Nuclear-translocated endostatin downregulates hypoxia inducible factor-1α activation through interfering with Zn(II) homeostasis

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Abstract. Hypoxia-inducible factor-1α (HIF-1α) is key in tumor progression and aggressiveness as it regulates a series of genes involved in angiogenesis and anaerobic metabolism. Previous studies have shown that the transcriptional levels of HIF-1α may be downregulated by endostatin. However, the molecular mechanism by which endostatin represses HIF-1α expression remains unknown. The current study investigated the mechanism by which nuclear-translocated endostatin suppresses HIF-1α activation by disrupting Zn(II) homeostasis. Endostatin was observed to downregulate HIF-1α expression at mRNA and protein levels. Blockage of endostatin nuclear translocation by RNA interference of importin α1/β1 or ectopic expression of NLS-deficient mutant nucleolin in human umbilical vein endothelial cells co-transfected with small interfering (si)-nucleolin siRNA compromises endostatin-reduced HIF-1α expression. Nuclear-translocated apo-endostatin, but not holo-endostatin, significantly disrupts the interaction between CBP/p300 and HIF-1α by disturbing Zn(II) homeostasis, which leads to the transcriptional inactivation of HIF-1α. The results reveal mechanistic insights into the method by which nuclear-translocated endostatin downregulates HIF-1α activation and provides a novel way to investigate the function of endostatin in endothelial cells.

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Introduction

Hypoxia-inducible factor-1α (HIF-1α) is a critical transcription factor that mediates cellular responses to hypoxia (1,2). By regulating a series of genes involved in angiogenesis, anaerobic energy metabolism and inflammation, HIF-1α is crucial in driving tumor progression and metastasis (3,4). Consistent with these reports, increased HIF-1α levels have been observed in various types of solid tumor (5). Moreover, elevated levels of HIF-1α protein are usually associated with poor prognosis and treatment-resistance in cancer patients (5). Since tumor progression and metastasis rely heavily on HIF-1α signaling, this pathway has become an attractive target for therapy. In the past decades, several small-molecule inhibitors of the HIF-1α pathway have been explored as potential therapeutic agents for tumors, including the heat shock protein 90 inhibitor 17-allyl-aminogeldanamycin, the topoisomerase I inhibitor topotecan and the thioredoxin inhibitor pleurotin (6). Agents such as camptothecin-11 and SN38 (7-ethyl-10-hydroxy-camptothecin) inhibit tumor angiogenesis, growth and metastasis by decreasing HIF-1α levels and inhibiting the expression of HIF-1α-modulated genes (7), including vascular endothelial growth factor receptor 2 (VEGFR2). Notably, endostatin, a potent endogenous inhibitor of angiogenesis, may also repress HIF-1α and HIF-1α-regulated gene expression (8). However, the molecular mechanism by which endostatin suppresses HIF-1α expression remains uncharacterized.

Endostatin, a 183-amino acid C-terminal proteolytic fragment of collagen XVIII, is a potent endogenous tumor angiogenesis inhibitor (9). It has been well documented that endostatin impairs angiogenesis and tumor progression by inhibiting the proliferation, migration and tube formation of endothelial cells (10). A number of studies reported that endostatin exerts its functions extracellularly (11,12). Notably, endostatin may be internalized and may translocate into the nucleus (13). Shi *et al* (13) reported that activated endothelial cells express high levels of nucleolin, which may associate with endostatin and mediate its internalization. In addition, the

internalized endostatin interrupted angiogenesis and tumor growth by inhibiting the phosphorylation of nucleolin. Besides nucleolin, the caveolae/lipid rafts and clathrin-coated pits were also crucial in endostatin internalization (14). Notably, cholesterol sequestration by nystatin increased the internalization and activity of endostatin in the endothelium, which was positively correlated with its antiangiogenic efficacy (14). More recently, Song *et al* (15) reported that endostatin internalization by endothelial cells was mediated by the integrin α5β1-nucleolin-uPAR receptor complex. Following the internalization of endostatin from the cell membrane to the cytoplasm, nucleolin and importin α1β1 mediate endostatin nuclear translocation (15). However, the detailed mechanism, particularly the contribution of the nuclear-translocated endostatin to HIF-1α signaling, has not been identified.

In the present study, nuclear-translocated endostatin was shown to inhibit HIF-1α expression, which is mediated by importin α1β1 and nucleolin. The nuclear-translocated endostatin disrupts the association of CREB-binding protein (CBP)/p300 with HIF-1α by impairing Zn(II) homeostasis. In conclusion, these results reveal how the nuclear-translocated endostatin downregulates HIF-1α expression in endothelial cells and also provides a novel explanation for the broad-spectrum anti-angiogenic activity of endostatin.

Materials and methods

Reagents. Antibodies against HIF-2α, GAPDH and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-VEGFR2 antibody was obtained from Abcam (Cambridge, MA, USA). Antibodies against HIF-1α, CBP and Oct-4 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-linked goat anti-mouse and goat anti-rabbit IgG antibody for immunoprecipitation and immunoblotting analysis, fluorescein isothiocyanate-linked goat anti-mouse and goat anti-rabbit IgG antibody for immunofluorescence and confocal microscopy were purchased from Jackson ImmunoResearch (Newmarket, UK). Small interfering (si) RNAs were obtained from Gene Pharma (Shanghai, China). Endostatin and N-4 endostatin (Δ2-5 endostatin) were obtained from Protgen Ltd. (Beijing, China).

Plasmids and construction. Wild type mouse nucleolin ($mNCL^{wt}$; NM_005381) was subcloned into the pCMV6-AN-GFP vector (Origene, Rockville, MD, USA) and NLS-deficient mutant nucleolin ($mNCL^{mut}$) was constructed using the Quick Change Mutagenesis kit (Stratagene, Santa Clara, CA, USA). All the plasmids were purified from *Escherichia coli* using PowerPrep Plasmid Purification kits (Origene, Rockville, MD, USA).

Cell transfection. The HUVEC cells in 24-well plates were transfected with the $mNCL^{wt}$ or the $mNCL^{mut}$ plasmid using a TurboFect reagent (Thermo Fisher Scientific, Waltham, MA, USA), then co-transfected twice (at 0 and 24 h) with 200 pmol scramble siRNA (or siRNA pool) or nucleolin siRNA.

Apo and holo sample preparation. Apo and holo samples (lab stock) preperation was performed as previously described (16) Briefly, HUVECs were incubated with bovine serum albumin, apo-endostatin (non-zinc-binding), BSA+$ZnCl_2$ or holo-endostatin (zinc-binding) for 12 h, all of which were lab stock.

Cell culture and RNAi. CRL-1730 human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured with 5% CO_2 in endothelial cell medium (Sciencell, Carlsbad, CA, USA) as previously described (17). For RNAi, oligofection of siRNA duplexes was performed using Oligofectamine (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, HUVECs were transfected twice (at 0 and 24 h) with 200 pmol of scramble siRNA or importin α1, importin β1 or nucleolin siRNAs. Following 24 h, the mRNA expression levels of target genes in transfected cells were detected by quantitative polymerase chain reaction (qPCR). The oligonucleotide sequences were as follows: importin α1, CGUUGUACCAGAAACUACC; importin β1, UCGGUUAUAUUUGCCAAGA; and nucleolin, GGCAAAGCAUUGGUAGCAA.

Luciferase assay. HUVECs were plated in 12-well plates at $4x10^5$ cells/well and transfected with pGL3 (empty vector) or pGL3-HIF-1α luciferase plasmid (lab stock) (15) using TurboFect (Thermo Fisher Scientific Inc., Waltham, MA, USA). Following 24 h, $mNCL^{wt}$ and NLS deficient $mNCL^{mut}$ were ectopically expressed in HUVECs cotransfected with si-nucleolin siRNA (avoiding the interference of endogenous nucleolin). The luciferase activity was measured in triplicate using the Bright-Glo Luciferase assay system (Promega Corporation, Madison, WI, USA).

qPCR. HUVECs were incubated with bovine serum albumin (BSA), apo-endostatin (non-zinc-binding), BSA+$ZnCl_2$ or holo-endostatin (zinc-binding) for 12 h. Total RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc.). qPCR amplification was performed using the SYBR-Green qPCR Master mix kit (Stratagene, Santa Clara, CA, USA). The primers used were as follows: Forward: 5'-CGTTCC TTCGATCAGTTGTC-3' and reverse: 5'-TCAGTG GTGGCAGTGGTAGT-3' HIF-1α; forward: 5'-CAAGCT ACTCAAGCTGCCAG-3' and reverse: 5'-CAACAG AGAAATGAATGCTG-3' for importin α1; forward: 5'-CAA GGCACAATATCAGC-3' and reverse: 5'-GCAGTC AGAATCTCATTGG-3' importin β1; forward: 5'-CCTTCT GAGGACATTCCAAGACA-3' and reverse: 5'-ACGGTA TTGCCCTTGAAATGTT-3' for nucleolin; and forward: 5'-CGGCTACCACATCCAAGGAA and reverse: 5'-ACC ACCCTGTTGCTGTAGCC-3' for GAPDH. Independent experiments were performed in triplicates.

Immunofluorescence and confocal microscopy. HUVECs were fixed with 4% paraformaldehyde, permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100, washed twice with PBS and blocked with PBS containing 10% normal goat serum for 15 min. Cells were stained with primary antibodies for 2 h, washed three times with PBS for 15 min and incubated with fluorescein

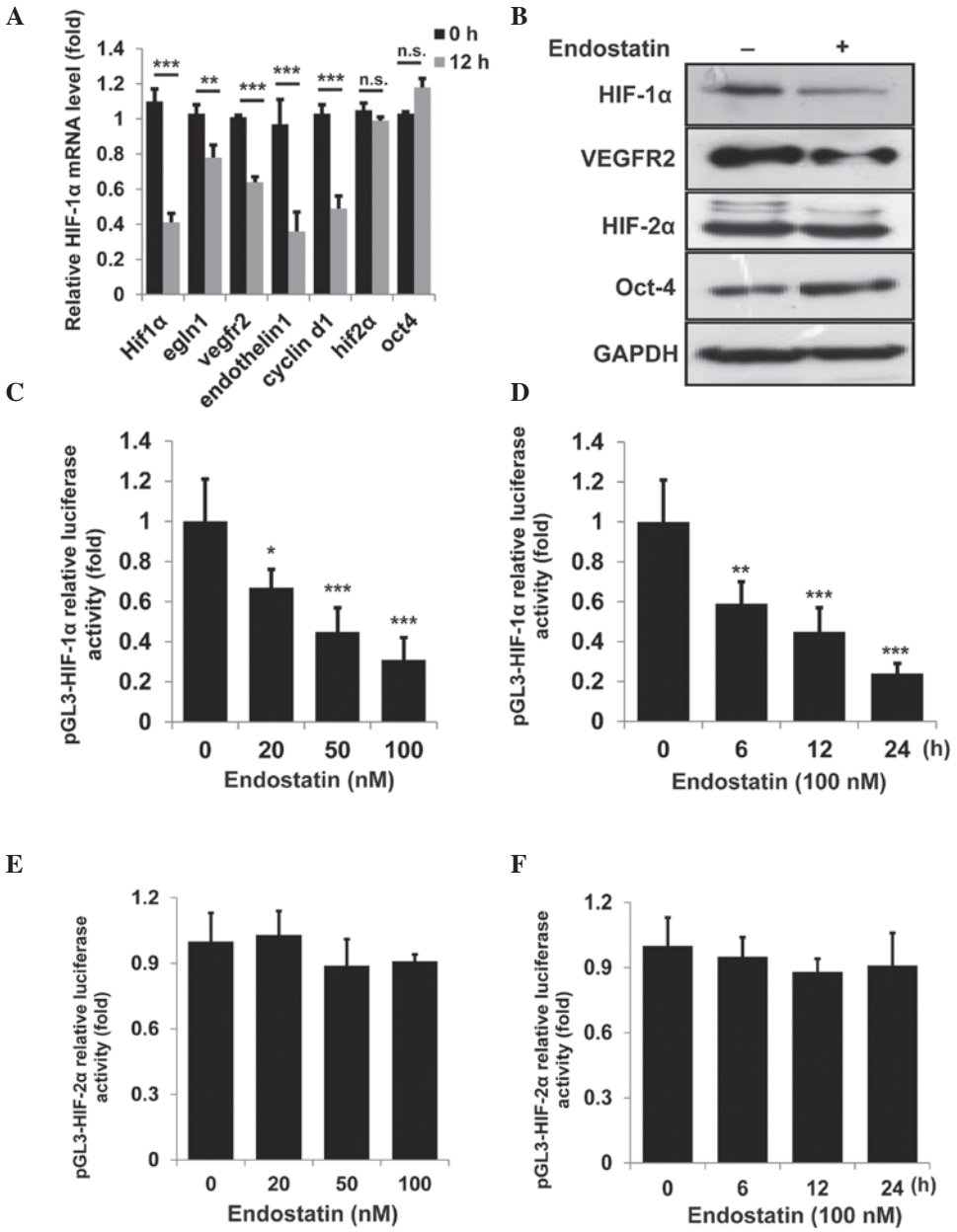

Figure 1. Endostatin represses HIF-1α expression. (A) HUVECs were treated with 100 nM endostatin for 12 h, the mRNA levels of indicated genes were detected by qPCR. (B) HUVECs were treated as shown in (A) and western blot analysis shows levels of indicated proteins in HUVECs. Endostatin suppressed the expression of the HIF-1α reporter gene in the luciferase activity assay. (C) HUVECs were treated with 20, 50 and 100 nM endostatin for 12 h or (D) with 100 nM for the indicated times. HUVECs were treated (E) with 20, 50 and 100 nM endostatin for 12 h or (F) 100 nM for indicated times to determine the effect of endostatin on the HIF-2α reporter gene in the luciferase activity assay. Data are expressed as relative luciferase activity (fold). All experiments were repeated at least twice. *P<0.05, **P<0.01 and ***P<0.005, vs. control. HIF-1α, hypoxia-inducible factor-1α; HUVECs, human umbilical vein endothelial cells; qPCR, quantitative polymerase chain reaction.

isothiocyanate FITC-linked anti-mouse and anti-rabbit IgG antibodies for 30 min at room temperature. Nuclei were stained with DAPI (Beyotime Institute of Biotechnology, Haimen, China). All immunofluorescence images were analyzed with a Nikon A1 laser scanning confocal microscope (63x/1.49 NA oil objective) and NIS-Elements AR software (Nikon Corporation, Tokyo, Japan).

Immunoprecipitation and immunoblotting analysis. Immunoprecipitation and immunoblotting assays were performed as previously described (15) and experiments were repeated at least twice.

Statistical analysis. For statistical analysis, the data are expressed as the mean ± standard deviation and compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Endostatin suppresses HIF-1α expression. Abdollahi *et al* (8) have reported that the impact of endostatin on genetic expression is widespread in 12% of the genome. Notably, a number of hypoxia-associated genes are significantly downregulated by endostatin. Since HIF-1 signaling has been well-documented

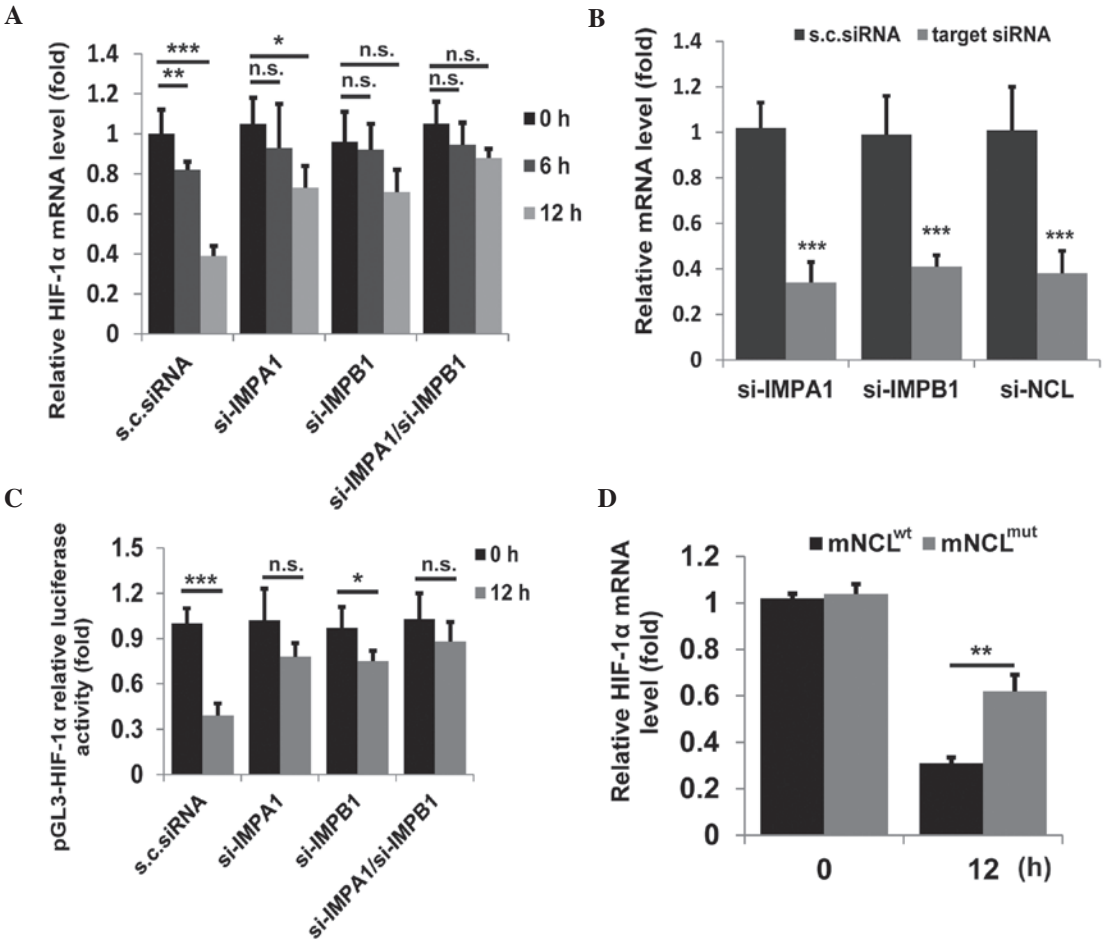

Figure 2. Effect of nuclear-translocated endostatin on HIF-1α expression. (A) The relative mRNA level (fold) of HIF-1α in HUVECs transfected with siRNA targeting indicated genes or scrambled siRNA, detected by qPCR. (B) HUVECs were transfected twice with indicated siRNAs and analyzed following 24 h. The knock-down efficiencies of targeted genes were detected by qPCR. (C) HUVECs were transfected with siRNA targeting indicated genes or scrambled siRNA and the pGL3-HIF-1α relative luciferase activity (fold) in cell lysates was measured. (D) The relative mRNA level (fold) of HIF-1α in HUVECs co-transfected with siRNA targeting human nucleolin and the vector encoding $mNCL^{wt}$ or $mNCL^{mut}$, detected by qPCR. Data are shown as the mean ±standard deviation. All experiments were repeated at least twice. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$, vs. control. HIF-1α, hypoxia-inducible factor-1α; HUVECs, human umbilical vein endothelial cells; qPCR, quantitative polymerase chain reaction; $mNCL^{wt}$, mouse wildtype nucleolin; $mNCL^{mut}$, NLS-mutant nucleolin; IMPA1, importin α1; IMPB1, importin β1.

as an angiogenic inducer (18-20), the endostatin-induced repression of several HIF-1 or HIF-2-associated genes were examined by qPCR. As shown in Fig. 1A, the expression of all HIF-1α associated genes in HUVECs, including *hif-1α*, *egln1*, *vegfr2*, *endothilin-1* and *cyclin D1* was significantly suppressed by endostatin, while the HIF-2α-associated genes, including *hif-2α* and *oct-4*, were not affected. The protein levels of HIF-1α, VEGFR2, HIF-2α and Oct-4 were further detected. Consistently, the expression of HIF-1α and VEGFR2, but not HIF-2α and Oct-4, were suppressed by endostatin (Fig. 1B). To further confirm whether endostatin may directly repress the transcriptional activity of HIF-1α, HIF-1α-luciferase activity was detected following endostatin treatment. As shown in Fig. 1C and D, endostatin suppressed HIF-1α-luciferase activity in a time- and dose-dependent manner, while having little effect on HIF-2α-luciferase activity (Fig. 1E and F). These results indicate that endostatin inhibits HIF-1α expression at the transcriptional level.

Downregulation of HIF-1α is modulated by nuclear-translocated endostatin. A number of studies have reported that nuclear translocation is essential for the antitumor effects of endostatin (13,15). As treatment of human umbilical vein endothelial cells (HUVECs) with endostatin for 30 min leads to endostatin in cell nucleus (15), endostatin in the cell nucleus was defined as nuclear-tanslocated endostatin. Downregulation of HIF-1α was hypothesized to be modulated by nuclear-translocated endostatin. The importin α1β1/nucleolin complex has been observed to mediate endostatin nuclear translocation (15). In the current study, siRNAs were used to block importin-dependent translocation. The results showed that siRNAs that targeted importin α1 or β1 significantly eliminated the endostatin-mediated suppression of HIF-1α expression (Fig. 2A and B). In addition, endostatin exhibited little effect on HIF-1α-luciferase activity upon knock-down of importin α1 or β1 (Fig. 2C). To further verify that the downregulation of HIF-1α was modulated by the nuclear-tanslocated endostatin, wild-type mouse nucleolin ($mNCL^{wt}$) and NLS-deficient mutant nucleolin ($mNCL^{mut}$) were ectopically expressed in HUVECs co-transfected with si-nucleolin siRNA. Compared with $mNCL^{wt}$, the ectopic $mNCL^{mut}$ in HUVECs significantly attenuated endostatin-mediated suppression of the HIF-1α mRNA level (Fig. 2D). These observations

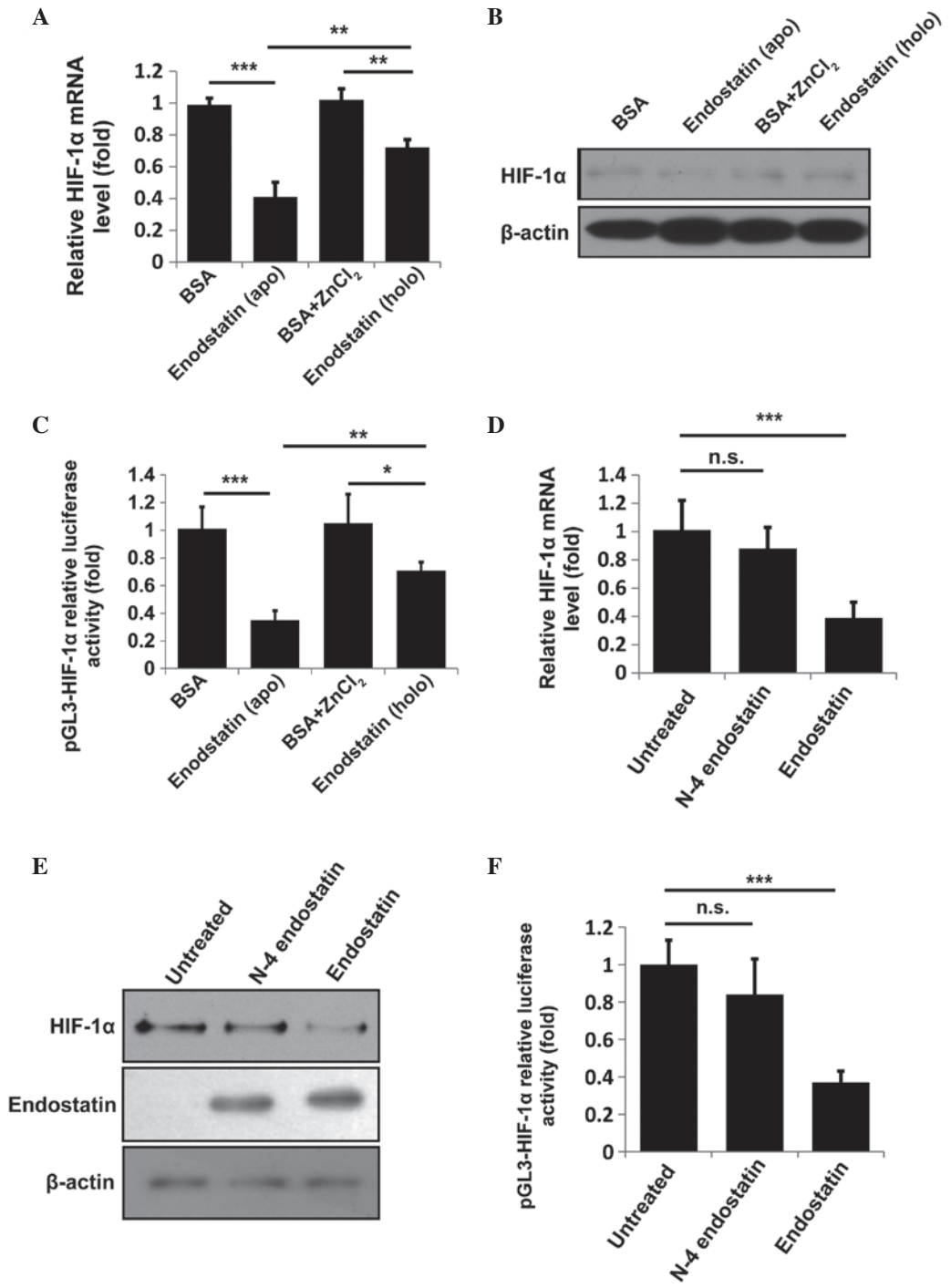

Figure 3. Zn(II)-binding capacity is indispensable for endostatin-mediated HIF-1α inactivation. HUVECs were treated with bovine serum albumin, apo-endostatin [endostatin (apo)], BSA+$ZnCl_2$ and holo-endostatin [endostatin (holo)] for 12 h and (A) the relative mRNA level (fold), (B) protein level and (C) relative luciferase activity (fold) of HIF-1α were measured. HUVECs were treated without or with N-4 endostatin, endostatin for 12 h and (D) the relative mRNA level (fold), (E) protein level and (F) relative luciferase activity (fold) of HIF-1α were measured. Data are shown as the mean ± standard deviation. All experiments were repeated at least twice. *P<0.05, **P<0.01 and ***P<0.005, vs. control. HIF-1α, hypoxia-inducible factor-1α; HUVECs, human umbilical vein endothelial cells.

demonstrate that nuclear-translocated endostatin is critical in HIF-1α suppression.

Zn(II)-binding capacity is indispensable for endostatin-mediated HIF-1α inactivation. The crystal structure of endostatin shows that endostatin is a Zn(II)-dependent protein (21) and other studies have reported that its Zn(II)-binding capacity is responsible for its anti-angiogenic activity (16,22). Moreover, the Zn(II) dissociation constant of endostatin was measured to be 6.7 nM (16), suggesting a marked Zn(II)-binding capacity. Thus, Zn(II) homeostasis in the nucleus was hypothesized to be regulated by the nuclear-translocated endostatin. To verify this hypothesis, HUVECs were incubated with BSA, apo-endostatin (non-zinc-binding), BSA+$ZnCl_2$ or holo-endostatin (zinc-binding) for 12 h. The cells were collected and analyzed, as shown in Fig. 3A-C. Compared with apo-endostatin, holo-endostatin had a reduced ability to suppress the mRNA and protein levels of HIF-1α and marginally downregulated

the HIF-1α-luciferase activity. Previously, Fu and Luo (22) reported that the N-terminal 2-5 amino acid residues (HSHR) of endostatin were critical for its zinc binding. N-4 endostatin (Δ2-5 endostatin) was used to further confirm this observation. Consistently, N-4 endostatin treatment had little effect on HIF-1α expression and HIF-1α-luciferase activity (Fig. 3D-F). In addition, no significant differences among BSA, apo-N-4 endostatin, BSA+$ZnCl_2$ or holo-N-4 endostatin groups in regulating HIF-1α expression or HIF-1α-luciferase activity were identified (Fig. 4A-C). These results indicate that the competition for Zn(II) by nuclear-translocated endostatin results in the transcriptional inactivation of HIF-1α.

Nuclear-translocated endostatin disrupts the interaction between CBP/p300 and HIF-1α by competing for Zn(II). In this study, the inhibitory effect of endostatin on HIF-1α expression was investigated. Although HIF-1α itself is not a Zn(II)-binding protein, it activates the transcription of adaptive genes by recruiting a well-known co-activator, CBP/p300 in a Zn(II)-dependent manner (23). As a result of a reduction in free Zn(II) induced by nuclear-translocated endostatin, the interaction between HIF-1α and CBP/p300 may be interrupted and the HIF-1α-mediated transcription is likely to be further disturbed. Since HIF-1α is a self-regulated gene, the disrupted association of CBP/p300 with HIF-1α may also be caused by endostatin-induced HIF-1α suppression. To exclude this possibility, HUVECs were treated with endostatin for the indicated times and mRNA were detected by qPCR. As shown in Fig. 5A, endostatin treatment for 3 h showed little effect on HIF-1α mRNA. Therefore, HUVECs were treated with BSA, apo-endostatin, BSA+$ZnCl_2$ or holo-endostatin for 3 h and the cell immunofluorescence imaging was captured by confocal microscopy. As shown in Fig. 5B and C, compared with apo-endostatin, holo-endostatin only marginally interfered with the co-localization of CBP/p300 and HIF-1α. To further confirm this result, HUVECs were treated as described in Fig. 4B and the interaction between HIF-1α and CBP/p300 was evaluated by immunoprecipitation assay. Consistently, holo-endostatin treatment exhibited a marginal effect on the association of CBP/p300 with HIF-1α compared with the apo-endostatin group (Fig. 5D). These findings demonstrate that competition for Zn(II) by nuclear-translocated endostatin disrupts the interaction between CBP/p300 and HIF-1α.

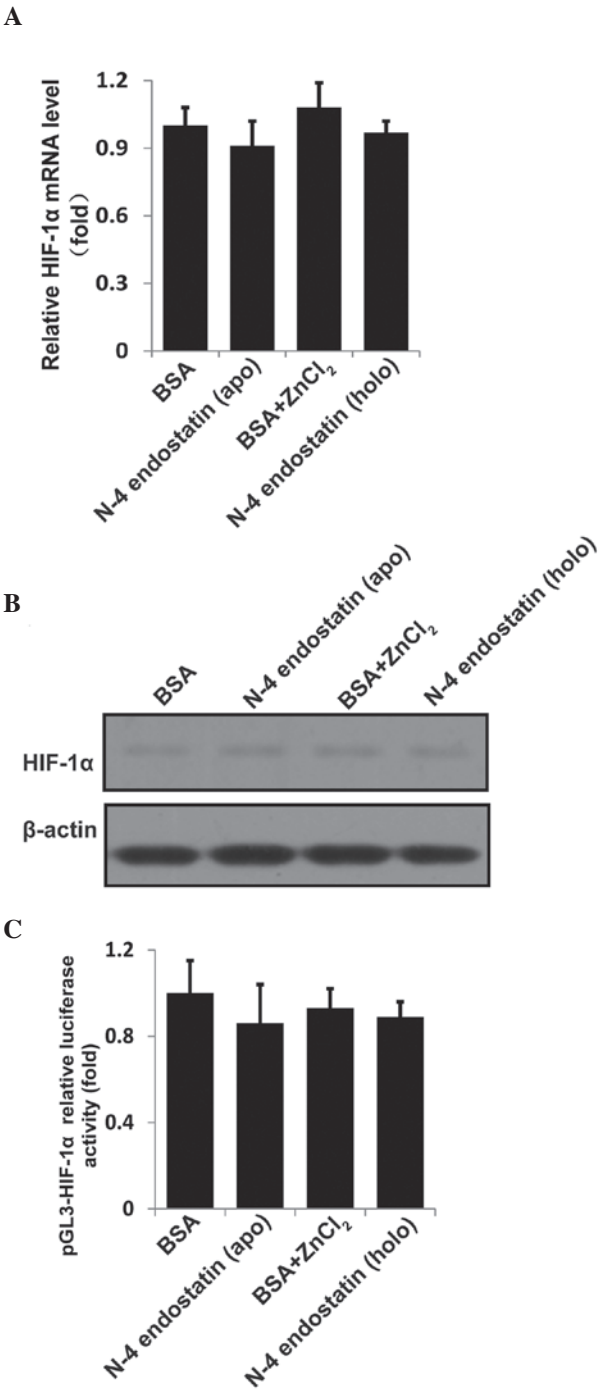

Figure 4. HUVECs were treated with BSA, N-4 endostatin (apo), BSA+$ZnCl_2$ and N-4 endostatin (holo) for 12 h. (A) The relative mRNA level (fold), (B) protein level and (C) relative luciferase activity (fold) of hypoxia-inducible factor-1α (HIF-1α) were measured. Data are shown as the mean ± standard deviation. All experiments were repeated at least twice. HUVECs, human umbilical vein endothelial cells; BSA, bovine serum albumin.

Discussion

Nuclear-translocated endostatin has been shown to downregulate the transcriptional activity of HIF-1α by disrupting the interaction between CBP/p300 and HIF-1α. Thus, a working model, based on the present results, is proposed as shown in Fig. 5E i.e., nuclear-translocated endostatin mediated by nucleolin and importin α1β1 disrupts the interaction between CBP/p300 and HIF-1α by competing for Zn(II) and then governs the HIF-1α signaling at transcriptional level.

Endostatin is a well-documented endogenous inhibitor of angiogenesis (9). Although the structure, function and molecular mechanism of endostatin have been extensively investigated, several controversial observations remain. It was unclear why *P. pastoris*-expressed endostatin was failed at phase II in the USA, whereas endostar, an N-terminal-modified endostatin expressed by *E. coli*, was approved by the State Food and Drug Administration (24). To determine the cause of this, *P. pastoris*- and *E. coli*-expressed endostatins were investigated. Notably, ~93% of *P. pastoris*-expressed endostatin was observed in the truncated form, which lost its zinc-binding capacity, leading to reduced stability and lowered anti-angiogenic capacity. Endostatin expressed by *E. coli* was shown to have an intact molecular structure with full antitumor activity (22). Therefore, *E. coli*-expressed endostatin was used in the current study.

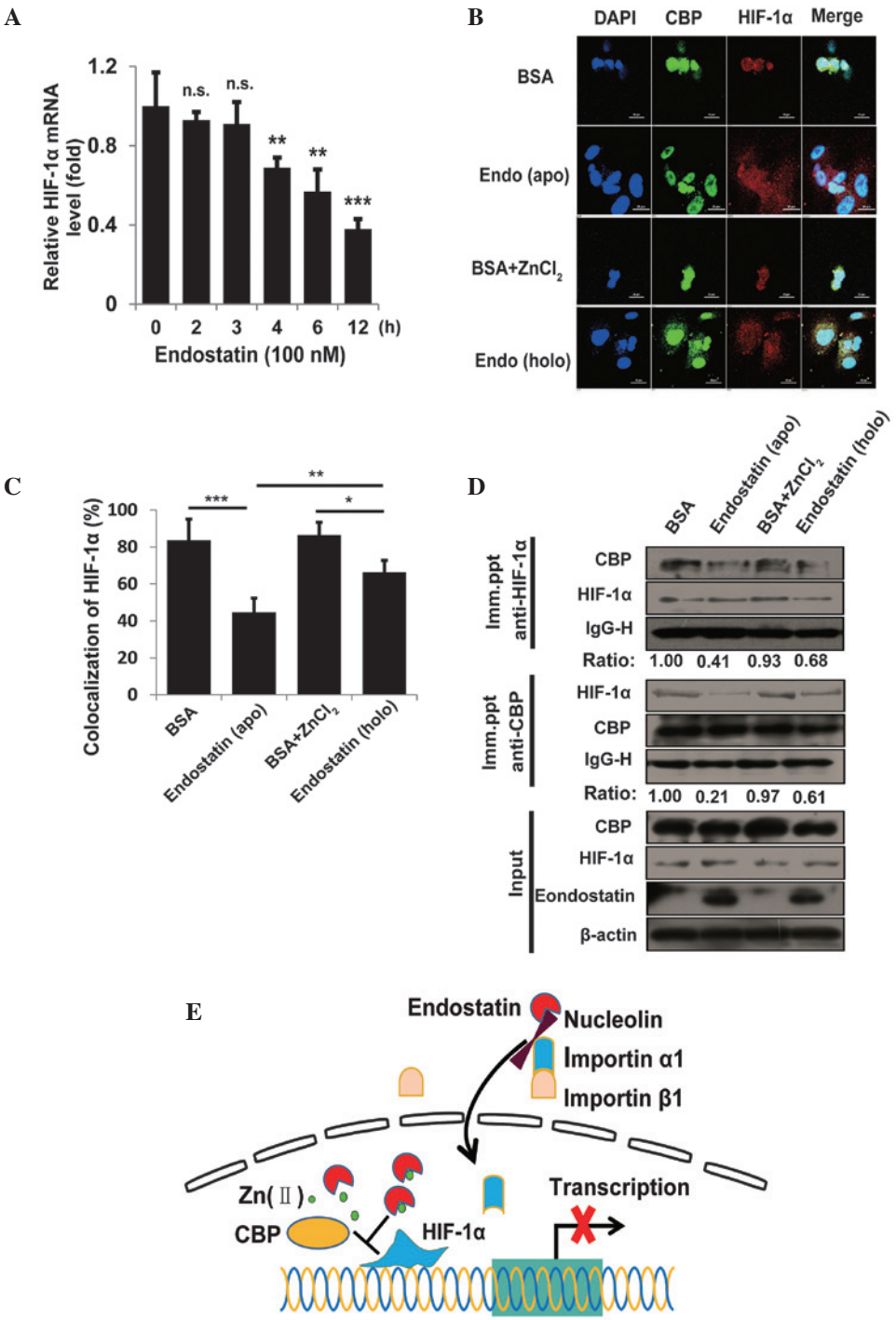

Figure 5. Effect of nuclear-translocated endostatin on the interaction between CBP/p300 and HIF-1α. (A) HUVECs were treated with endostatin (100 nM) for the indicated times and the relative mRNA level of HIF-1α (fold) was detected by qPCR. (B) Immunofluorescence staining of CBP/p300 (green) and HIF-1α (red) in HUVECs treated with BSA, apo-endostatin, BSA+$ZnCl_2$ and holo-endostatin. Digital images were captured by the Nikon A1 fluorescence microscope using 63x/1.49 NA oil objectives. Images were captured with NIS-Elements AR 3.0 software. Nuclei (blue) were stained by DAPI. Scale bar, 20 μm. (C) The levels of colocalization (Fig. 4B) were quantified by calculating the percentage of red pixels (HIF-1α) that colocalized with light blue pixels (green, blue merged) from eight random fields per well of three experiments. (D) Following incubation with BSA, apo-endostatin, BSA+$ZnCl_2$ and holo-endostatin, cells were lysed and immunoprecipitated with agarose-conjugated anti-HIF-1α and anti-CBP to detect the binding pattern between HIF-1α/CBP. The indicated proteins were detected by immunoblotting. (E) Model for the nuclear-translocated endostatin effect on HIF-1α inactivation: Nuclear-translocated endostatin mediated by nucleolin and importin α1β1 disrupts the interaction between CBP/p300 and HIF-1α though interfering with Zn(II) homeostasis and then governs the HIF-1α signaling at the transcriptional level. Data are presented as the mean ± standard deviation. All experiments were repeated at least twice. *P<0.05, **P<0.01 and ***P<0.005, vs. control. CBP, CREB-binding protein; HIF-1α, hypoxia-inducible factor-1α; HUVECs, human umbilical vein endothelial cells; qPCR, quantitative polymerase chain reaction; BSA, bovine serum albumin.

Increasing evidence demonstrated that endostatin inhibited endothelial cell proliferation, migration and tube formation by blocking a number of well-known pathways associated with angiogenesis, including HIF-1α, nuclear factor (NF)-κB, activator protein 1 and Stats (8). In the current study, nuclear-translocated endostatin was observed to downregulate

HIF-1α activation at the transcriptional level. Since HIF-1α expression was partly regulated in an NF-κB-dependent manner, endostatin inhibition of the NF-κB pathway may augment the inhibition of HIF-1α. In addition, VEGFR2, a downstream gene of HIF-1α, was also downregulated by endostatin (Fig. 1A and B), which was consistent with previous studies (8). The aforementioned mentioned results provide substantial evidence that endostatin downregulates the HIF-1α pathway in endothelial cells.

Exclusive internalization of endostatin in endothelial cells has been observed and was demonstrated to exhibit an integral role in inhibiting angiogenesis and tumor growth (13-15). More recently, Song *et al* (15) reported that endostatin was transported into the nucleus by the importin α1β1/nucleolin complex. Similarly, in the current study, the nuclear-translocation of endostatin mediated by the importin α1β1/nucleolin complex was shown to be critical for the regulation of HIF-1α transcription (Fig. 2A-E). Notably, endostatin is a Zn(II)-binding protein and the Zn(II)-binding site consists of three histidine residues (His1, His3 and His11) and an aspartic acid residue (Asp76) at the N-terminus (16,21). Neither double mutation in H1/3A nor site mutation in His11 or Asp76 significantly impaired its anti-angiogenic activity, suggesting that the Zn(II)-binding capacity is central in the bioactivity of endostatin. In addition, N-4 endostatin exhibited a reduced zinc-binding capacity (22), which led to decreased stability and impaired antitumor capacity of endostatin, also indicating that the Zn(II)-binding capacity was indispensable for its function. Moreover, Song *et al* (15) proposed that nuclear-translocated endostatin may block a number of well-known pathways associated with angiogenesis. Consistently, the current results have shown that nuclear-translocated endostatin impairs the interaction between CBP/p300 and HIF-1α through the competition for Zn(II), which results in downregulation of HIF-1α expression. Based on the current studies, these observations provide solid evidence to support the proposed working model (Fig. 5E). In conclusion, the present study shows that nuclear-translocated endostatin disrupts the interaction between CBP/p300 and HIF-1α through the competition for Zn(II), which leads to the transcriptional inactivation of HIF-1α. This study identifies a novel molecular mechanism by which nuclear-translocated endostatin inhibits HIF-1α expression and provides a novel explanation for understanding the contribution of Zn(II) to the bioactivity of endostatin.

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