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Forkhead transcription factors regulate expression of the chemokine receptor CXCR4 in endothelial cells and CXCL12-induced cell migration

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Abstract

Foxc1 and Foxc2 transcription factors are required for vascular development. However, the molecular mechanisms by which Foxc1 and Foxc2 control angiogenesis, the growth of new blood vessels from pre-existing vessels and capillaries, remain unknown. CXC chemokine ligand 12 (CXCL12) and its receptor, CXCR4, are critical for the process of angiogenesis, including the migration and tube formation of endothelial cells. Here we show that Foxc1 and Foxc2 directly induce *CXCR4* expression by activating its promoter in endothelial cells. Furthermore, *Foxc1*-deficient endothelial cells show a significant reduction in *CXCR4* expression as well as CXCL12-stimulated migration. Taken together, these results provide novel evidence that Foxc transcription factors are important regulators of the chemotactic motility of endothelial cells through the induction of *CXCR4* expression.

Keywords

Forkhead/Fox proteins; Foxc1; Foxc2; CXCR4; CXCL12/SDF-1; Migration; Endothelial cells

Introduction

The CXCR4-CXCL12 chemokine axis is required for normal development of the cardiovascular, hematopoietic, gonadal and nervous systems [1–6]. As such, the CXCR4 receptor is detected in various types of cells, including hematopoietic and vascular endothelial cells, while it is important to note that CXCR4 expression in endothelial cells of the developing embryo is restricted to arteries such as the dorsal aorta and mesenteric arteries [2,7]. Previous studies also demonstrate the function of the CXCR4-CXCL12 pathway in the process of angiogenesis such as endothelial cell migration and capillary tube formation [8–12]. Several extracellular signals and transcriptional factors such as CXCL12, vascular endothelial growth factor (VEGF), hypoxia and NF-κB have been shown to induce *CXCR4* expression in different cell types, including monocytes, cancer cells and endothelial cells [2,10,11,13–17]. However, the molecular basis of transcriptional regulation of *CXCR4*, in particular in vascular endothelial cells, is still not fully understood.

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We have recently shown that Foxc1 and Foxc2, two closely related Fox transcription factors, are important for vascular development [19,20] and that they act upstream of Notch signaling in arterial gene expression by directly regulating the *Dll4* promoter via a Fox-binding element [20]. Since overexpression of Foxc proteins induces multiple arterial markers in cultured endothelial cells [20], we hypothesized that *CXCR4* is a novel downstream target of Foxc transcription factors in endothelial cells In this study, we show that Foxc transcription factors directly transactivate the *CXCR4* promoter in endothelial cells. Endothelial cells lacking *Foxc1* exhibit impaired migration stimulated by CXCL12. Together, we demonstrate that Foxc transcription factors play an important role in regulating the transcription of *CXCR4* in endothelial cells and thereby control CXCL12-dependent cell motility.

Materials and methods

Plasmid construction

A genomic fragment of the murine *CXCR4* locus between −2.7 kb to the transcription initiation site was cloned by PCR from an bacterial artificial chromosome (BAC) clone (BACPAC Resource Center at Children's Hospital Oakland Research Institute) and inserted into pGL3-basic reporter (Invitrogen) at the KpnI and MluI sites (designated as FULL-LUC), followed by sequence confirmation. A series of deletion constructs from the 5′-end of the promoter were made by restriction enzyme digestion using SpeI (−1.9 kb), SacII (−1.2 kb), SacI (−0.9 kb) and PmlI (−0.4 kb), respectively.

Transient transfection and luciferase assay

Mouse embryonic endothelial cells (MEECs), which were isolated from early mouse embryos and immortalized [21], were obtained from Dr. Marie-Jose Goumans (The Netherlands Cancer Institute) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection of plasmid DNA into MEECs was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For luciferase reporter assay, pRL-CMV reporter plasmid (Promega) containing the *Renilla* luciferase gene as an internal control was co-transfected with Foxc expression vectors and the firefly luciferase reporter constructs. All transfections were carried out in triplicate in 1% gelatin coated 24-well plates. Luciferase assays were carried out using the Dual-Luciferase assay kit (Promega). Data are expressed as the means +s.d. of three independent experiments in triplicates (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

Quantitative real-time RT-PCR

Isolation of total RNA from endothelial cells and cDNA synthesis were performed using RNeasy mini kit (Qiagen) and iScript (Bio-Rad), respectively. Real-time PCR were carried out using the SYBR GREEN PCR Master Mix (ABI) and i-Cycler (Bio-Rad) according to the manufacturers' instructions. Each data was normalized by expression levels of *ppi*, a house keeping gene. The sequences of PCR primers used are *CXCR4*-sense; 5′-GGTCTGGAGACTATGACTCC-3′, *CXCR*-antisense; 5′-CACAGATGTACCTGTCATCC-3′, *Foxc1*-sense; 5′-CGGCACTCTTAGAGCCAAAT-3′, *Foxc1*-antisense 5′-TTTGAGCTGATGCTGGTGAG-3′, *Ppi*-sense; 5′-CAAATGCTGGACCAAACACA-3′, *Ppi*-antisense; 5′-TGCCATCCAGCCATTCAGTC-3′. Results are reported as mean +s.d. of triplicate experiments from 3 samples per each group. P values were determined by Student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

Chromatin immunoprecipitation (ChIP) assay

Cultured MEECs were washed with PBS and treated with formaldehyde to cross-link protein to DNA. Cellular lysates were obtained by scraping, followed by pulse ultrasonication to shear

cellular DNA. After centrifugation, supernatants containing sheared chromatin were incubated with anti-Foxc2 antibody or control IgG (Abcam), followed by addition of Protein A+G Sepharose (Santa Cruz), overnight. After elution, immune complexes were subsequently treated with proteinase K at 55 °C for 1.5 h and extracted with phenol/chloroform and chloroform. Immunoprecipitated DNA was analyzed by PCR. The sequences of PCR primers used are sense; 5′-AATTTTGTTGCCTGGTGCAG-3′, antisense; 5′-TTGCACGTTTACTATAAAGT-3′.

Endothelial-specific conditional Foxc1 mutant mice

Full details of generation of the conditional allele for *Foxc1* (designated as *Foxc1*^{flx}) in mice using the Cre/loxP system will be described elsewhere (Seo, S., H.H., and T.K., in preparation). *Foxc1*$^{flx/flx}$ mice were viable and did not show any abnormalities. *Foxc1*$^{+/-}$ mice crossed with *Tie2-Cre* transgenic mice [22] were crossed with *Foxc1*$^{flx/flx}$ mice to generate endothelial-specific conditional knockout (CKO) mice (*Foxc1*$^{flx/-}$; *Tie2-Cre*).

Cell migration assay

Transwells with 8 μm pores (Costar) were pre-coated with collagen I (20 mg/ml) overnight at 4°C. The filters were subsequently blocked with 3% BSA in PBS to inhibit nonspecific migration. The lower wells of the chamber were filled with medium containing 0.1% BSA and murine CXCL12α (R & D Systems) at 100 ng/ml. Pulmonary microvascular endothelial cells (PMVECs) were isolated from adult lungs of conditional *Foxc* mutant (*Foxc1*$^{flx/-}$*; Tie2-Cre*) and control (*Foxc1*$^{flx/flx}$) mice, as described [23]. Subconfluent PMVECs cultured in serum-free medium containing 0.1% BSA were removed, and a total of 5×10^4 suspended cells were added to the upper chambers and then incubated for 5 h at 37°C. Non-migratory cells on the upper side of the filters were removed by a cotton swab, and the filters were fixed in 4% formaldehyde in PBS. Migratory cells attached to the bottom side of the filters were stained with 1% crystal violet and counted under microscopy. Results are reported as mean +s.e. of triplicate experiments from 3 samples per each group (N=9). P values were determined by corresponding sample indicated using Student's t-test (*$p<0.05$, **$p<0.01$).

Results

Foxc1 and Foxc2 induce CXCR4 expression in endothelial cells

In an effort to identify a new downstream target of Foxc1 and Foxc2, we first tested whether Foxc1 and Foxc2 can induce *CXCR4* expression in endothelial cells. We have recently shown that overexpression of Foxc1 and Foxc2 upregulate expression of several arterial genes, including *Dll4* and *Hey2* in mouse embryonic endothelial cells (MEECs) [20]. As shown in Fig. 1, MEECs were transfected with an expression vector for Foxc1 or Foxc2, and quantitative real-time RT-PCR was performed to determine the levels of *CXCR4* mRNA. Significantly, Foxc1 and Foxc2 increased the expression of *CXCR4* in endothelial cells by approximately 3.5-fold.

Foxc1 and Foxc2 directly activate CXCR4 promoter activity

Previous studies have characterized the promoter activity of *CXCR4* [11,14–17], although the effect of Fox transcription factors on the regulation of the *CXCR4* promoter remains unknown. Therefore, to evaluate the direct involvement of Foxc1 and Foxc2 in *CXCR4* expression at the transcriptional level, we first generated a luciferase reporter containing a 2.7 kb region upstream of the murine *CXCR4*. By transfection of this reporter plasmid along with Foxc expression vectors into MEECs, we found that Foxc1 and Foxc2 markedly transactivated the *CXCR4* promoter in a dose-dependent manner (up to 7- and 8-fold, respectively) (Fig. 2A). Consistently, a constitutively active form of Foxc2 (caFoxc2)[24], which only includes the N-

terminal activation domain and the DNA binding domain, further transactivated the *CXCR4* promoter by 10-fold. Since there are several putative Foxc-binding sites within the promoter region (data not shown), we next generated a series of deletion constructs with different regions of the *CXCR4* promoter (Fig. 2B). Importantly, the shortest promoter (0.4 kb) maintained almost the same activity induced by Foxc as the longer constructs. This observation indicates that the 0.4 kb upstream region is responsible for Foxc-mediated activity of the *CXCR4* promoter. Indeed, there is a conserved Fox-binding element (FBE) [25] between human and mouse that is adjacent to the TATA box (Fig. 2C). ChIP assays using MEECs further demonstrated that Foxc2 bound to the endogenous promoter region of *CXCR4* containing the FBE in vivo (Fig. 2D). Taken together, these results suggest that Foxc transcription factors induce the transcription of *CXCR4* in endothelial cells.

Foxc1-deficient endothelial cells show a reduction in CXCR4 expression and CXCL12-induced cell migration

In order to further validate the function of Foxc transcription factors in the regulation of *CXCR4* transcription, we generated endothelial-specific conditional knockout (CKO) mice for *Foxc1* crossed with *Tie2-Cre* mice. *Foxc1*-CKO (*Foxc1*$^{flx/-}$; *Tie2-Cre*) mice were born at a normal Mendelian ratio and viable until adulthood (data not shown). Using adult lungs of control (*Foxc1*$^{flx/flx}$) and *Foxc1*-CKO mice, we isolated pulmonary microvascular endothelial cells (PMVECs). Almost complete absence of *Foxc1* expression in PMVECs from *Foxc1*-CKO mice was confirmed by quantitative real-time RT-PCR (Fig. 3A). Moreover, we found that the expression of *CXCR4* was notably decreased in *Foxc1*-deficient endothelial cells compared with the control cells (Fig. 3A). These finding indicate that the lack of Foxc1 results in significant downregulation of *CXCR4* expression.

We next examined whether Foxc1 deficiency affected the chemotactic migration of endothelial cells toward CXCL12 using PMVECs isolated from *Foxc1*-CKO and control mice (Fig. 3B). While CXCL12 stimulated the migration of the control cells, *Foxc1*-mutant endothelial cells had a significant reduction in the chemotactic migration. Taken together, these data suggest that Foxc1 regulates endothelial cell migration through the induction of *CXCR4* expression.

Discussion

Compelling evidence shows the importance of the CXCR4-CXCL12 signaling pathway in angiogenesis, although the basis for its function at the cellular level was not fully elucidated. A recent study demonstrates that mutant mice for *CXCR4* have impaired filopodial extension from the larger superior mesenteric arterial endothelial cells, leading to a lack of interconnecting vessels between the larger artery and the neighboring capillary plexus [2]. This observation reinforces the critical role of CXCR4 in sprouting and migration of endothelial cells. Our results in this paper show that Foxc transcription factors are pivotal regulators for *CXCR4* expression in vascular endothelial cells, thereby controlling CXCL12-stimulated migration. Interestingly, expression of *CXCR4* is also regulated by another Fox transcription factor, Foxo1, which is required for vascular development and postnatal neovascularization [26], although whether Foxo1 directly control the activity of the *CXCR4* promoter remains to be elucidated. Therefore, to our knowledge, our work is the first report that describes the functional link of Fox transcription factors to *CXCR4* expression and CXCL12-stimulated endothelial migration. It should also be noted that the VEGF pathway induces CXCR4 expression in endothelial cells [10,11] and acts upstream of Foxc transcription factors in arterial gene expression [27]. Therefore, it will be of significance to test the relationship between VEGF signaling and Foxc transcription factors on the induction of *CXCR4* expression in endothelial cells in the future.

The CXCR4-CXCL12 signaling pathway has been shown to induce neovascularization under pathological conditions such as tumor growth and myocardial infarction by promoting the growth of pre-existing blood vessels and/or recruitment of CXCR4+ endothelial progenitor cells (EPCs) from the bone marrow [28,29]. Accordingly, we have found that human FOXC1 and FOXC2 are expressed in tumor blood vessels as well as EPCs (unpublished results). Further studies need to be performed to elucidate the function of Foxc transcription factors in CXCR4-mediated postnatal neovascularization.

In conclusion, our results indicate that Foxc transcription factors play an important role in the chemotactic movement of endothelial cells through the control of *CXCR4* expression.

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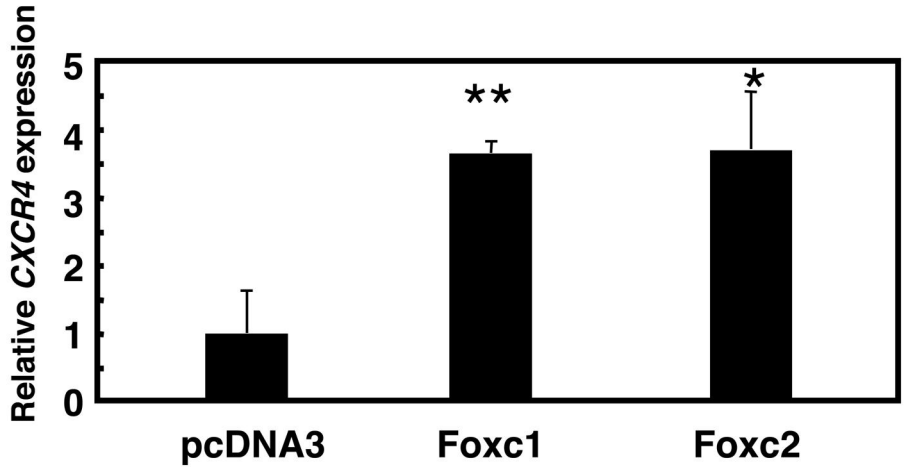
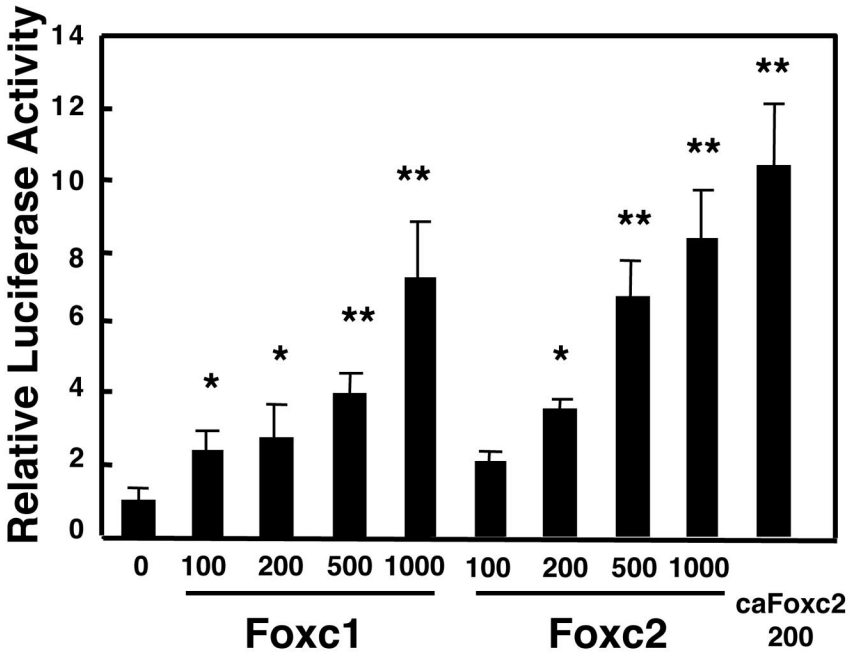


Fig. 1.

Foxc1 and Foxc2 upregulate expression of *CXCR4* in endothelial cells. MEECs were transfected with Foxc expression vector or control (pcDNA) vector. Levels of *CXCR4* mRNA were measured by quantitative real-time RT-PCR using RNA isolated from the tranfected cells. *p<0.05, **p<0.01 versus control.

Fig. 2A



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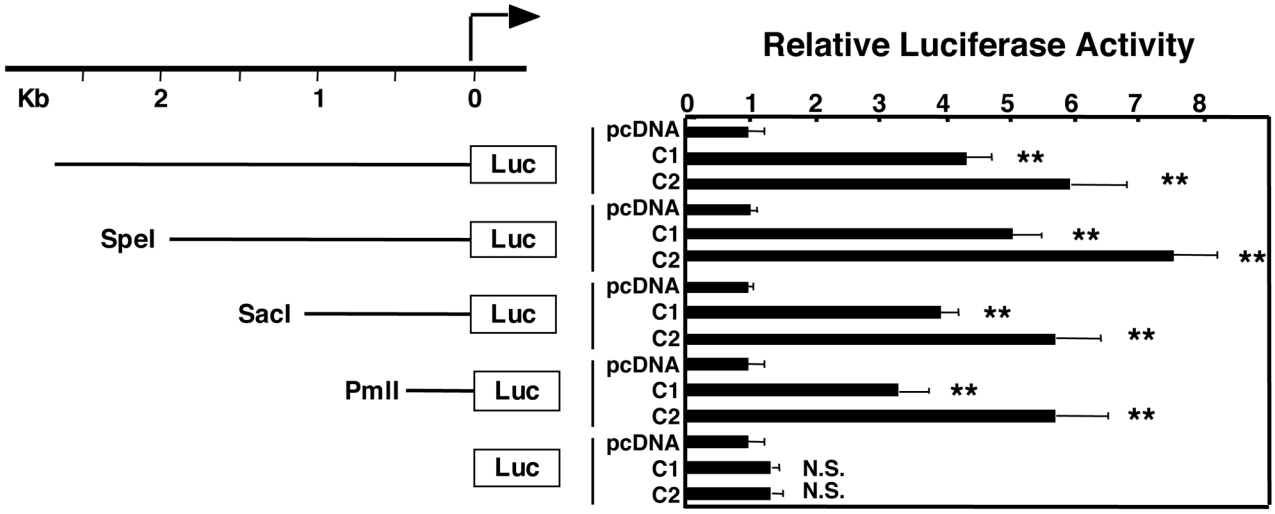

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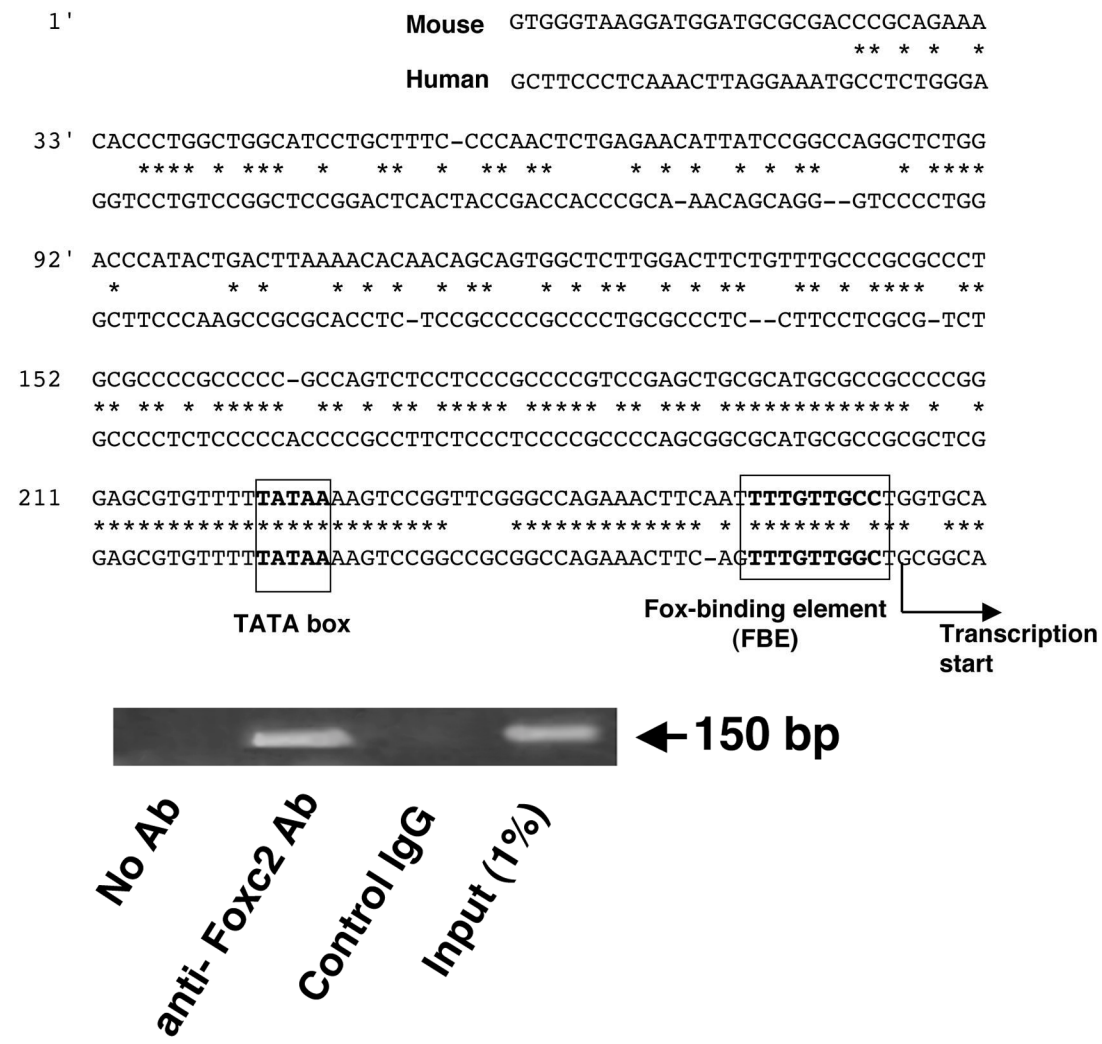
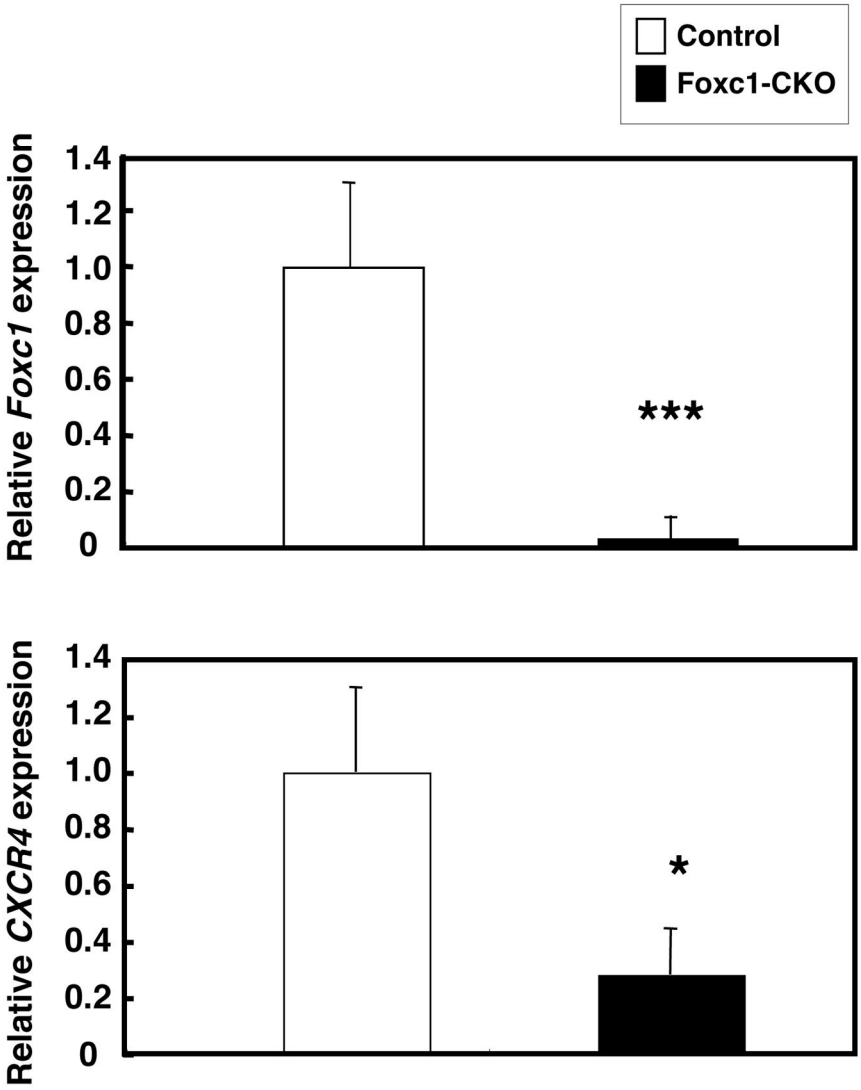


Fig. 2.

Foxc1 and Foxc2 directly regulate *CXCR4* promoter activity in endothelial cells. (A) Foxc1 and Foxc2 transactivate the *CXCR4* promoter in a dose-dependent manner. MEECs were transfected with Foxc expression vector or control (pcDNA3) vector (0–1000 ng) along with luciferase reporter containing the murine *CXCR4* promoter (2.7 kb). Expression vector for a constitutively active form of Foxc2 (caFoxc2) (200 ng) was used as a positive control. *$p<0.05$, **$p<0.01$ versus control. N.S., non-significant. (B) Identification of a minimum region in the *CXCR4* promoter that is sufficient to mediate Foxc-induced promoter activity. MEECs were transfected with a series of deletion constructs along with Foxc expression vector or control (pcDNA3) vector for luciferase assays. Note that Foxc-induced promoter activity remained almost intact in the shortest region (0.4 kb). *$p<0.05$, **$p<0.01$ versus control. (C) Alignment of the promoter regions of mouse and human *CXCR4* including a conserved Fox-binding element (FBE). (D) ChIP assay to detect specific binding of Foxc2 to the *CXCR4* promoter in MEECs. DNA isolated from MEECs was immunoprecipitated with PBS (No Ab), control IgG,

or anti-Foxc2 antibody and amplified by PCR with specific primers for the promoter region of *CXCR4* including the FBE.

Fig. 3A



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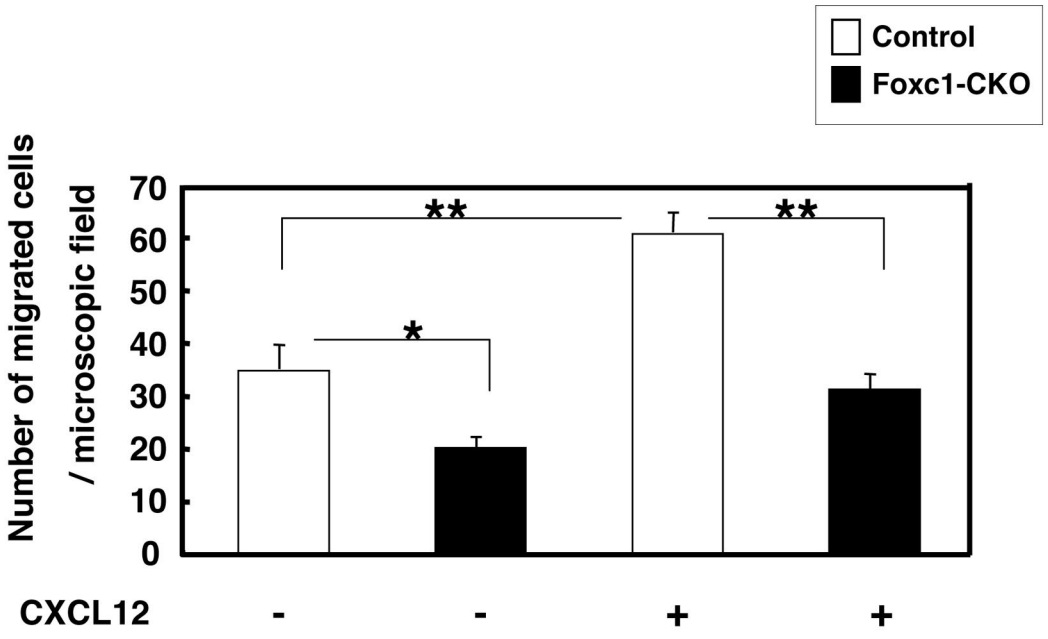

Fig. 3.

Foxc1 mediates endothelial cell migration through *CXCR4* expression. (A) Expression of *CXCR4* is remarkably reduced in *Foxc1*-deficient endothelial cells. Quantitative real-time RT-PCR to detect mRNA levels of *Foxc1* and *CXCR4* were performed using RNA from PMVECs isolated from *Foxc1*-CKO and control mice. *p<0.05, ***p<0.005 versus control. (B) *Foxc1*-deficient endothelial cells show a significant reduction in CXCL12-induced migration. PMVECs isolated from *Foxc1*-CKO and control mice were subjected to transwell migration assays in the presence or absence of CXCL12 (100 ng/ml). *p<0.05, **p<0.01 versus either control or with CXCL12.