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Homeobox D1 regulates angiogenic functions of endothelial cells via integrin β1 expression

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ABSTRACT

Homeobox (HOX) family genes, major transcription factors for embryonic development, have been also implicated in vascular development and angiogenesis, particularly with regulation of genes involved in cell–cell or cell–extracellular matrix (ECM) interactions. However, the cellular and molecular functions of HOXD1 in endothelial cells (ECs) are yet to be explored. We here report that HOXD1 is prominently expressed in human ECs and regulates angiogenic activities. Knockdown of HOXD1 in ECs resulted in significant inhibition of migration and adhesion as well as tube like structure formation. These effects were correlated with the reduced expression of integrin β1 (ITGB1), an important signaling component of angiogenesis. Consistently, ITGB1 promoter activity was decreased by HOXD1 knockdown in ECs. Furthermore, we identified the putative HOXD1-binding sites in the promoter region of ITGB1. Together, these findings suggest that HOXD1 plays a significant role in EC functions by regulating the expression of ITGB1.

1. Introduction

Homeobox (HOX) genes are key regulatory transcription factors that play important roles in organogenesis during development and hematopoiesis also have been implicated in the control of vasculogenesis and angiogenesis [1]. Several known HOX target genes have been identified in endothelial cells (ECs), regulating EC proliferation, migration, and adhesion through interactions with the extracellular matrix (ECM) during angiogenesis. Depletion of the HOXA9 protein results in a significant decrease in tumor necrosis factor alpha-induced E-selectin, which is a cell adhesion molecule expressed in ECs [2]. In addition, expression of HOXA9 specifically activates the E-selectin promoter in ECs [2]. Antisense constructs of HOXD3 have been shown to inhibit expression of integrin α5; HOXD3 binds directly to the integrin α5 and β3 promoters [3]. These findings suggest that the HOX genes specifically expressed in ECs may influence angiogenesis and vascular remodeling by regulating the expression of genes that encode cell surface molecules, including integrins. The HOX genes are therefore great candidates for the transcriptional control of genes that are responsible for angiogenesis and vascular remodeling [4].

Angiogenesis is an enormously dynamic process that is intimately influenced by interactions between perivascular ECM and vascular integrins. In fact, several integrin receptors for provisional ECM proteins play crucial roles in angiogenesis [5,6]. Integrin αvβ3 and αvβ5 are upregulated in ECs during wound healing, and inhibition of these integrins blocks angiogenesis *in vivo* [7]. In addition, heterodimeric β1 integrins are expressed in ECs. When ECs are undergoing remodeling, various ECM components are also changing at the same time [6]. ECs upregulate the expression of integrins in response to stimulation with angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Integrin α1β1 and α2β1 are upregulated by VEGF, and antibody-based inhibition of these integrins inhibits tumor angiogenesis [8]. However, it is unclear how integrins are regulated in ECs.

In the present study, we demonstrated that the HOXD1 gene is specifically expressed in mature ECs and outgrowth endothelial cells (OECs). By employing *in vitro* angiogenic assays, we identified that

Abbreviations: bFGF, basic fibroblast growth factor; EC, endothelial cells; EPC, endothelial progenitor cell; ECM, extracellular matrix; FBS, fetal bovine serum; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HOX, homeobox; HUVEC, human umbilical vein endothelial cell; ITGB1, integrin β1; OEC, outgrowth endothelial cell; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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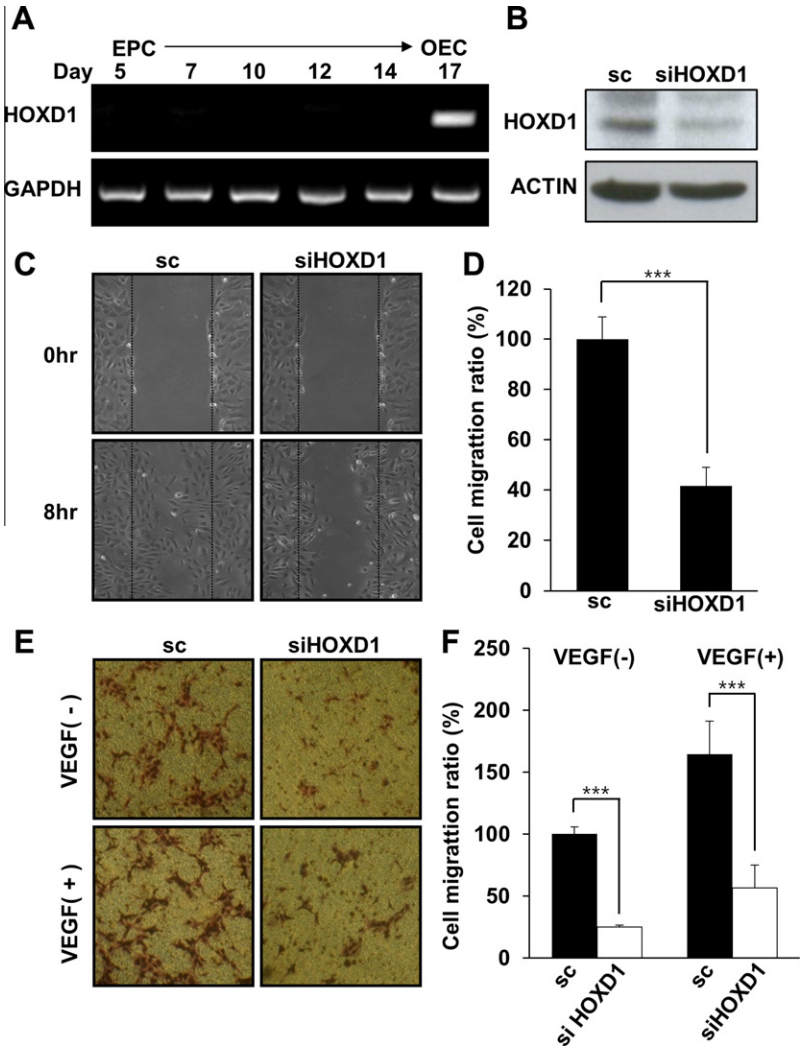

Fig. 1. HOXD1 is expressed in OECs and HUVECs; silencing of HOXD1 controls EC migration. (A) RT-PCR analysis of HOXD1 in EPCs (days 5–14) and OECs (day 17). Gene expression was normalized to GAPDH. (B) Protein expression levels were measured by Western blot analysis after siRNA [control (sc) and HOXD1 (siHOXD1)] transfection. (C and D) EC migration on FN after siRNA transfection was assessed by wound healing assay (C) and quantified (D). $^{***}P < 0.001$ versus scrambled siRNA control after 8 h. (E and F) The chemotactic motility of HOXD1-deficient HUVECs was evaluated by Boyden chamber assay on FN (E) and quantified (F). $^{***}P < 0.001$ versus scrambled siRNA control untreated and VEGF-treated cells.

the knockdown of HOXD1 adversely affects EC migration, adhesion, and tube like structure formation. Furthermore, we provide the evidence that ITGB1 is a transcriptional target of HOXD1 in ECs.

2. Materials and methods

2.1. Isolation and culture of endothelial progenitor cells (EPCs) and ECs

EPCs were isolated from human umbilical cord blood as previously described [9]. Briefly, human umbilical cord blood samples (approximately 50 mL each) were collected by gravity flow from fresh placentas with attached umbilical cords. The study protocol was approved by the local ethics committee. EPCs were isolated by Ficoll-Paque gradient centrifugation (Biochrom, Berlin, Germany) for 30 min at 400 *g* and then washed three times in phosphate-buffered saline (PBS; Biochrom). Cells (1×10^6) were seeded onto 6-well plates coated with human FN (Sigma, St. Louis, MO) in endothelial growth medium-2 (Clonetics, Cell Systems, St. Katharinen, Germany). After 3 days, non-adherent cells were removed, and fresh culture medium was added. Phenotypic analyses of the cells were performed on days 5, 7, 11, and 13. EPC identification and estimation of culture purity (90–95%) were determined by staining cells with progenitor- and EC-specific markers, including anti-CXCR4 and anti-CDH5 (VE-cadherin) antibodies (BD Pharmingen, San Jose, CA).

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase as described previously [10], and cells from passages 3 to 6 were used. HUVECs were cultured on 2% gelatin-coated dishes at 37 °C in a 5% CO_2-humidified atmosphere using M199 medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL bFGF (Upstate Biotechnology, Billerica, MA), and 5 U/mL heparin.

2.2. Transfection of siRNAs

ECs were transfected with scrambled and HOXD1 siRNAs using lipofectamine (Invitrogen) for 3 h. Cells were assayed 36 h after transfection. HOXD1 siRNA was designed by Dharmacon Inc. using the sequence 5′-CGAGAUAGCCAACUGCUUG-3′.

2.3. EC migration assay

The chemotactic motility of HUVECs was assayed in Transwell chambers (Corning Costar, NY, NY) using polycarbonate filters

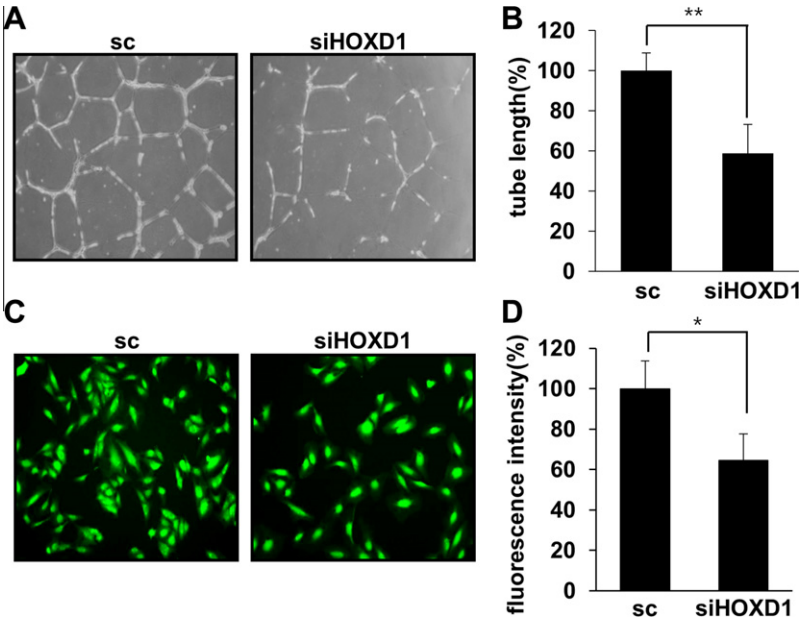

Fig. 2. Inhibition of EC tube formation and adhesion in HOXD1-silenced cells. (A and B) Tube-like structure formation on Matrigel in HOXD1-silenced (siHOXD1) HUVECs was photographed (A) at 24 h and quantified (B). $^{***}P < 0.001$ versus scramble siRNA control (sc) group. (C and D) Adhesion assay on FN of HOXD1-silenced cells were photographed (C) at 1 h and quantified (D). $^{*}P < 0.05$ versus FN-coated plate.

(8-μm pore size, 6.5-mm diameter). Briefly, the lower surface of the filter was coated with 3 μg/mL FN. Fresh M199 medium containing 1% FBS and 20 ng VEGF was added to the lower wells. HUVECs were trypsinized and suspended in M199 containing 1% FBS (final concentration (1×10^6 cells/mL). A 100-μL aliquot of the cell suspension was added to each of the upper wells and incubated at 37 °C for 4 h. Cells were then fixed and stained with hematoxylin and eosin. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy (200× magnification). Ten fields were counted for each assay. Each sample was assayed in triplicate, and the assays were repeated twice.

The wound healing assay is performed by scratching confluent HUVECs on FN-coated, 35-mm dishes with micropipette tips, and images were captured at 0 and 8 h after wounding. For quantitative analysis, five fields per plate were photographed, and distances between the front lines were measured using Image J software (National Institutes of Health, Bethesda, MD). Each assay was repeated three times.

2.4. In vitro tube formation assay

Tube formation was assayed as previously described [11]. In brief, 250 μL Matrigel (BD) was added to a 16-mm diameter tissue culture well and allowed to polymerize for 30 min at 37 °C. HUVECs were incubated in M199 containing 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL bFGF (Upstate Biotechnology), and 5 U/mL heparin. After trypsinization, the harvested cells were resuspended in M199 and plated onto a layer of Matrigel (1.5×10^5 cells/well). Matrigel cultures were incubated at 37 °C, and cultures were photographed at various time points (200× magnification). The area covered by the tube network was determined with an optical imaging technique: pictures of the tubes were scanned into Adobe Photoshop and quantified using Image J software.

2.5. EC adhesion assay

HUVECs transfected with siRNA were trypsinized and washed twice in 0% M199 (without phenol). Before seeding, a 96-well, optical bottom, culture plate was coated with FN (3 μg/mL in PBS), and the plate was incubated at 37 °C for 1 h in a humidified incubator. Just before use, the wells were rinsed twice with PBS. HUVECs were labeled by calcein AM, and 1.5×10^4 cells/well were seeded into 96-well, optical bottom, culture plates. Cells were incubated for 1 h at 37 °C, and non-adherent, calcein-labeled cells were removed by washing four times. M199 (20%) was added to the HUVECs in each well, and the fluorescence was measured (absorbance max. of 494 nm and emission max. of 517 nm) using a fluorescein filter set by FLUO star Omega (BGM LABTEC, Offenburg, Germany).

2.6. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Semi-quantitative RT-PCR was performed with 1× SYBR Green mix (Invitrogen) in an iCycler (Bio-Rad, Hercules, CA). Gene expression was determined as previously described [12], and all results were normalized to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used are shown in Supplementary Table 1.

2.7. Analysis of the ITGB1 promoter and HOXD1-binding sites

A 1-kb upstream sequence of ITGB1 was obtained from Biomart [13], scanned using MotifLocator [14], and the position weight matrix of HOXD1 was obtained from the UniPROBE database [15]. The HOXD1-binding sites larger than the cutoff of 0.7 were obtained after scanning.

2.8. Construction of luciferase reporters and luciferase assay

For construction of ITGB1-Luc, a PCR fragment was amplified using a human bacterial chromosome clone (RP11-479G22; BACPAC Resource center) as template DNA. We cloned genomic fragments extending from positions −980 to +7, −941 to +7, and −778 to +7 relative to the ITGB1, flanked by restriction sites CTCGAG (*Xho*I) and AAGCTT (*Hin*dIII), respectively. PCR-based mutagenesis of the −980 ITGB1 in Mut1-Luc (TACTAAATTACTGGG to TACTAAAGTACTGGG), Mut2-Luc (TACTAAATTACTGGG to TACT-

AACGTACTGGG), and Mut4-Luc (TACTAAATTACTGGG to TACTAACGGCCTGGG) was performed using the −980 ITGB1-Luc reporter as template DNA. All fragments are cloned into the pGL3 basic luciferase expression vector (Promega, Madison, WI). The primers used for plasmid construction are shown in Supplementary Table 2.

2.9. Transfection and luciferase assay

HUVECs were transiently transfected using lipofectamine (Invitrogen). For luciferase assays, the pRL-CMV reporter plasmid (Promega) containing *Renilla* luciferase as an internal control was co-transfected with the firefly luciferase reporter constructs described above. All transfections were carried out in triplicate. The transfected cells were harvested 24 h after transfection, and luciferase activity was measured using the dual luciferase reporter assay system (Promega).

3. Results

3.1. HOXD1 is expressed in OECs and ECs

We previously performed Affymetrix gene chip analysis during EPC differentiation as well as in HUVECs, completely differentiated ECs (GEO Accession No. GSE12891) [9]. The analysis revealed that HOXD1 is highly expressed in OECs and HUVECs compared to early stage EPCs, which was confirmed by RT-PCR analysis (Supplementary Fig. 1A and Fig. 1A). We also examined the expression of other HOX family members in ECs and other cells (Supplementary Fig. 1A). HOXD1 showed relatively specific expression in differentiated ECs compared to other gene members, suggesting that HOXD1 may play a significant role in EC function.

3.2. Loss of HOXD1 function diminishes EC migration and adhesion on FN

To investigate whether HOXD1 has important roles in ECs, we silenced endogenous HOXD1 using siRNA (Fig. 1B and Supplementary Fig. 1B) and evaluated its effects on EC properties important for function such as migration and adhesion. Knockdown of HOXD1 showed inhibition of wound healing migration on FN-coated plates (Fig. 1C and D). A Transwell migration assay also showed that both basal and VEGF-induced migration on FN were significantly reduced in HOXD1 knockdown cells (Fig. 1E and F). EC tube formation assay on Matrigel revealed that scrambled siRNA-transfected cells formed well-organized, tube-like structure at 24 h, whereas tube-like structure formation in HOXD1-silenced ECs was severely impaired (Fig. 2A and B). These data suggest that HOXD1 may be involved in EC migratory function on FN, which is important for angiogenesis *in vitro*. To further investigate the direct effect of HOXD1 on EC–FN interactions, we performed an adhesion assay on FN-coated plates. Interestingly, knockdown of HOXD1 in HUVECs decreased adhesiveness compared to control siRNA (Fig. 2C and D). However, both scrambled siRNA- and HOXD1 siRNA-treated cells did not change their adherence to gelatin- and collagen-coated wells (data not shown). Taken together, these results suggest that HOXD1 may have an important role in angiogenesis through the regulation of EC–ECM interactions.

3.3. HOXD1 transcriptionally regulates expression of ITGB1

To elucidate the mechanism by which HOXD1 affects EC–ECM interactions, we evaluated the expression of integrin family members in ECs transfected with HOXD1 siRNA by RT-PCR. Knockdown of HOXD1 decreased ITGB1, which is a component of heterodimeric integrin receptors for FN (Fig. 3A and B). Semi-quantitative PCR results validated the diminution of the ITGB1 gene by HOXD1 knockdown compared with control transfected cells (Fig. 3C and D). To determine whether ITGB1 transcription is regulated by HOXD1, we first constructed luciferase reporters using the pGL3 promoter vector, which carries the ITGB1 promoter. We silenced the endogenous HOXD1 gene and determined the resulting luciferase activity using the pGL3-ITGB1 promoter vector. The basal promoter activity of ITGB1, which is significant compared to the pGL3 control vector, was substantially reduced by HOXD1 knockdown (Fig. 3E). This finding indicates that HOXD1 likely regulates ITGB1 transcription and EC–FN interactions.

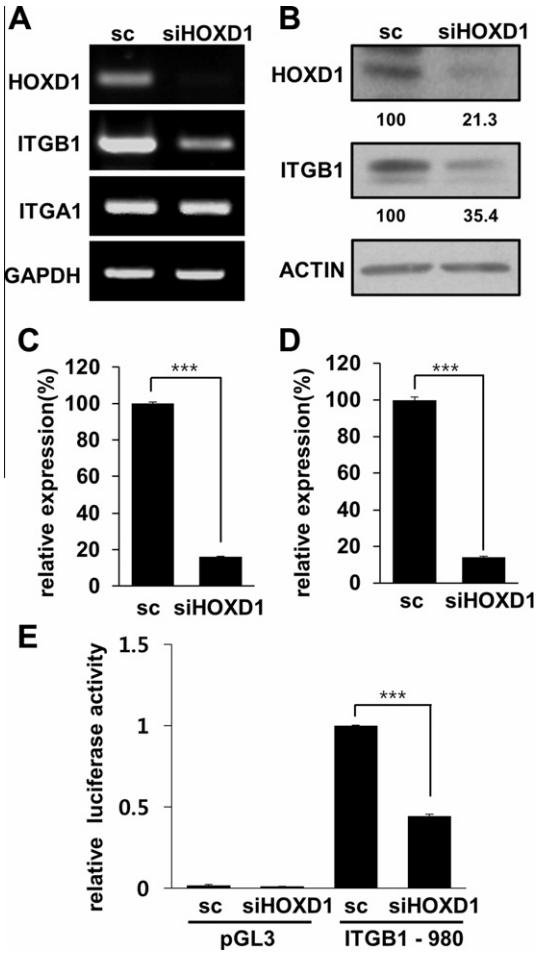

Fig. 3. HOXD1 regulates ITGB1 promoter activity. (A) mRNA levels of HOXD1, ITGB1, ITGA1, and GAPDH were determined by RT-PCR. (B) Protein expression levels were measured by Western blot analysis and quantified using Image J software. (C and D) Relative mRNA levels of each gene were determined by semi-quantitative RT-PCR. All results were normalized to GAPDH. $^{***}P < 0.001$ versus scrambled siRNA control cells (sc), (C) HOXD1, and (D) ITGB1. (E) Relative luciferase activity of the ITGB1 promoter was measured after siRNA [control (sc) and HOXD1 (siHOXD1)] transfection. $^{***}P < 0.001$ versus scrambled siRNA control cells.

3.4. Identification of HOXD1 binding site in the ITGB1 promoter

We next investigated which region of the IGTB1 promoter is responsible for HOXD1 regulation. To identify the responsible promoter region, we generated ITGB1 promoter deletion constructs and evaluated their promoter activity (Fig. 4A and B). The activity of the −980 ITGB1 promoter was significantly higher than the −941 and −778 ITGB1 promoters, suggesting that the promoter region 980–941 bp upstream is likely to contain HOXD1-binding

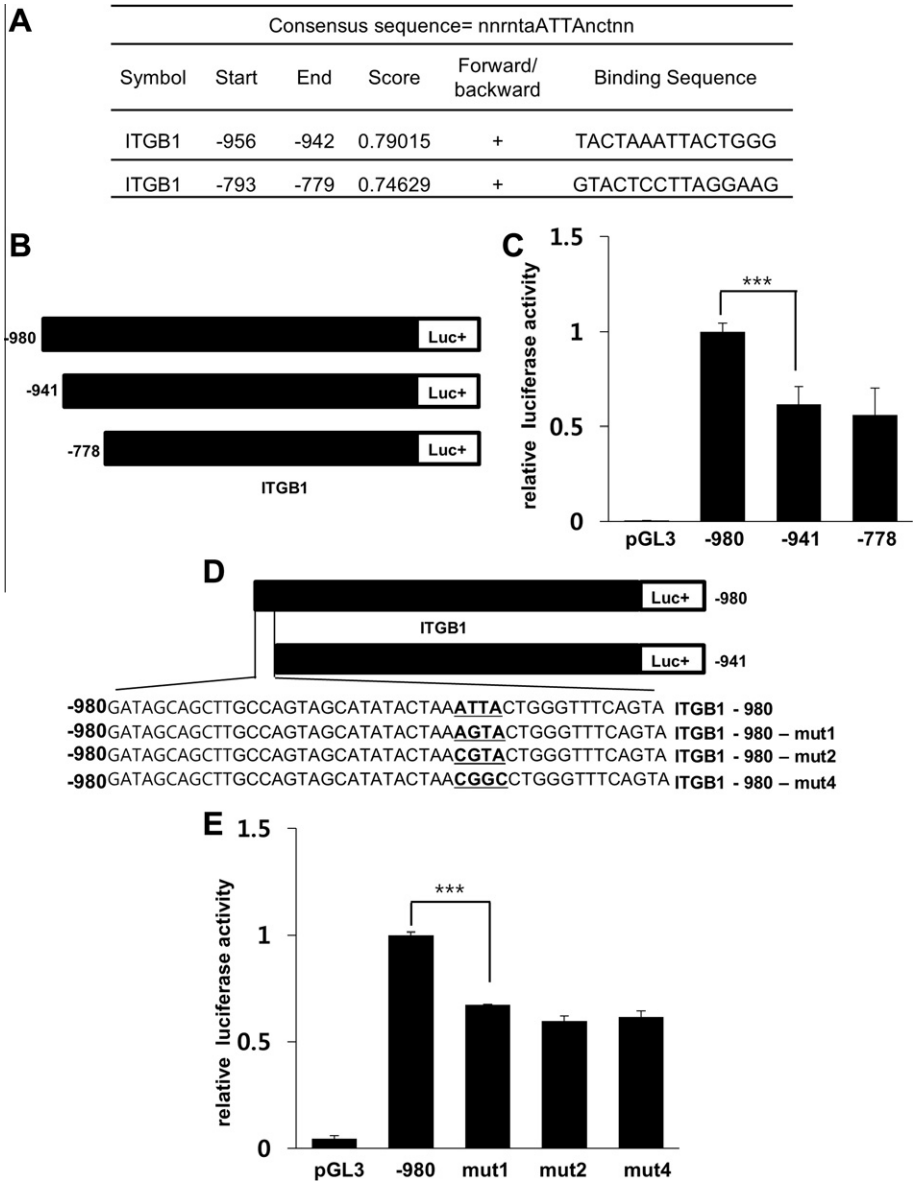

Fig. 4. HOXD1 and ITGB1 transcriptionally interact with each other. (A) The putative HOXD1-binding consensus sequence was evaluated using bioinformatics (see Section 2). (B) Deletion constructs of designed ITGB1 promoters which mutate HOX-binding consensus sequence. (C) Relative luciferase activity of the deletion constructs shown in (B) was evaluated after transient transfection. $^{***}P < 0.001$ versus ITGB1 promoter. (D) The corresponding mutated ITGB1 promoter sequences are shown with mutated base pairs bold and underlined. (E) Relative luciferase activity of the mutated ITGB1 promoters was measured after transient transfection. $^{***}P < 0.001$ versus ITGB1 promoter.

sites (Fig. 4C). To identify the potential HOXD1-binding sites in this promoter region, we scanned the promoter sequence using a HOX-binding consensus sequence and found one potential binding site that contains the minimal HOX-binding consensus sequence ATTA (TAAT) [16]. We constructed ITGB1 mutants with one, two, or four bases mutated in the putative HOX-binding site (Fig. 4D). Transcriptional activity of the ITGB1 mutant promoters was significantly reduced (Fig. 4E), suggesting that HOXD1 regulates ITGB1 transcription via direct binding to the promoter in ECs.

4. Discussion

A number of HOXA, HOXB, and HOXC clusters are expressed in the cardiovascular system during embryogenesis; interestingly, several HOXA, HOXB, and HOXD clusters have been detected in vascular ECs [1,17]. The expression of HOXA9, known for its important role in endothelial stem cell determination [18], is restricted to ECs [17]. HOXB cluster genes are specifically induced by differentiating factors, such as tissue plasminogen activator (TPA) and VEGF in ECs [19]. Reduction of HOXB3 expression with antisense impairs capillary morphogenesis of dermal microvascular ECs cultured on basement membrane ECM [20]. In addition, the orphan HOX gene, hematopoietically expressed homeobox (HEX), is expressed in the blood islands where the vascular and hematopoietic stem cells are located, and manipulation of its expression in ECs results in defects in angiogenesis-related properties *in vitro* by the regulation of genes that are important to angiogenesis [4]. These findings indicate that HOX family genes have crucial roles in regulating angiogenesis. Previously, we evaluated the expression of several HOX genes during EPC differentiation. Among them, HOXD1 was found to be highly expressed in differentiated ECs. Although the functional role of HOXD1 in regulating EPC differentiation needs to be further investigated, its expression pattern during EC differentiation suggests a possible role in both the differentiation and function of ECs.

The potential involvement of HOXD1 in angiogenesis was suggested by the present study using migration, Matrigel tube formation, and adhesion assays. Interestingly, EC migration and adhesion on FN was significantly reduced after transfection with HOXD1 siRNAs, leading to the hypothesis that HOXD1 may regulate EC–ECM interactions, particularly via FN, through the transcriptional regulation of FN receptors. Various integrins, including FN receptors ($\alpha5\beta1$, $\alpha v\beta1$, and $\alpha v\beta5$) are expressed in ECs and are responsible for mediating the interaction between ECs, the ECM, and intracellular signaling pathways, thereby affecting EC migration, proliferation, and adhesion [21,22]. Particularly, FN, a component of the basement membrane, affects vessel formation via EC interactions during embryogenesis [23,24]. Mutation of FN blocks blood vessel formation in the mouse yolk sac, whereas aortic ECs in the embryo are disorganized and scattered [25]. ITGB1, a major component of the FN receptors, expressed in ECs has been reported to be essential for angiogenesis. EC-specific deletion of ITGB1 results in defects in angiogenic sprouting and vascular branching morphogenesis in mice [26,27], suggesting a requirement of this integrin in vascular development and patterning. Consistently, ITGB1-null ECs are deficient in adhesion and migration [24]. Despite of its critical role in angiogenesis, the transcriptional regulation of ITGB1 in EC remains unclear. We for the first time identified HOXD1 as a key regulator of ITGB1 in ECs. Our results together imply that ITGB1 up-regulated by HOXD1 in differentiated ECs may play a significant role in angiogenesis possibly through interaction with FN.

Previously, the genes regulated by HOX family transcription factors were reported to contain the minimal HOX-binding consensus sequence, ATTA, in their proximal promoter regions [16]. The mutation of a potential binding site (ATTA) in the ITGB1 promoter, which contains the minimal HOX-binding consensus sequence, led to a significant reduction in ITGB1 promoter activity in ECs. These results suggest that the HOX-binding consensus sequence (ATTA), which was previously reported to be important in the regulation of integrin $\beta3$ by HOXD3 in ECs [3], is crucial for the regulation of ITGB1 by HOXD1 in ECs.

HOX gene regulation is involved in cardiovascular remodeling; its deregulation results in defects in angiogenesis, lymphangiogenesis, atherosclerosis, and wound healing [4]. However, the roles of HOX genes in ECs have not been fully elucidated. A further understanding of these roles is crucial for the identification of the molecular processes involved in vascular cell differentiation and angiogenesis, as well as for the design of future therapeutic strategies. The current report suggests that HOXD1 plays a crucial role in EC function, including migration and adhesion, through the transcriptional regulation of IGTB1. ITGB1 mediates the interactions between ECs and the ECM that are important for cell invasion and proliferation, processes necessary for new blood vessel growth. Therefore, these studies provide insight for both the role of HOXD1 in the transcriptional regulation of ITGB1 in ECs and a further understanding of the mechanisms of angiogenesis and blood vessel formation.

5. Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.017.

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