Supplemental information

Supplemental Table 1. Sequence of primers for cloning, mutating and realtime PCR

Sense primer (5'-3')	Antisense primer (5'-3')
(1) CGGGGTACCTGCCGATTGCACCACATCGCC	CTAGCTAGCCCTGTCCGCCGCCCTTCACGCCG
(2) CGGGGTACCGCGCTGAGTCAGACAGACCT	CTAGCTAGCCAACCCATCCTGTGTAAATGG
(3) CGGGGTACCGGATGCACAGGCCCAGTCACTT	CTAGCTAGCCCTGTCCGCCGCCCTTCACGCCG
(4) TCTCTGAGACTCTAGGTTGCTCACGTAAAAT	ATTTTACGTGAGCAACCTAGAGTCTCAGAGA
(5) GTTCCCTGAAGGTGGAAAGTATTATGGAAA	TTTCCATAATACTTTCCACCTTCAGGGAAC
(6) CTAATGCTACAAAGGTGGAGGTAGATAATA	TATTATCTACCTCCACCTTTGTAGCATTAG
(7) GCGCTGAGTCAGACAGACCT	CAACCCATCCTGTGTAAATGG
(8) CTGCCTCCTTAGGCTGGAAT	TAAAACTAGGGCAGGCGATG
(9) CGGGCTAACCGACCAGGTGTCCCG	CCTGCATGATGGCGTCAAAG
(10) GCAACCCAACAGCAAACTTTC	GACGGCGTAGCTCGATAGG
(11) TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC
(13) CTCATCAGCATCCAGCAG	GGGAGCAGGTCAGGAATG
(14) CAAATGCTGGACCAAACACA	TGCCATCCAGCCATTCAGTC
(15) GATGGGGACGTCAACATTCT	TGTGGTTAAGACGCTACCAAAG
(16) TGGCAACAATGAAGCTATCG	ATGTCGGGACCAGTAGGACA
(17) GGTGTGGATCGAGCTGTCTT	CAAGGCCAGCATTTACAGTG
(18) CACCATTCAATTTGACAGCAA	TCCTCTCCCTTGGCACTGTA

(1)-(3) Primer sets for cloning of Proximal Itgb3 HHR-LUC, Distal Itgb3 HHR-LUC, and -793 Itgb3-LUC, respectively. Underlined sequences are adaptor *Kpn*I sites for sense primers, and *Nhe*I sites for antisense primers.
(4)-(6) Primer sets for construction of 1Mut-LUC, 2Mut-LUC and 3Mut-LUC, respectively. Underlined sequences are mutated FBEs.
(7)-(8) Primer sets for ChIP assays for the Distal HHR and Proximal HHR, respectively.
(9)-(18) Primer sets for real-time RT-PCR for *Itgb3*, *Foxc2*, *Itgb5, Fibronectin*, *ppi, Itga1, Itgb1, Itgav* and *Itga5,* respectively.

Materials and Methods

Construction of recombinant adenovirus for Foxc1

The complete coding sequence of mouse Foxc1 with Xba I sites at 5' and 3' ends was first generated using Pfu DNA polymerase (Stratagene) and primers including XbaI sites (5'-ATCTAGAGCCGGGGCC**ATG**CAGGCG-3' and 5'-CTCTAGAAGGG**TCA**GAATTTGCTACAG-3'). The Xba I sites and the Kozak sequence upstream of the start codon for efficient translation are underlined and double underlined, respectively. The initiation and stop codons are shown in bold. The PCR product was subcloned into the Eco RV site of pBluescript II KS+ (Stratagene). Generation of recombinant adenovirus for Foxc1 was performed as described in Experimental Procedures.

Isolation of CD31+ Flk1+ endothelial cells from mouse embryos by flow cytometry

Mouse embryos at E12.5 were washed in PBS supplemented with 1% FCS, minced manually by scissors and then digested with 0.1% collagenase and 0.1% dispase for 1 h at 37°C. Single cells after dissociation by processing through Cell Strainer (BD Falcon) were subjected to FACS analysis as described in Experimental Procedures. Antibodies used for staining were PE-conjugated anti–mouse CD61 (Itgb3) (eBioscience), FITC-conjugated anti-mouse CD31 (PECAM1) (eBioscience), and biotin-conjugated anti-mouse Flk1 (BD Pharmingen) followed by StreptAvidin-APC (eBioscience).

Supplementary Figure legends

Fig. 1. Foxc1 increases mRNA levels of Itgb3, Itgb5 and FN as detected by real-time RT-PCR. MEECs were infected with either Ad-control or Ad-Foxc1. The experiment was independently repeated three times, and statistical significance was determined by Student's t-tests (*$p<0.05$, **$p<0.01$, ***$p<0.005$ versus Ad-control infected cells).

Fig. 2. Reduced mRNA expression of Itgb3 and Itgb5 in PMVECs isolated from control ($Foxc1^{flx/flx}$) and endothelial-specific Foxc1 mutant (Foxc1-CKO) mice (Hayashi and Kume, 2008). Relative mRNA levels of Foxc1, Itgb3, Itgb5 and FN were measured by real-time RT-PCR. Results are presented as the means +/- s.d. from triplicate experiments. Asterisks indicate statistical significance determined by Student's t-tests (*$p<0.05$ versus control mice). N.D., not detected. N.S., non-significant.

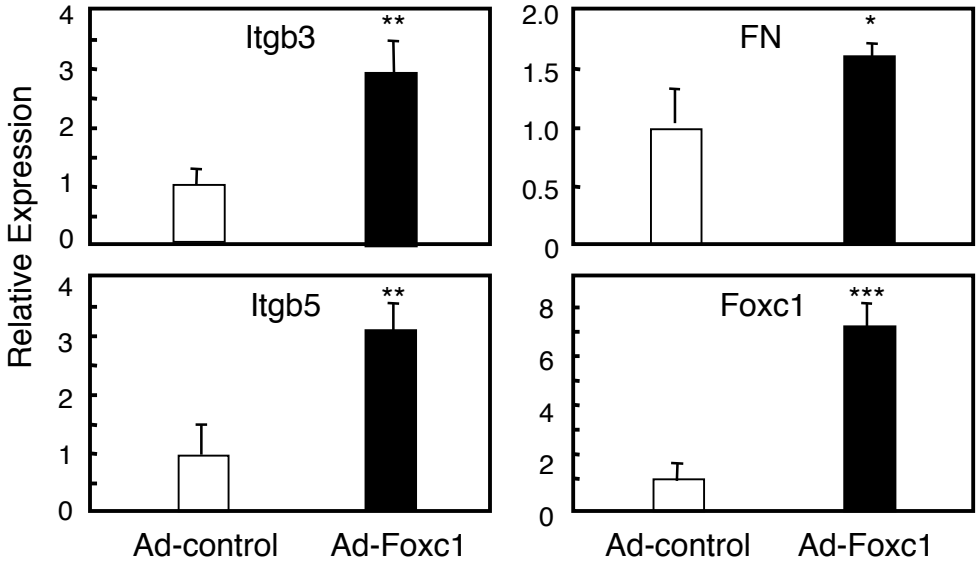
Fig. 3. Foxc1 induces promoter activity of Itgb3. (A) Foxc1 activates the proximal Itgb3 promoter in a dose-dependent manner. Luciferase reporter assays were performed using MEECs transfected with Foxc1 expression vector (0-500 ng/ml) and either Proximal HHR-Itgb3-LUC or its deletion construct (-793 Itgb3-LUC). Fold-increase in normalized luciferase activity is shown. Data are shown as the means +/- s.d., N=6 from two independent experiments (*p<0.05 versus cells transfected with Foxc1 at 0 ng/ml). N.S., non-significant. (B) Foxc1 activates the distal Itgb3 promoter in a dose-dependent manner. Data are shown as the means +/- s.d., N=8 from two independent experiments (*p<0.05 versus cells transfected with Foxc1 at 0 ng/ml).

Fig. 4. Expression of Itgb3 and Foxc1 in CD31+ Flk1+ endothelial cells isolated from Foxc2-mutant embryos by flow cytometry. (A) Surface levels of Itgb3 expression in wild-type, Foxc2+/- and Foxc2-/- endothelial cells at E12.5. Replicate analyses of three independent embryos for each genotype are shown. (B) Real-time RT-PCR analysis to detect expression of Itgb3 and Foxc1 in Foxc2-mutant endothelial cells.

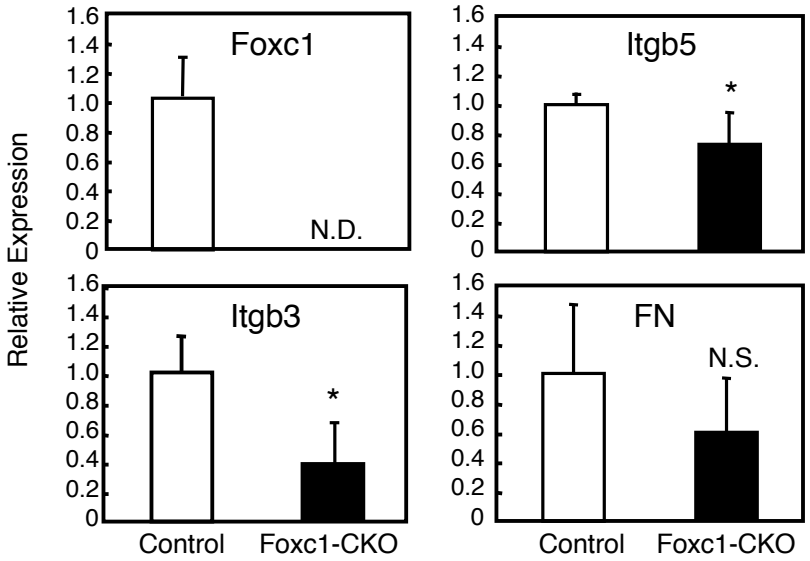
Reference

Hayashi, H. and Kume, T. (2008) Forkhead transcription factors regulate expression of the chemokine receptor CXCR4 in endothelial cells and CXCL12-induced cell migration. Biochem. Biophys. Res. Commun. 367: 584-589.

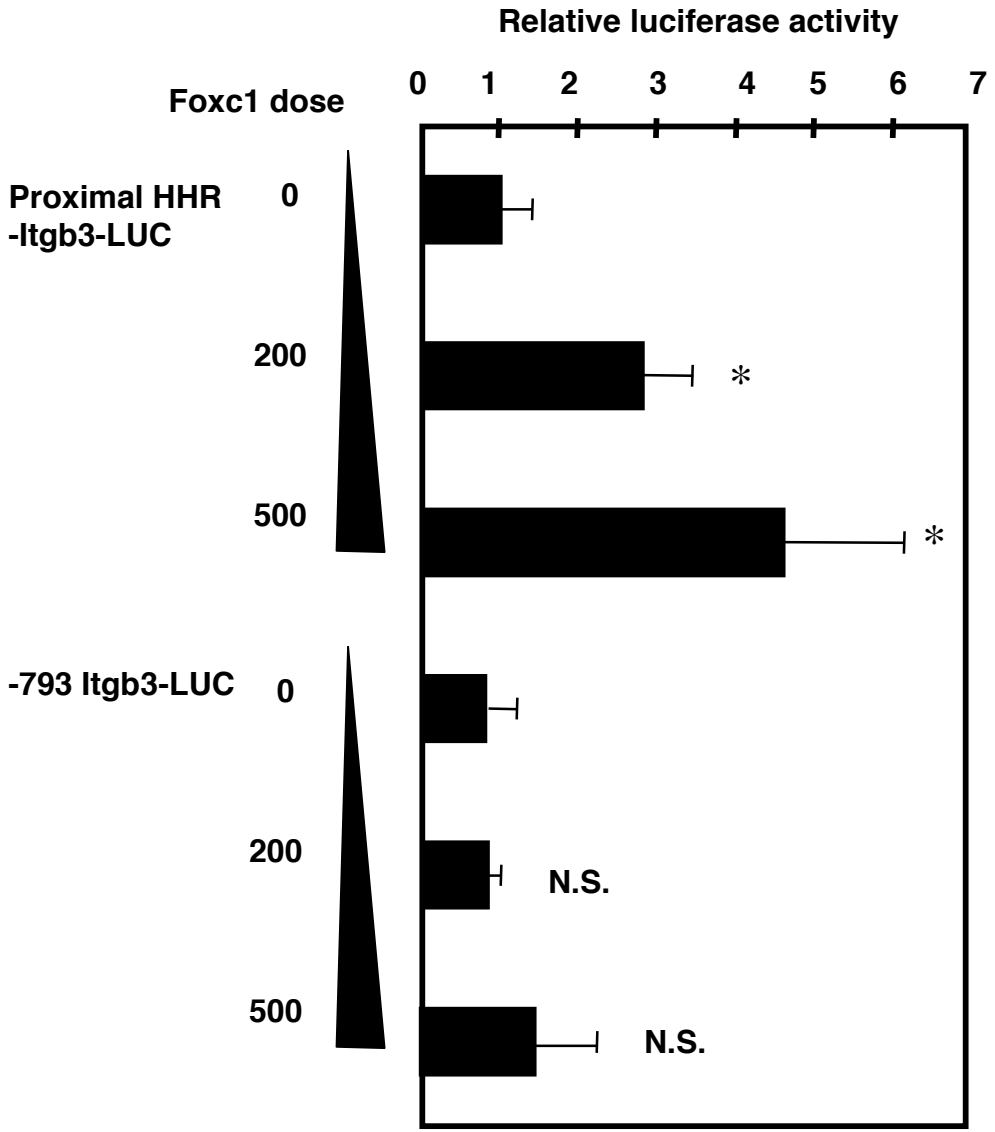
Supplemental Fig. 1



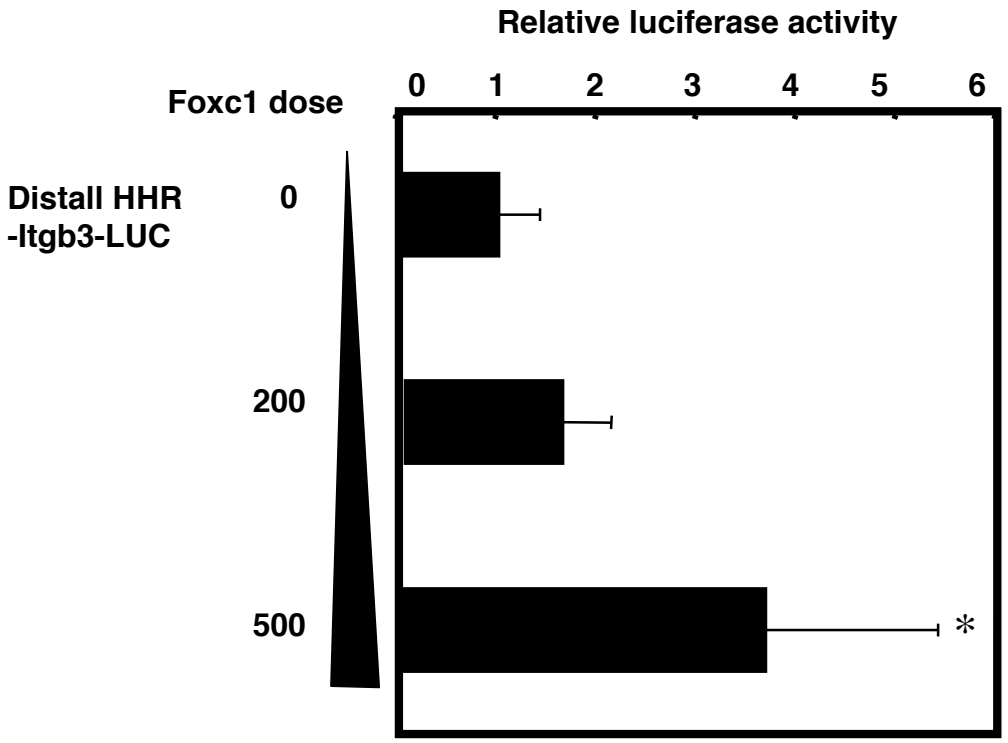
Supplemental Fig. 2



Supplemental Fig. 3A



Supplemental Fig. 3B



Supplemental Fig. 4