detectable EGFP signal and DSCR-1s protein expression in HMVECs but not in B16-F1 melanoma cells (Supplemental Figure 8, A and B).

In the next series of experiments, mice were injected intravenously with Ad-Flt1-DSCR-1s or control adenovirus. Seven days later, organs were harvested and assayed for DSCR-1s and EGFP expression by real-time PCR and immunofluorescence microscopy, respectively. Ad-Flt1-DSCR-1s resulted in significantly increased DSCR-1s mRNA expression in the heart (278-fold), lung (953-fold), and liver (264-fold) (Supplemental Figure 8C). Immunostaining revealed colocalization of EGFP and PECAM-1 in endothelial cells of multiple organs (Supplemental Figure 8D, and data not shown). Thus, in vivo delivery of Ad-Flt1-DSCR-1s results in overexpression of DSCR-1s in the intact endothelium of mice.

Endotoxemia in mice is associated with a reduction in heart rate, blood pressure, and body temperature and an increase in circulating IL-6 levels (12, 13). This effect was accentuated in Dscr1–/– mice (Supplemental Figure 9) and attenuated in DSCR-1s–overexpressing animals (Supplemental Figure 9). Although DSCR-1 deficiency has been linked to cardiac hypertrophy (14, 15), there was no gross evidence of cardiac abnormalities in control or LPS-treated mice (data not shown).

We previously reported that endotoxemia in mice is associated with increased circulating levels of VEGF (16, 17). Interestingly, resting levels of plasma VEGF were 5.0-fold higher in Dscr1–/– mice compared with wild-type littermates (Figure 8A). In response to LPS treatment, Dscr1–/– mice demonstrated super-induction of circulating VEGF levels (2.2-fold higher vs. wild-type mice) (Figure 8A). Ad-Flt1-DSCR-1s–injected mice had no change in resting VEGF levels. However, in response to endotoxemia, DSCR-1s–overexpressing mice demonstrated a
LPS-mediated induction of cell adhesion molecules was attenuated by overexpression of DSCR-1s (Figure 8B).

Next, we employed Mac1 staining to assay for leukocyte infiltration in the heart, lung, and liver. As shown in Figure 9, DSCR-1–null mutation resulted in increased numbers of activated leukocytes in the heart and lung (2.7- and 2.0-fold, respectively) compared with wild-type littermates. No such changes were observed in the liver, where inflammatory mediators failed to upregulate endogenous Dscr1s (see Figure 3B). In contrast to the effect of DSCR-1 knockout, overexpression of DSCR-1s resulted in a marked reduction in leukocyte infiltration in the heart, lung, and liver.

We have recently shown that hyperactivation of the VEGF/calcineurin/NFAT pathway triggers apoptosis in DSCR-1–deficient tumor endothelial cells (18). Given that Dscr1–/– mice have elevated circulating levels of VEGF (Figure 8A), we hypothesized that endotoxemia may result in increased endothelial cell apoptosis in Dscr1–/– mice. To test this hypothesis, we carried out TUNEL assay in tissue sections from the heart and lung of LPS-treated DSCR-1–null mice and their wild-type littermates. As shown in Figure 10A, endotoxemic wild-type mice demonstrated a small number of TUNEL-positive endothelial cells in the heart and even fewer in the lung. However, in Dscr1–/– mice, LPS administration resulted in a marked increase in the number of TUNEL-positive cells in both organs.

Finally, in survival studies, LPS-treated Dscr1–/– mice demonstrated markedly increased mortality compared with endotoxemic wild-type littermates (Figure 10B, left). In contrast, Ad-mediated overexpression of DSCR-1s conferred a survival advantage compared with Ad-Flt1-control (Figure 10B, right).

**Discussion**

In the present study, we show that expression of the DSCR-1s isoform is temporally and spatially regulated in the intact endothelium, and we provide new evidence for a critical role of DSCR-1s in the host response. The intergenic Dscr1s promoter exhibited robust and uniform endothelial expression during the critical embryonic stages of vasculogenesis and angiogenesis. However, the Dscr1s promoter was markedly downregulated after E14 and in the adult exhibited only low-level, vascular bed–restricted activity in the heart, brain, lung, and kidney. These findings suggest that the DSCR-1s isoform plays a particularly important role during embryonic development. In contrast to DSCR-1s, the DSCR-1L isoform is preferentially expressed in the fetal brain, brain, lung, and kidney. These differences in expression between DSCR-1s and DSCR-1L may indicate that the two isoforms have unique and differing functions.

We and others have previously reported that VEGF induces expression of DSCR-1s in cultured endothelial cells (8, 20). Here, we have extended these results by showing that systemically administered VEGF stimulates DSCR-1s expression and promoter activity in selected vascular beds in vivo, including the heart, brain, lung, and kidney. In contrast, DSCR-1L expression was unchanged. Vascular bed–specific induction of DSCR-1s did not simply reflect local differences in VEGF signaling. Indeed, VEGF administration resulted in phosphorylation of the VEGF receptor Flk-1 in otherwise DSCR-1s–negative organs such as the liver. These findings suggest that differential expression of DSCR-1s in response to VEGF is mediated at a post-receptor level.

The systemic administration of LPS also induced organ-specific expression of DSCR-1s. However LPS, compared with VEGF, treat-
**Figure 8**

DSCR-1 levels influence inflammatory marker expression in sepsis. Wild-type mice, Dscr1–/– (KO), or mice injected with Ad-Flt1-Control (Ctrl) or Ad-Flt1-DSCR-1 (DSCR-1) were injected i.p. with or without 16 mg/kg LPS for 20 hours. (A) Blood was harvested for plasma, and VEGF levels were measured using ELISA. Data are expressed as mean ± standard deviation; n = 10. *P < 0.001, **P < 0.01 compared with wild-type or Ad-Flt1-control. (B) Heart and lung were harvested and assayed by real-time PCR for E-selectin, ICAM-1, and VCAM-1 mRNA expression. Data are expressed as mean ± standard deviation; n = 4. †P < 0.001, ‡P < 0.01, §P < 0.05 compared with wild-type or Ad-Flt1-control injected with LPS.
ment of cultured endothelial cells had little effect on DSCR-1s expression. This discordance suggests that endotoxemia-associated induction of DSCR-1s results not from direct LPS-mediated Toll-like receptor 4 signaling in endothelial cells, but rather from secondary effects of LPS. For example, endotoxemia is associated with elevated levels of VEGF and thrombin, both of which have been implicated in sepsis pathophysiology (17, 21) and shown to induce expression of DSCR-1s in endothelial cells (8, 10). Further studies are needed to determine the molecular basis for vascular bed–specific regulation of DSCR-1s expression under both basal and inducible conditions. The DSCR-1s–lacZ mice generated in this study should serve as a useful tool for dissecting these mechanisms.

Using siRNA or adenoviral miRNA approaches to downregulate transcription factor expression in endothelial cells and mice, respectively, we have shown that the DSCR1 promoter is activated via NFATc1, c2, and c3 and GATA2. There is evidence that DSCR-1L, which lacks NFAT consensus elements, is under the control of the Notch pathway. For example, during keratinocyte differen-

---

**Figure 9**
DSCR-1 levels influence inflammatory leukocyte infiltration rate in sepsis. Heart, lung, and liver of wild-type mice, Dscr1−/− mice, or mice injected with Ad-Flt1-control or Ad-Flt1-DSCR-1 were harvested, cryosectioned, and stained with anti-Mac1 antibody. Mac1-positive activated leukocytes (brown) were quantified based on the analysis of 6 independent sections (bar graphs). Data are expressed as mean ± standard deviation. *P < 0.01, compared with wild-type or Ad-Flt1-control injected with LPS. Scale bars: 50 μm.
Figure 10
DSCR-1 modulates endothelial cell survival and sepsis mortality. (A) LPS-treated heart and lung were harvested and processed for TUNEL (green), PECAM-1 (red), or DAPI (blue) staining. Arrows indicate TUNEL-reactive apoptotic cell nuclei. Scale bars: 50 μm. (B) Survival studies were carried out in Dscr1−/− (KO) or wild-type littermates administered 16 mg/kg LPS i.p. (left panel). Alternatively, mice were administered 5 × 10⁹ PFU of Ad-Flt1-control or Ad-Flt1-DSCR-1 i.v. and 3 days later received 16 or 18 mg/kg LPS i.p. (middle and right panels). Percentages of mice (surviving/total) are indicated.
tiation, increased Notch activity was shown to downregulate DSCR-1L by promoting binding of Hes-1 to the exon 1 promoter (7). In non-endothelial cells, DSCR-1L functions like DSCR-1s in inhibiting calcineurin (22–24). Indeed, in keratinocytes, Notch 1/Hes-1–mediated reduction of DSCR-1L levels led to an accentuation of calcineurin/NFAT signaling, which in turn is predicted to activate the DSCR1s promoter. It is interesting to speculate that a similar mechanism of gene regulation is operative in endothelial cells. However, a recent study demonstrated that DSCR-1L does not inhibit, but rather activates calcineurin/NFAT in endothelial cells and promotes angiogenesis (25). Clearly, further studies are required to delineate the regulatory mechanisms and functions of DSCR-1s and DSCR-1L in the endothelium.

As an endogenous inhibitor of calcineurin, DSCR-1s acts in a negative feedback regulatory loop to attenuate endothelial cell activation. For example, DSCR-1s has been shown to suppress VEGF induction of E-selectin, VCAM-1, tissue factor, and COX-2 in cultured endothelial cells (20). Conversely, siRNA-mediated knockdown of DSCR-1 accentuated VEGF stimulation of tissue factor, E-selectin, and VCAM-1, while knockdown of NFATc1 blocked gene expression (20). In other studies, DSCR-1s overexpression in HUVECs inhibited VEGF or thrombin induction of tissue factor, IL-8, VCAM-1, E-selectin, monocyte chemoattractant protein–1 (MCP-1), and ICAM-1 (8, 10). To determine whether DSCR-1s plays an antinflammatory role in vivo, we employed an endotoxia model in DSCR-1–null mice and in mice overexpressing DSCR-1s. Because endotoxia stimulates DSCR-1s expression primarily in the endothelium, we used the human Flt1 promoter (spanning the region between –748 and +284) for adenosivirus-mediated expression of DSCR-1s in vivo. This promoter has been shown to direct strong and uniform activity in the endothelium of Hprt locus–targeted mice (26). Moreover, the Flt1 promoter has been used successfully in an adenoviral system to direct expression in lung endothelium (27). Consistent with these data, we observed strong expression of DSCR-1s and EGFP in the lung of Ad-Flt1-DSCR-1s–injected mice.

Importantly, we have demonstrated reciprocal effects of DSCR-1 deficiency and DSCR-1s overexpression on sepsis morbidity/mortality. Our data suggest that DSCR-1s protects against LPS-mediated bradycardia, hypotension, and hypothermia; attenuates circulating levels of inflammatory mediators and mRNA expression of endothelial activation markers; and inhibits leukocyte infiltration in the heart, lung, and liver. Together with our findings in cultured endothelial cells (8, 10), these in vivo data suggest that DSCR-1s is protective in sepsis, in part by inhibiting NFAT-mediated endothelial cell activation and secondary inflammation. Interestingly, DSCR-1 deficiency was associated with increased circulating levels of VEGF. Given that VEGF activates NFAT, among other signaling pathways, and has been shown to play a pathophysiological role in sepsis (17), this effect may further contribute to the increased sepsis morbidity and mortality in Dscr1–/– mice.

We have previously shown that the overexpression of DSCR-1s inhibited neovascularization in Matrigel plugs and xenografts in mice (8). In contrast to these findings, Dscr1–/– mice demonstrated reduced blood vessel formation in Matrigel, corneal micropocket, and tumor xenograft assays (18). Dscr1–/– endothelial cells displayed hyperactivation of the calcineurin/NFAT pathway and increased sensitivity to VEGF signaling (18). However, rather than inducing cell proliferation, VEGF-mediated activation of calcineurin/NFAT in Dscr1–/– endothelial cells “re-routed” downstream signaling, resulting in increased apoptosis, which thus explains the paradoxical reduction in neovascularization (18). Consistent with these findings, we found that endotoxia promoted endothelial apoptosis in Dscr1–/– mice. Thus, DSCR-1s plays a dual role in the host response to infection by protecting against excessive endothelial activation and promoting endothelial survival. Based on these data, we propose that DSCR-1s represents a novel therapeutic target in inflammatory diseases.

**Methods**

Generation and analysis of Hprt-targeted transgenic mice. Hprt-targeted ES cells containing the DSCR-1–lacZ transgene were used to generate chimeric mice as previously described (26). Chimeric males were bred to C57BL/6J females to obtain agouti offspring. Female agouti offspring were then bred to wild-type males to generate hemizygous male mice. Analysis of embryos and hemizygous adult male tissues was carried out as previously described (26, 28). The level and pattern of transgene expression were compared with those of the littermate control (same genetic background) mice. All animal studies were approved by the Institutional Animal Care and Use Committees at the University of Tokyo and Beth Israel Deaconess Medical Center.

**Materials and cell culture.** Human and murine VEGF were obtained from R&D Systems. Thrombin and CsA were obtained from Calbiochem. LPS and polyinosinic acid potassium salt [poly(I)] were from Sigma-Aldrich. Antibodies against PECAM-1, VCAM-1, E-selectin, and Mac1 were obtained from BD Biosciences — PharMingen. Goat anti-GATA2 antibody was from R&D Systems. Rabbit anti-EGFP and anti-lacZ antibodies were from Medical & Biological Laboratories Co. Mouse monoclonal anti-GATA2 and DSCR-1 antibodies were generated with baculovirus glycoprotein 64–GATA2 and baculovirus glycoprotein 64–DSCR1 fusion proteins, respectively, as the antigen. HMVECs (Clontech) were grown in Endothelial Growth Medium-2-MV (EGM-2-MV) Bullet Kit (Clontech). HMVECs were used within the first 10 passages. Mouse B16-F1 melanoma (CRL-6323; ATCC), mouse LLC (CRL-1642; ATCC), human skin fibroblast (Clontech), and mouse MS-1 (CRL-2279; ATCC) cells were grown in DMEM plus 10% FBS.

Construction of plasmids and adenoviruses. The DSCR-1 intergenic promoter was cloned as described previously (8). To generate the Hprt targeting vector, the Dscr1 promoter fragment (–1,664/+84) was subcloned into pSDF-lacZ, then ligated with pMop8L. For luciferase assays, DSCR-1 promoter was subcloned into pGLA4.12 (luc2cp) (Promega). For construction of adenosivirus expressing EGFP alone (control) or DSCR-1 plus EGFP driven by the Flt1 promoter, a 1,026-bp fragment of the human Flt1 promoter was inserted into MfeI/Hind-digested pBluescript (Clontech). The resulting vector was used to insert IRES2-EGFP or DSCR-1-IRES2-EGFP gene cassettes. The resulting plasmids were transferred to Adeno-X DNA (Clontech) using the Adeno-X adenoviral expression system (Clontech).

To generate the Ad-miGATA2, mirRNA sequences (Supplemental Table 1) against mouse GATA2 were ligated into pcDNA6.2-GW/EmGFP-miR (Invitrogen), then transferred to pAd/CMV/VS-DEST gateway vector according to the manufacturer’s instructions (Invitrogen).

Real-time PCR. RNA was extracted from endothelial cells using the TRIzol reagent (Invitrogen). For harvesting the RNA from mouse organs, tissues were quickly soaked in RNAzol solution (Ambion), then immersed with TRIzol and homogenized using a tissue grinder (Polytron). Two micrograms of total RNA were reverse transcribed using SuperScript III enzyme and oligo-dT primer as specified by Invitrogen. Real-time PCR including SYBR Green reagent was performed on an instrument according to instructions provided by the manufacturer (Applied Biosystems). Primer pairs are shown in Supplemental Table 1. Expression values are represented as mean ± standard deviation relative to cyclophilin A expression.
Transient transfection of endothelial cells with plasmid DNA and siRNA and analysis of luciferase activity. HMVECs were transfected with plasmid DNA using FuGENE HD reagent (Roche), and luciferase activities were calculated using the Dual-Luciferase assay kit (Promega) as previously described (29). The transfected cells were serum starved, preincubated for 30 minutes with 1 μM CsA or anti-Flik-1/KDR neutralizing antibody (R&D Systems), and then incubated for 2 hours with conditioned medium from confluent B16-F1 or LLC tumor cells cultured for 2 days in DMEM with 2% FBS or incubated with DMEM plus 2% FBS in the presence or absence of 50 ng/ml VEGF for 2 days. For siRNA transfection, HMVECs were incubated with a mixture of 40–120 nM Stealth siRNA (Invitrogen) and Lipofectamine Max (Invitrogen) for 24 hours. After serum starvation (EBM-2 plus 0.5% FBS) for 16 hours, cells were treated with 50 ng/ml VEGF, 5 U/ml thrombin, 10 ng/ml TNF-α, or 20 μg/ml LPS for 2 hours and then processed for luciferase activity, real-time PCR, or Western blotting. Targeted siRNA sequences are shown in Supplemental Table 1.

Administration of CsA and GATA2 miRNA to mice. Matrigel (Clontech) containing 1 × 10^6 B16-F1 cells with vehicle or 20 μg CsA was implanted s.c. into transgenic mice. The mice were subsequently treated with 1 mg/kg CsA i.p. every second day. After 20 days, tumor xenografts were harvested and washed in PBS, mounted in ProLong Gold Anti-Fade Reagent with DAPI and incubation with first antibody diluted by Block Ace (in PBS) (Dai-Irin) with or without VEGF. Organs were removed for whole-mount lacZ staining. Alternatively, mice were treated with CsA (1 mg/kg) every second day for 14 days and then injected with vehicle or VEGF. Organs were removed for whole-mount lacZ staining. Alternatively, mice were injected i.v. with 0.2 mg poly(I) (30) and 1 × 10^10 PFU Ad-miGATA2 or Ad-miControl was implanted s.c. into mice. After 14 days, the same amounts of adenoviruses were injected into the tumor. After 3 days, xenografts were harvested and examined. Alternatively, mice were injected i.v. with 0.2 mg poly(I) (30) and 1 × 10^10 PFU Ad-miGATA2 or Ad-miControl on days 0, 2, and 7. On day 7, the mice were injected with or without VEGF. Organs were removed for whole mount lacZ staining.

Immunohistochemistry. Mice were perfused with 2% paraformaldehyde in PBS. Tissues were then harvested and fixed with 2% paraformaldehyde for 2 hours and immersed in 30% sucrose for 20 hours at 4°C. Cryosectioned slides were treated with ice-cold acetone for 10 minutes and then processed for blocking with Protein Block Serum-Free (Dako) for 30 minutes. The same slides were coimmunostained with anti-Mac1 antibody. To detect apoptosis, detection kit was used according to the instructions provided by the manufacturer. Male C57BL/6 mice at 8 weeks of age and male Dicer−/− mice and their age-matched wild-type littermates at 7–9 weeks of age were used in this study. In a study involving adenovirus administration, 5.0 × 10^10 PFU of Ad-Fli-1-HDSCR-1 or Ad-Fli-1-control were intravenously injected 3 days prior to LPS administration.

Measurement of IL-6 and VEGF levels in plasma. Systemic blood samples were collected and centrifuged. Plasma was harvested and stored at −80°C until all samples were prepared. thawed plasma was diluted and the IL-6 and VEGF level measured using Quantikine ELISA kit (R&D Systems).

Statistics. Data are presented as mean ± standard deviation or SEM as indicated. Pvalues were calculated using 2-tailed unpaired Student’s t test. The Wilcoxon rank–sum test was used for mouse survival studies. Statistical tests and graphing were done with KaleidaGraph (Synergy Software). A P value less than 0.05 was considered significant.

Acknowledgments
This study was supported by the Fund for Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology–Japan (T. Minami); the Takeda Science Foundation, Japan (T. Minami); the Garrett B. Smith Foundation (S. Ryeom); the Smith Family Medical Foundation (S. Ryeom); and NIH grants HL082927 (to W.C. Aird) and HL076540 (to W.C. Aird).

Received for publication March 25, 2008, and accepted in revised form May 27, 2009.

Address correspondence to: Takashi Minami, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan. Phone: 81-3-5452-5403; Fax: 81-3-5452-5232; E-mail: minami@med.rcast.u-tokyo.ac.jp. Or to: William C. Aird, Department of Molecular and Vascular Medicine, Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, Massachusetts 02215, USA. Phone: (617) 667-1031; Fax: (617) 667-1035; E-mail: waird@bidmc.harvard.edu.


