PRC2 Components Maintain DNA Hypermethylation of the Upstream Promoter and Regulate Robo4 Expression in Endothelial Cells

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Roundabout4 (Robo4) is an endothelial cell-specific protein that stabilizes the vasculature in pathological angiogenesis and inflammation. We previously determined a 3-kb Robo4 promoter and demonstrated the importance of the upstream region for nuclear factor-kappaB (NF-κB)-mediated promoter activation induced by tumor necrosis factor α (TNFα). This region contains unique genomic features, including promoter region-specific DNA hypermethylation and chromatin condensation; however, the function of the region remains poorly understood. In this study, we analyzed the DNA sequences of the region and identified a motif for polycomb repressive complex 2 (PRC2). Chromatin immunoprecipitation assay indicates the binding of the PRC2 component, SUZ12, to the motif. A mutation in the motif decreased DNA methylation in embryonic stem cells and increased Robo4 promoter activity in endothelial cells. An inhibitor for the PRC2 component, EZH2, induced the promoter activity and expression of Robo4 in endothelial cells treated with or without TNFα. Taken together, these results indicate that the PRC2 components maintain DNA hypermethylation and suppress Robo4 expression via the PRC2 binding motif in the upstream promoter.

Key words Roundabout4; polycomb repressive complex 2; DNA methylation; endothelial cell; gene regulation

INTRODUCTION

Roundabout4 (Robo4) is a single-pass transmembrane protein that is specifically expressed in the endothelial cells (ECs). Robo4 stabilizes the vasculature and suppresses pathological angiogenesis by repressing EC migration, proliferation, and hyperpermeability induced by the vascular endothelial growth factor (VEGF). In addition, Robo4 also suppresses inflammatory hyperpermeability by stabilizing the vascular endothelial (VE)-cadherin mediated cell adhesion and impairs the pathological phenotypes of the mouse models of inflammation. Thus, Robo4 is expected to be a potential therapeutic target against angiogenic and inflammatory diseases.

Expression of human Robo4 is regulated by a 3 kb promoter. The proximal promoter regulates the EC-specific expression of Robo4 via DNA demethylation during stem cell differentiation into ECs. In contrast, the upstream region at around –2.5 kb activates the promoter. Transcription factors, including nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), bind to the motifs in this region and activate the promoter. This region is hypermethylated during cell differentiation while the other regions are completely demethylated. In addition, the chromatin structure of this region is specifically condensed. However, the mechanism that induces this region-specific DNA hypermethylation and chromatin condensation as well as the function of this region in mediating Robo4 expression is unclear.

Polycomb repressive complex 2 (PRC2), a class of polycomb-group proteins, consists of components such as EZH2, EED, and SUZ12. PRC2 suppresses gene expression via epigenetic mechanisms, including chromatin condensation and DNA methylation. SUZ12 binds to the specific DNA motif under the existence of the long non-coding RNA, HOTAIR. EZH2 is a histone methyltransferase that induces trimethylation of histone H3 lysine 27 (H3K27me3) via the SET domain at its carboxy-terminal region. In addition to the methyltransferase activity, EZH2 in the PRC2 recruits DNA methyltransferases (DNMTs) via its amino-terminal region and induces DNA methylation and gene suppression. EZH2 also regulates tumor necrosis factor α (TNFα)-NF-κB signaling and regulates EC differentiation and the maturation of vasculature. In this study, we investigated whether the PRC2 components regulate DNA hypermethylation of the upstream promoter and Robo4 expression.

MATERIALS AND METHODS

Cell Culture Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2MV medium (Lonza, Basel, Switzerland). Mouse embryonic stem (ES) cells were cultured on mouse embryonic fibroblasts in KnockOut-Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Scientific, Waltham, MA, U.S.A.) containing 1000 U/mL ES-GRO mLIF (Millipore, Burlington, MA, U.S.A.), 0.1 mM 2-mercaptoethanol, 15% fetal bovine serum (FBS), 1X Glutamax (Invitrogen, Carlsbad, CA, U.S.A.), 0.1 mM MEM amino acids (Invitrogen), and 1% penicillin–streptomycin. All cells were cultured at 37°C in 5% CO2.

Plasmid Construction The reporter plasmid containing 3 kb Robo4 promoter and the firefly luciferase gene (pGL3-Robo4) was described previously. To prepare a reporter plasmid containing mutated promoter (pGL3-Robo4mut), the −2521 PRC2 motif in pGL3-Robo4 was mutated (CTGTCCCT to CaGaatCT) by site-directed mutagenesis.
using PCR with specific primers (Table S1). To prepare the targeting vector for ES cell line (pMP8II-Robo4mut), the mutated promoter was purified from pGL3-Robo4mut and cloned into the Hprt-targeting vector, pMP8II containing LacZ gene.5)

ES Cell Lines Preparation of the hprt-targeted ES cell line containing the wild type Robo4 promoter was described previously.5,8) An ES cell line containing the mutant Robo4 promoter was prepared using a similar method. Briefly, a targeting vector containing this mutant promoter followed by LacZ gene (pMP8II-Robo4mut) was electroporated into Hprt-deficient ES cells. Transgenic ES cells were selected in the culture medium containing HAT (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Chromatin Immunoprecipitation (ChIP) Assay HUVECs (1.0 × 10⁷ cells) were crosslinked with a solution containing 50 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N″,N‴-tetraacetic acid (EGTA), and 1% formaldehyde. The cells were sonicated using the Sonifer Model 250 (Branson, Danbury, CT, U.S.A.) and incubated for 24 h with Dynabeads (Thermo Scientific) pretreated with 3 µg antibodies against SUZ12 (ab12073; Abcam, Cambridge, U.K.) or control immunoglobulin G (IgG). DNA-protein complexes were collected using a magnet and de-crosslinked in a solution containing 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 1% sodium dodecyl sulfate (SDS). The resulting DNA sample was analyzed by real-time PCR using specific primers (Table S1).

Reporter Assay HUVECs (1.0 × 10⁵ cells) were transfected with 1 µg each reporter plasmid (wild type or mutated pGL3-Robo4) and 50 ng Renilla luciferase vector (pRL-CMV, Promega, Madison, WI, U.S.A.) using FuGENE 6 (Promega) and cultured. The resulting DNA sample was analyzed by real-time PCR using specific primers (Table S1).

Bisulfite Sequencing The genomic DNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan) from ES cell lines with Robo4 promoter with or without a mutation in PRC2 motif and HUVECs treated with or without GSK-126. The resulting genomic DNA was processed using the MethyEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures, Sydney, Australia). The Robo4 promoter fragments were then amplified by PCR from the bisulfite-treated DNA using specific primers (Table S1), cloned into a vector, and propagated in DH5α cells. Plasmids isolated from 10 randomly selected colonies were sequenced. Percentages of methylation for each CpG site and for total 7 CpG sites were calculated.

Treatment of HUVECs with an EZH2 Inhibitor and TNFα HUVECs (1.0 × 10⁵ cells) were cultured for 72 h with or without GSK126 (5 µM; BioVision, Milipitas, CA, U.S.A.). For the assay with TNFα treatment, HUVECs (1.0 × 10⁵ cells) were cultured for 16 h in EBM-2 medium (Lonza) containing 0.5% FBS with or without the EZH2 inhibitor, GSK126 (5 µM), and then treated with TNFα (80 ng/mL; WAKO, Osaka, Japan) for 12 h.

Real-Time RT-PCR The total RNA from cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was reverse-transcribed with SuperScript VILO Master Mix (Invitrogen). Real-time PCR was performed using the cDNA, specific primers (Table S1), and QuantiTect SYBR Green PCR Kit (Qiagen). The copy numbers were calculated using the standard curve prepared using known amounts of plasmids including the target sequences. The expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

RESULTS

**PRC2 Binds to the Upstream Region of the Robo4 Promoter** We have previously shown that the chromatin structure of the upstream promoter region is highly condensed and the DNA is hypermethylated1) (Fig. 1A). To investigate the mechanism by which this region-specific epigenetic modification was induced, we searched for the HOTAIR-dependent binding motif of the PRC2 core component, SUZ12, in the upstream promoter region and identified CTG TCC CT sequence at −2521. Furthermore, ChIP assay demonstrated that SUZ12 was bound to the motif (Fig. 1B). These results suggest that PRC2 binds to the −2521 motif and may regulate DNA hypermethylation and chromatin condensation.

**PRC2 Motif Maintains DNA Hypermethylation in the Upstream Region** Seven CpG sites in the upstream promoter region were found to be specifically hypermethylated (Fig. 1A), and this hypermethylation is maintained during ES/iPS cell differentiation into ECs.7,8) To investigate whether the −2521 PRC2 motif contributes to this hypermethylation, we analyzed the methylation pattern of the seven CpG sites in the promoter with or without a mutation in the PRC2 motif in mouse ES cells (Fig. 2A). The CpG sites in the wild type promoter were highly methylated (89%), while those in the mutant promoter showed decreased methylation (47%). These results indicate that the PRC2 motif contributes to the main-
To investigate whether activity of EZH2, a PRC2 component, contributes to the DNA hypermethylation, we analyzed the methylation pattern of the promoter in HUVECs treated with or without GSK126, which inhibits histone methyltransferase activity of EZH2. GSK126 hardly affected the methylation patterns of the upstream promoter (Fig. 2B). This result indicates that EZH2 activity is not essential for the maintenance of DNA hypermethylation.

EZH2 Suppresses Promoter Activity and Expression of Robo4 through the $-2521$ PRC2 Motif

To investigate whether activity of EZH2, a PRC2 component, contributes to the DNA hypermethylation, we analyzed the methylation pattern of the promoter in HUVECs treated with or without GSK126, which inhibits histone methyltransferase activity of EZH2. GSK126 hardly affected the methylation patterns of the upstream promoter (Fig. 2B). This result indicates that EZH2 activity is not essential for the maintenance of DNA hypermethylation.

Fig. 2. The $-2521$ PRC2 Motif Maintains DNA Methylation of the Upstream Region Independent of EZH2 Activity

(A) Methylation pattern of seven CpG sites in the upstream region of the wild type or mutant Robo4 promoter targeted in the Hprt locus of mouse ES cells. DNA methylation was analyzed by bisulfite sequencing. (B) Methylation pattern of the seven CpG sites in HUVECs treated with or without GSK126 for 72 h. The data shows the percentage of methylation at each CpG site. The percentage at the right bottom indicates the total percentage of methylation of the seven CpG sites.

PRC2 Motif and EZH2 Activity Are Not Necessary for TNF-$\alpha$-Induced Robo4 Upregulation

We previously demonstrated that TNF-$\alpha$ induces Robo4 upregulation via NF-$\kappa$B activation and binding motifs that are located close to the $-2521$ PRC2 motif (10) (Fig. 1A). Since EZH2 has also been shown to regulate TNF-$\alpha$-NF-$\kappa$B signaling, we speculated that the PRC2 components regulate TNF-$\alpha$-induced Robo4 upregulation. To test this hypothesis, we measured Robo4 expression...
in HUVECs treated with or without GSK126 and TNFα by real-time PCR (Fig. 4A). TNFα increased Robo4 expression in both HUVECs treated with or without GSK126. In reporter assays using Robo4 promoter with or without a mutation in the PRC2 motif, the TNFα treatment increased the activity of both wild type and mutant promoters (Fig. 4B). These results indicate that the PRC2 motif and EZH2 are not necessary for TNFα-induced Robo4 upregulation in ECs.

**DISCUSSION**

PRC2 is well known to suppress gene expression by inducing DNA methylation and chromatin condensation. However, few reports indicate the function of PRC2 in ECs. The current study demonstrated that SUZ12 binds to the upstream motif of Robo4 promoter and that EZH2 suppresses the promoter activity through the same motif. These findings strongly suggest the PRC2 binds the −2521 PRC2 motif, which was further demonstrated to regulate DNA hypermethylation independent of EZH2 activity. Thus, we successfully demonstrated the functions of PRC2 components in mediating Robo4 gene regulation and DNA methylation, which provides insights into the functions of PRC2 components in mediating Robo4 gene expression.

A previous report demonstrated that EZH2 in PRC2 induces DNA methylation by interacting with DNMTs. The catalytic domain of EZH2 that induces H3K27me3 is not necessary for this interaction. Consistent with this, our results indicate that the PRC2 motif maintains DNA hypermethylation of the Robo4 upstream promoter independent of the EZH2 activity. This suggests that PRC2 and the recruited DNMT(s) induce DNA hypermethylation of the upstream promoter and suppress Robo4 expression by decreasing promoter activity.

In contrast, EZH2 activity can induce H3K27me3, which might further induce Robo4 promoter suppression. As H3K27me3 is associated with chromatin condensation, it is suggested that EZH2 in the PRC2 suppresses Robo4 promoter activity by inducing chromatin condensation of the upstream promoter. It is speculated that EZH2-induced chromatin condensation alters accessibility of upstream enhancer, including the binding motifs of AP-1 and NF-κB, to the proximal promoter and suppresses promoter activity. The PRC2 motif and EZH2 activity also suppresses promoter activity in ECs treated with TNFα, but they are not necessary for TNFα-induced Robo4 upregulation. Further study on the structure of the Robo4 promoter should reveal the detailed mechanism of PRC2-mediated Robo4 suppression. In conclusion, our current study demonstrated the novel mechanism of Robo4 gene regulation by PRC2 components and suggests the importance of PRC2 for endothelial gene regulation.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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