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HIF-2 directly activates CD82 gene expression in endothelial cells

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ABSTRACT

Hypoxia inducible factor (HIF)-1 and HIF-2 are transcription factors that mediate the cellular response to hypoxia. Although HIF-1 and HIF-2 share the same target genes, both proteins activate a distinct subset of genes. To identify the target genes preferentially activated by HIF-2 in endothelial cells, DNA microarray analysis was performed to human umbilical vein endothelial cells (HUVECs) with forced expression of either HIF-1α or HIF-2α. In the present study, which is the first comparative study of target genes induced by either HIF-1 or HIF-2 in HUVECs, HIF-1 (and not HIF-2) stimulated mainly glycolytic, hexose metabolic and alcohol metabolic gene expression. However, HIF-2 (but not HIF-1) induced developmental gene expressions such as Fms-like tyrosine kinase 1 (*Flt-1*) and angiopoietin 2 (*Angpt2*). Furthermore, *CD82* was up-regulated by HIF-2, but not by HIF-1, in response to hypoxia. HIF-2 regulated *CD82* gene expression by binding to its HRE consensus sequence located within its first intron. Assessing the function of CD82 in HUVECs forced its expression. This result revealed that CD82 negatively regulates the HUVECs cell migration. The induction of *CD82* gene expression in endothelial cells provided new insights into a specific function of HIF-2.

1. Introduction

Adaptive transcriptional responses to oxygen deprivation (hypoxia) are mediated by hypoxia inducible factors (HIFs), which are heterodimeric transcription factors composed of two basic helix-loop–helix-PAS family proteins, α-subunits and a common β-subunit. The transcriptional activity of HIF is determined by the hypoxic stabilization of the HIF-α proteins. The α-subunits have been identified as HIF-1, HIF-2 and HIF-3. Following the identification of HIF-1α, it was characterized as a structurally related protein; HIF-2α also has been described, but has only been partially characterized [1]. HIF-1α and HIF-2α are highly conservative proteins, that share a similar domain structure, are heterodimerized with HIF-1β, and bind to the same DNA sequence called hypoxia response element (HRE). HIF-1α is ubiquitously expressed in all tissues, whereas HIF-2α expression is limited to tissue in the endothelium, kidney, lungs, heart, and small intestine [2].

HIF activity is essential during embryogenesis. For example, the deletion of *Hif-1β* (*Arnt*), *Hif-1α*, and *Hif-2α* in mice leads to embryonic lethality [3–9]. To dissect the unique and over lapping roles of HIF-1α and HIF-2α, "knock-in" mice in which a cDNA encoding for HIF-2α was targeted to the *Hif-1α* locus, generated a *Hif-1α*$^{Hif\text{-}2aKI}$ allele [10]. The replacement of HIF-1α with HIF-2α resulted in an expanded expression of HIF-2-specific target genes, but the *Hif-2α KI* allele failed to compensate for the loss of HIF-1α expression. Expanded HIF-2α is deleterious to embryonic development. HIF isoforms are believed to function differently from one another. In studies designed to identify unique HIF-1 and HIF-2 target genes, it was reported that HIF-1 and HIF-2 activate a number of common genes; however, HIF-1 exclusively induces the hypoxic transcription of glycolytic genes such as phosphoglycerate kinase I (*Pgk1*) [11] and aldolase A (*Alda*) [12]. A number of putative HIF-2 target genes were identified, including erythropoietin (*Epo*) [13], Fms-like tyrosine kinase 1 (*Flt-1*) [14] and the receptor tyrosine kinase for Ang1 and Ang2 (*Tie2*) [15].

CD82 (KAI1, kangai, or C3) is a member of the tetraspanin family, which was originally identified as a metastasis suppressor that would not affect primary tumorigenicity *in vivo*. Although the mechanism of action for CD82 remains unclear, recent studies have shown that CD82 may inhibit cell motility by regulating the biological activities of its associated proteins and/or reorganizing plasma membrane microdomains [16,17]. The association of CD82 with integrins regulates the motility and invasiveness of various cell types [18]. Furthermore, CD82 associates with several growth factor receptors [19,20] and other tetraspanins such as CD9 in

Abbreviations: HUVECs, human umbilical vein endothelial cells; HIF-1α, -2α and -β, hypoxia inducible factor-1α, -2α and -β; HRE, hypoxia response element; ARNT, aryl hydrocarbon receptor nuclear translocation-like; PGK1, phosphoglycerate kinase 1; FLT1, Fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor.

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the plasma membrane [17]. In addition to their motility-dependent function, tetraspanins regulate cell–cell functions [21], trafficking, and processing of the associated molecules [22] and can influence the lipid composition of the plasma membrane [23].

In the present study, DNA microarray analysis was used to compare HIF-1α with HIF-2α-overexpressed HUVECs to dissect the unique and over-lapping roles of HIF-1 and HIF-2. In particular, the focus was on CD82 as differentially expressed endothelial cell molecules with an expression that is strongly up-regulated by HIF-2. Furthermore, the functional HRE was found within intron1 of the CD82 locus, and CD82 was shown to function as a regulator of cell motility in endothelial cells. These results show that CD82 is involved in the HIF-2 specific target gene and may be responsible for the specific role of HIF-2 in endothelial cells.

2. Material and methods

2.1. Cell culture

HUVECs were purchased from Kurabo (Kurashiki, Japan) and used for experiments before passage 7. Confluent HUVECs plated on a collagen-coated dish were incubated under either normoxic (20% O_2) or hypoxic (2% O_2) conditions, which were created by cultivating cells in a CO_2–N_2-incubator (Taitec, Nagoya, Japan) flushed with a mixture of 2% O_2, 5% CO_2, and 93% N_2.

2.2. Antibodies and siRNAs

The following antibodies were used in the present study: anti-CD82 antibody (TS82b, 59509-100) (Abcam, Cambridge, UK), anti-HIF-1α antibody (NB100-449), and anti-HIF-2α antibody (NB100-122) (Novus biologicals, Littleton, CO). Stealth small interfering RNAs (siRNAs) for *HIF-2α* (HSS103261) were purchased from Invitrogen Corp. (Carsbad, CA).

2.3. Plasmids and adenoviruses

A full-length cDNA encoding human CD82 was inserted in-frame into pEGFP-N1 (Clonetech, Mountain View, CA) at the XhoI and AgeI sites to produce an expression vector (pEGFP–CD82) for CD82 fused with EGFP at its COOH terminus. Human HIF-1α and HIF-2α cDNAs were purchased from OpenBiosystems (Huntsville, AL) and were subcloned into pcDNA 3.1(−) at the EcoRV and BamHI sites. Constitutive active forms of HIF-1α(3Mut; P405A, P546G, N803A) and HIF-2α(3Mut; P402A, P531G, N847G) were developed using the QuickChange mutagenesis protocol (Stratagene, La Jolla, CA). After sequence verification, the XbaI–KpnI fragment was subcloned into a pShuttle vector at the same site. Adenoviruses for expressing CD82–EGFP, HIF-1α(3Mut) or HIF-2α(3Mut) were prepared according to the manufacturer's protocol. Infection efficiency, determined by immunocytochemistry, was consistently >80% of the cells.

2.4. RT-PCR

Cellular RNA was extracted using Trizol (Invitrogen Corp.) and reverse transcription was performed using a Super Scripts III kit (Invitrogen Corp.) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using a QuantiFast SYBR Green PCR kit (Qiagen, Valencia, CA). Primers used in the present study were as Supplemental methods. Quantitative PCR was performed with realplex[4] (Eppendorf, Hamburg, Germany) at 60 °C with annealing and extension for 40 cycles. For normalization, expression of human β-*ACTIN* was determined in parallel as an endogenous control.

2.5. Microarray analysis

Confluent HUVECs on a collagen-coated dish were changed with HuMedia-EB2 medium (Kurabo) containing 0.2% fetal calf serum (FCS) or HuMedia-EG2 (Kurabo) and infected GFP, HIF-1α(3Mut) or HIF-2α(3Mut) adenovirus for 24 h. Total RNA were purified using RNeasy (Qiagen) and were reverse-transcribed to cDNAs. Biotin-labeled RNAs derived from cDNAs were fragmented according to the manufacturer's instructions (Affymetrix, Santa Claea, CA). Labeled cRNA probes were hybridized to an Affymetrix U133plus 2.0 array (Affymetrix). Only genes with at least one present call, at least one expression value above 100 were retained and those with a P value of <0.01 were included.

2.6. siRNA-mediated knockdown experiment

The cells were infected with adenovirus vectors at the appropriate multiplicity of infection. For siRNA-mediated gene silencing, HUVECs were transfected with 20 nM siRNA duplexes using Lipofectamine RNAi MAX reagent (Invitrogen Corp.), and were cultured for 48 h.

2.7. Immunoblot

The cells were lysed in RIPA buffer containing 50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (Roche Applied Science, Indianapolis, IL). The cell lysates were centrifuged, and the supernatants were used for immunoblotting. Proteins reacting with primary antibodies were visualized using the ECL system (GE Healthcare Life Science, Piscataway, NJ) and analyzed using the Las-1000 system (Fuji Film, Tokyo, Japan). Protein concentration was determined by a dye method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.8. Wound closure assay

The cells were seeded on 12-well plates (16×10^4 cells/ well) and incubated for 24 h to confluence. The medium was replaced with fresh HuMedia-EB2 containing 2% FBS and hydrocortisone 12 h before the onset of the assay. The assay was started by scratching the confluent cells with a pipette tip to inflict wounding. Wounded cells were incubated in the same medium at 37 °C for 24 h to heal. Cells were photographed at 0 and 24 h after the onset of the assay using a phase-contrast microscope (model IX-81; Olympus Corp., Tokyo, Japan). The migrated distance for % control is presented the average of four independent experiments. One experiment was done at three dishes with two field photos per dish.

2.9. Cell motility assay

The motility of individual cells was quantified by time-lapse videomicroscopy. The transfectants were re-plated on 35-mm-diameter collagen-coated glass-base dish (Asahi Techno Glass, Chiba, Japan) at 3×10^3 cells/dish in HuMedia-EG2, and were allowed to adhere to the substrates. Twelve hour after re-plating, the medium was replaced with HuMedia-EG2. Cell migration was monitored using an inverted microscope (model IX-81; Olympus) equipped with a built-in CO_2 moist incubator. Video images were collected at 3 min intervals using MetaMolph 6.1 software (Molecular Devices Corporation). The positions of nuclei were tracked to quantify the cell motility. Total distance that cells traveled for 10 h was estimated in pixels using the same software.

2.10. ChIP assay

The ChIP assay was performed according to the protocol provided with the ChIP assay kit (Active motif, Carsbad, CA). Briefly, HUVECs were exposed to hypoxia for 6 h or infected with GFP or HIF-2α (3Mut) adenovirus 24 h prior to harvest. The primary antibody used for immunoprecipitation was anti-HIF-2α. The primer sequences that were designed to amplify the HRE-containing region of the *CD82* promoters were as follows; forward, 5′-AAACAAACGGAGAGGGGAGT-3′ and reverse, 5′-CAGTGAGTGGG AGGTGGTTT-3′.

2.11. Statistics

Assays were statistically evaluated using a two-tailed Student's *t* test. *P* values of <0.05 were considered significant.

3. Results

3.1. Identification of CD82 as a HIF-2 specific target gene

To assess the function of HIF-2 in endothelial cells, we first compared a target profile, which was induced by either HIF-2 or HIF-1. HUVECs were infected with either adenovirus containing EGFP (control), a constitutively active form of HIF-1α(3Mut) or that of HIF-2α(3Mut). The expression levels of HIF-1α(3Mut) and HIF-2α(3Mut) were equivalent, as evaluated by immunocytochemistry (data not shown). Differential gene expression profiles were determined by microarray analysis using an Affymetrix GeneChip probe array. A total of 300 genes were identified that showed more than a 2-fold up-regulation from HUVECs infected with HIF-2α(3Mut) compared with those infected with GFP, and less than a 2-fold up-regulation from HUVECs infected with HIF-1α(3Mut) compared with those infected with GFP. The difference in normalization value of GFP infected HUVECs compared with those infected with HIF-1α(3Mut) and HIF-2α (3Mut) was 0.5 or more (Supplemental data). Among them, *CD82* expression was strongly up-regulated in HIF-2α(3Mut)-over-expressed HUVECs compared with HIF-1α(3Mut). To confirm the microarray data, CD82 levels were analyzed using real-time RT-PCR. In the present experiment, the expression levels of *PGK*, *VEGF* and *FLT1* were also analyzed as positive controls (Fig. 1A–C): *PGK*; the HIF-1 regulated gene, *VEGF*; the both HIF-1 and HIF-2 regulated gene, and *FLT1*; the HIF-2 regulated gene. The expression levels of *CD82* were 16-fold higher in the HIF-2α (3Mut) adenovirus infected HUVECs than in the control cells (Fig. 1D). Furthermore, Fig. 1E shows that HIF-2 induced CD82

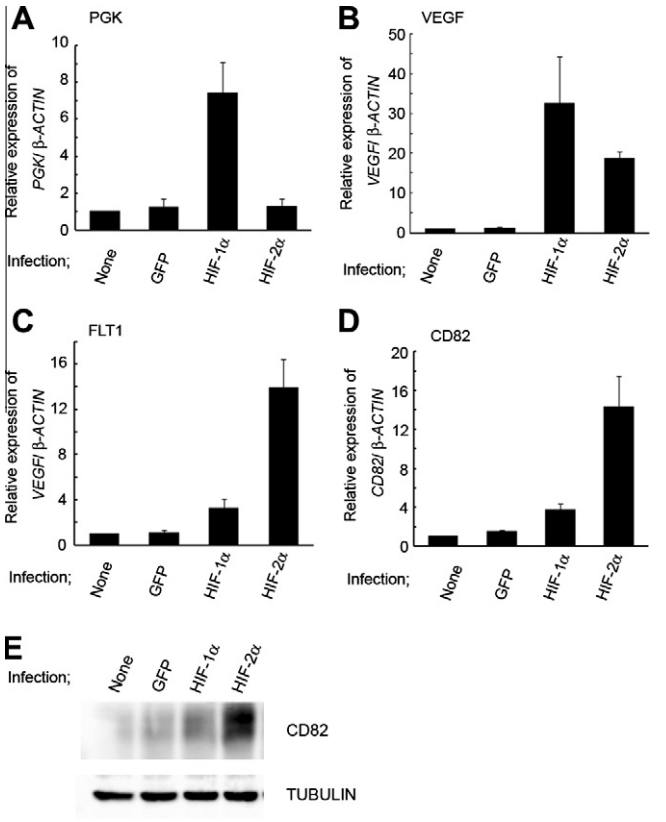

Fig. 1. Gene expression patterns in HIF-overexpressed cells. HUVECs were infected with adenovirus expressing either HIF-1α (3Mut) or HIF-2α (3Mut) under the medium condition of 0.2%FCS-HuMedia-EB2. The expression levels of PGK (A), VEGF (B), FLT1 (C) and CD82 (D) were analyzed by quantitative RT-PCR analysis. (E) HUVECs were infected with adenoviruses for 24 h. Cell lysates were subjected to immunoblot analysis of anti-CD82 antibody.

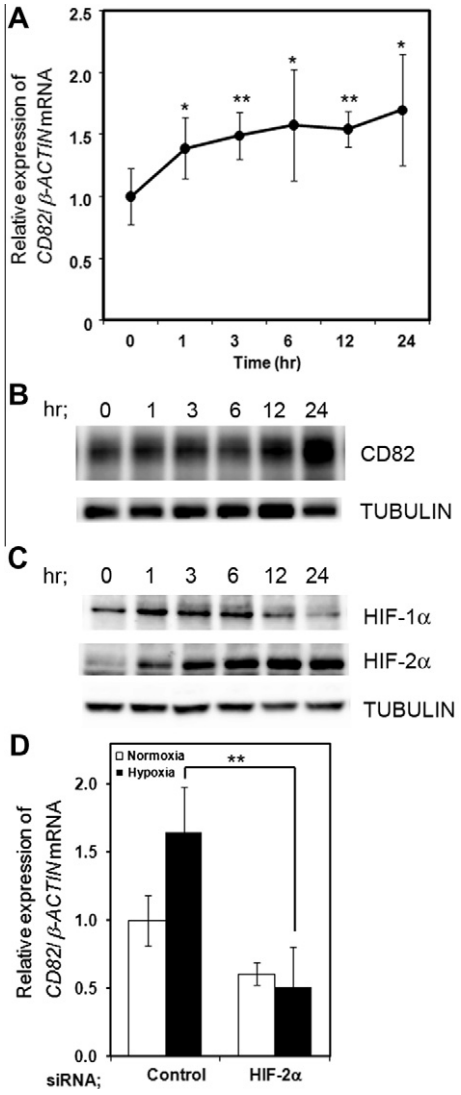

Fig. 2. Effect of hypoxia on the expression of CD82. (A) HUVECs were exposed to hypoxia for the indicated periods. After exposure, total RNA was extracted and subjected to real-time RT-PCR analysis to determine the expression level of CD82 mRNA (B and C) HUVECs were exposed to hypoxia for the periods indicated at the top. After exposure, the cells were collected, and the cell lysates were subjected to immune blot analysis with anti-CD82, anti-HIF-1α and HIF-2α. β-Tubulin was used as loading control. (D) HUVECs were transfected with either control or HIF-2α siRNAs, and then exposed to hypoxia for 12 h. Quantitative RT-PCR was carried out using a primer set for CD82.

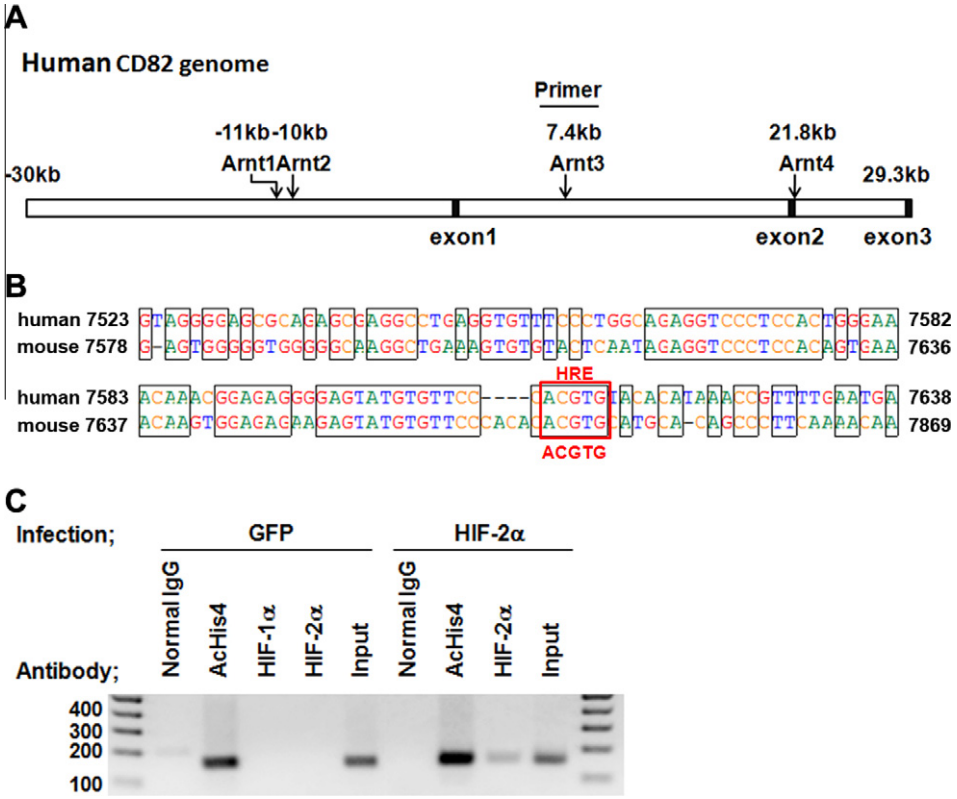

Fig. 3. HIF-2 binds to the HRE on CD82 intron1. (A) Schematic diagram of the location of the four putative ARNTs in the CD82 locus, with extra 30 kbp upstream where the transcription star site is located at bp. (B) The sequences of the ARNT3 regions are aligned between human and mouse. Sequences in the box are conserved. (C) The binding of HIF-2 to ARNT3 was investigated by ChIP assay in HUVECs. HUVECs were infected with adenovirus for 24 h. The cells were fixed with formaldehyde, and the cross-linked chromatin was immunoprecipitated with anti HIF-2 and control antibodies; IgG served as a negative control and Ac-His4 served as a positive control. Input (input) and co-immunoprecipitated DNA were used as a template for PCR with primers to amplify ARNT3. Representative gel data are shown.

at the protein level. These data indicated that HIF-2 stimulated *CD82* expression in HUVECs.

3.2. CD82 expression is up-regulated under hypoxic conditions in HUVECs

Under low O_2 conditions, HIF-α proteins were stabilized and HIF transcriptional ability was activated. Next, the question of whether hypoxia could induce CD82 expression via HIF-2 in HUVECs was tested. The up-regulation of CD82 protein was observed during exposure to hypoxia (Fig. 2A and B). Surprisingly, HIF-1α was stabilized immediately after hypoxia (0–3 h), whereas HIF-2α was stabilized at a later stage (6–24 h) (Fig. 2C). The maximum induction of CD82 expression was observed following 24 h of exposure to hypoxia. These data suggested that induction of CD82 expression was due to HIF-2α stabilization. To explore the role of HIF-2, an HIF-2α knockdown experiment was performed using HIF-2α siRNA with HUVECs exposed to hypoxia. HIF-2α knockdown HUVECs did not up-regulate CD82 protein levels under hypoxic conditions (Fig 2D). These results demonstrate that HIF-2α is required to induce CD82 expression under hypoxia.

3.3. HIF-2 binds to the CD82 HRE in HUVECs

Many HIF targets that contain HIF responsive elements (HREs) have been identified. The TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) was used for analysis of candidate HREs in the *CD82* gene and 30 kbp upstream of the gene, and four potential binding sites were found for ARNTs (ARNT1–4, in Fig. 3A). Since HIF may act as the same regulatory mechanism, the possibility that these four sites might be conserved in a mouse genome was examined. ARNT3, which was located at 7.4 kb within intron1 of the *CD82* genome, was conserved between a mouse and a human genome (Fig. 3B). In order to examine whether the predicted ARNT3 in the *CD82* locus was associated with the HIF complex, chromatin immunoprecipitation (ChIP) analysis was performed on HUVECs transfectants. HUVECs were infected with HIF-2α(3Mut) or GFP adenovirus for 24 h and then harvested for ChIP assay. HIF-2 was associated with the endogenous *CD82* HRE in HUVECs transfectant (Fig. 3C). These data showed that HIF-2 was associated with the *CD82* genome, and demonstrated a mechanism whereby CD82 was regulated by HIF-2.

3.4. CD82 suppressed the migration of HUVECs

To investigate the physiological function of CD82 in endothelial cells, the effect of the forced expression of CD82 in HUVECs was examined. First, the possibility that CD82 could regulate cell migration was investigated, because CD82 is known to be a tumor suppressor gene by inhibiting the invasive and migratory activities of tumor cells [17]. In wound–healing experiments, the wound–healing ability was reduced in CD82-overexpressed HUVECs compared with control cells (Fig. 4A and B). The process of wound–healing involved several factors such as proliferation, directed migration, cell motility and cell–cell coordination [24]. The individual mechanisms of action were determined for CD82. In cell proliferation, no significant difference could be found in the growth rates of overexpressed HUVECs, neither for CD82 nor for GFP (Fig. 4C). CD82 may stabilize or strengthen E-cadherin-dependent intercellular adhesion, reportedly by regulating

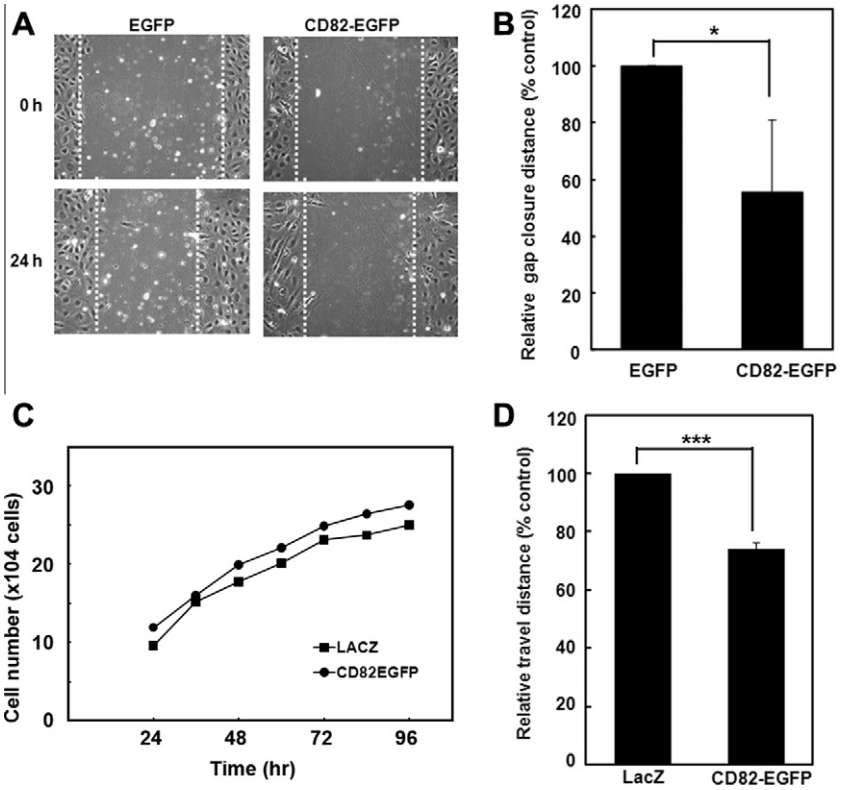

Fig. 4. The effect of the overexpression of CD82 on cell migration. (A) Wound closure assay. Wounded HUVECs were cultured for 24 h and then photographed under a light microscope. Cells were allowed to migrate in the EGF, and FGF-free, medium. (B) Average gap closure distance. Bars represent the average ± SD from four independent experiments. (C) Growth curve. HUVECs-transfectants were re-plated at 3×10^4 cells per 35 mm dish. Cells were collected and counted during the periods indicated at the top. (D) Individual cells within HUVECs-transfectants; LacZ or CD82–EGFP were tracked by time-lapsed microscopy for 10 h. The total length of cell-movement distance for 10 h was compared within cell types for an average of 15 or more tracks per cell type. Values are recorded as the mean ± SD of three independent experiments.

β-catenin-mediated signal transduction on cancer cells [25]. However, in the present study, CD82 did not reduce the tyrosine phosphorylation of β-catenin during growth factor stimulation, and had no effect on VE-cadherin localization in HUVECs (data not shown). To eliminate the possibility of cell–cell coordination, movement at the single-cell level was monitored. Single-cell tracking revealed that CD82-transfectants was reduced the total cell distance for 10 h (Fig. 4D). These data strongly indicated that CD82 suppressed cell motility in HUVECs.

4. Discussion

In the present study, HIF-2 specifically regulated CD82 gene expression in HUVECs via a binding to HRE in its intron1. Several papers have suggested that HIF-1 and HIF-2 may play different roles in different cell types and HIF-2 is especially important in endothelial cells. The fact that HIF-2 regulates the expression of VEGF Receptor-1 (*Flt-1*) [14], VEGF Receptor-2 (*Flk-1*) [26], and *Cadherin5* [27] suggests that HIF-2 plays an important role in angiogenesis. Although both HIF-1 and HIF-2 can bind to the same HRE [28], it is unknown why only HIF-2, and not HIF-1, induces these genes. HRE generally consists of HIF-binding and HIF-ancillary sequences, and there is a degree of variation in both among different genes. Furthermore, both subunits differ in their transactivation domains, implying they may have unique target genes and require distinct transcriptional cofactors [29].

The function of CD82 in endothelial cells was unknown. In the present study, CD82 may have regulated cell migration in HUVECs, similar to its effect in tumor cells. Elevated CD82 levels are reported to have drastically increased integrin αvβ3/vitronectin-dependent ovarian cancer cell adhesion. Since an inter-mediate level of cell adhesive strength is required for optimal cell migration, in ovarian cancer motility, CD82 inhibited integrin αvβ3/vitronectin-provoked tumor cell motility [30]. CD82 may play the role of a key molecule during cell migration through an association with integrins and the regulation of its function in endothelial cells.

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is crucial for many physiologic and pathologic situations. It involves coordinated endothelial proliferation, migration, and tube formation, with integrin-type adhesion receptors playing major roles. Interaction between endothelial cells and extracellular matrix has a central role in the regulation of angiogenesis processes. Endothelial cells can contact either basement-membrane or provisional matrix proteins, and can adopt either a resting or a motile and proliferative behavior, respectively [31]. Endothelial cells sense these environmental cues through integrin-adhesion receptor signal that determine cell behavior. Another of the tetraspanin family, molecule CD151, which is abundant on endothelial cells, supports pathologic angiogenesis, most likely by modulating essential endothelial cell functions [32]. CD151 affects signaling, and associates downstream functions, by providing a physical link between laminin-binding-integrins and key molecules on endothelial cells. An examination of all the evidence presented thus far suggests that overexpression of CD82 may also cause alterations in extracellular matrix-binding integrin, and also may support the pathologic angiogenesis of CD151. Additional studies are needed to investigate whether CD82 regulates pathological angiogenesis.

HIF-2α is highly expressed in embryonic vascular endothelial cells. HIF-2 activates the expression of target genes, the products of which help modulate vascular function and angiogenesis. Mice with HIF-2α-deficient endothelial cells developed normally but

displayed a variety of phenotypes including increased vessel permeability, aberrant endothelial cell ultrastructure, and pulmonary hypertension [33]. Further experimentation will focus on determining whether CD82, the specific HIF-2 target gene, contributes to the phenotype that was caused by HIF-2α deficiency in endothelial cells.

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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.2011.03.017.

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