Life Sciences 118 (2014) 185–190

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# Endothelin-1 regulates hypoxia-inducible factor-1α and -2α stability through prolyl hydroxylase domain 2 inhibition in human lymphatic endothelial cells

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ARTICLE INFO

*Article history:*
Received 1 November 2013
Accepted 21 February 2014
Available online 6 March 2014

*Keywords:*
Endothelin-1
Prolyl hydroxylases enzymes
Lymphatic endothelial cells
Hypoxia-inducible factor 1
Endothelin B receptor

ABSTRACT

*Aims:* Lymphangiogenesis, the formation of new lymphatic vessels, is thought to constitute a route for the tumor cells to metastasize. We previously demonstrated that endothelin-1 (ET-1) induces the expression of lymphangiogenic factors through hypoxia-inducible factor (HIF)-1α and HIF-2α. The stability of these transcriptional factors is essential for lymph/angiogenesis and tumor progression. Here we analyze the molecular mechanism through which ET-1 regulates HIF-1α and HIF-2α protein levels and how these transcriptional factors are implicated in controlling lymphatic endothelial cell (LEC) behavior.
*Main methods:* Using Western blotting assay and a reporter gene containing the HIF-1α oxygen-dependent degradation domain we monitored the capacity of ET-1 to increase HIF-1α and HIF-2α stability and nuclear accumulation. In addition, using siRNA against HIF-1α or HIF-2α, we investigated the implication of these transcriptional factors in ET-1-mediated tube-like structure formation. As HIF-1α proteosomal degradation is controlled by site-specific hydroxylation carried out by HIF-prolyl hydroxylase domain (PHD) enzymes, we analyzed the expression of PHD-2 isoform.
*Key findings:* We show that ET-1 through its receptor, $ET_BR$, controls HIF-α stability and nuclear accumulation by inhibiting prolyl hydroxylation and reduces PHD2 mRNA and protein levels. Transfection with HIF-1α or HIF-2α siRNA abrogated the capacity of ET-1 to induce tube-like structure formation.
*Significance:* These results reveal a PHD2-mediated mechanism through which ET-1 stabilizes HIF-1α and HIF-2α pathway thereby regulating LEC behavior and lymphangiogenesis.


## Introduction

Lymphangiogenesis refers to the formation of new lymphatic vessels that occurs during tumor growth and is thought to constitute conduits for the tumor cells to metastasize (Alitalo and Detmar, 2012). Therefore, elucidation of mechanisms regulating lymphatic vessel growth is a critical step toward developing therapeutic interventions aimed at preventing lymphatic metastases (Alitalo et al., 2005). Recently, several growth factors have been shown to be implicated in mediating lymphangiogenesis (Cao et al., 2006; Cao et al., 2012; Alitalo and Detmar, 2012). In particular we demonstrated that endothelin (ET)-1 through its binding with ETB receptor ($ET_BR$) expressed on lymphatic endothelial cells (LEC), induced cell growth and invasiveness directly and in a vascular endothelial growth factor (VEGF)-mediated manner (Spinella et al., 2009; Garrafa et al., 2012). ET-1-induced effects on LEC were abolished by $ET_BR$ antagonists, suggesting that this receptor may represent a potential target to inhibit lymphangiogenesis (Rosanò et al., 2013; Cueni et al., 2010).

Hypoxia-inducible factor-1α (HIF)-1α and HIF-2α are transcription factors that play a central role in coordinating angiogenesis (Semenza, 2012). In normoxic conditions, HIF-1α and HIF-2α are degraded by a mechanism involving hydroxylation of 2 proline residues, by HIF-prolyl hydroxylase domain (PHD), ubiquitylation, and proteosomal degradation (Metzen et al., 2005). Hypoxia and angiogenic stimuli stabilize HIF-1α, enabling dimerization with HIF-1β, nuclear translocation, and activation of HIF-1α/β-responsive elements in target genes. In this manner, the cascade of target gene transcription by HIF-1α coordinates diverse physiological events, such as cellular migration, proliferation, and tubule formation,

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http://dx.doi.org/10.1016/j.lfs.2014.02.030

which ultimately results in vasculogenesis (Semenza, 2012; Kaelin and Ratcliffe, 2008).

The PHD family is comprised of three bona fide HIF prolyl hydroxylases (PHD1, PHD2, and PHD3), among these PHD2 has been proposed to be the key oxygen sensor for setting steady-state levels of HIF-1α (Berra et al., 2003). In this regard we previously demonstrated that PHD2 mediates ET-1-induced HIF-1α and HIF-2α stability in melanoma cells (Spinella et al., 2010). More recently it has been shown that ET-1 induces a pulmonary arterial endothelial and smooth muscle cells (PASMC)-specific increase in HIF-1α levels by upregulation of HIF-1α synthesis and downregulation of PHD2-mediated degradation, thereby amplifying the induction of HIF-1α in PASMCs during moderate, prolonged hypoxia (Pisarcik et al., 2013). To date, relatively little is known about the ability of the individual members of the PHD family to control HIF-1α and HIF-2α in LEC.

Here we demonstrate that in LEC ET-1 induces HIF-1α and HIF-2α stabilization and nuclear accumulation by impairing their degradation. Using siRNA against HIF-α subunits, we show that HIF-1α and HIF-2α play a key role in coordinating and initiating lymphangiogenesis by regulating migration and sprouting of LEC. Therefore in this study we defined the molecular mechanism through which ET-1 controls HIF-1α and HIF-2α and identified PHD2 as potential regulator of HIF-1α and HIF-2α-mediated ET-1 lymphangiogenic activities.

## Material and methods

### *Lymphatic endothelial cell purification and cell cultures*

Human lymph node specimens were obtained according to the guidelines of Helsinki Declaration from patients undergoing surgical procedures for non-infectious or neoplastic conditions. Lymph nodal LEC were isolated as previously described (Garrafa et al., 2005; Garrafa et al., 2006; Spinella et al., 2009) from a pool of three patients. Cells were grown on collagen type I-coated flasks (5 μg/cm$^2$; Boehringer Mannheim) in EGM-2 MV (Lonza, Basel Switzerland). For the HIF-1α and HIF-2α stability study cycloheximide (CHX, 100 μM, SIGMA) was added after 24 h of ET-1 or hypoxic treatment, and the cells were further incubated under normoxia for varying times in the presence of CHX. To expose cells to hypoxia, a modular incubator was used with an atmosphere setting of 5% $CO_2$, 95% $N_2$, and 1% $O_2$. ET-1 (Peninsula Laboratories, Belmont, CA) was used at 100 nM. When BQ788 (1 μM; Peninsula Laboratories) was tested, it was added 30 min before the stimuli.

### *Transfection and luciferase assay*

Transfection experiments employed the Lipofectamine reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. For the silencing, small interfering RNA (siRNA) duplexes against human $ET_BR$, HIF-1α or HIF-2α mRNA (100 nM) (SMARTpool; Dharmacon, Lafayette, CO) were used. Non-target control siRNAs (Dharmacon) were used as control. After 48 h cells were used for 24 h treatment with ET-1 or hypoxia. Each knockdown experiment described herein was detected for a specific reduced expression of $ET_BR$, HIF-1α, or HIF-2α mRNA by quantitative real time PCR. For plasmid 1 μg of vectors encoding CMV-Luc-ODDD (Dr. R. K. Bruick, University of Texas Southwestern Medical Center, TX) in combination with the pCMV-β-galactosidase plasmid (Promega, Corporation, Madison, WI) were used. Luciferase activities were measured with Luciferase assay system (Promega).

### *Western blot analysis*

Cells were lysed in lysis buffer [250 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, protease inhibitors]. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Illkirch-Cedex France) were used to separate cytoplasmic and nuclear fractions. Whole cell lysates or nuclear extracts were subjected to SDS-PAGE and analyzed by Western blotting. Blots were developed with the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). Antibody against PHD2 was from Novus Biologicals (Littleton, CO); β-actin, used as loading control, was from Oncogene (CN Biosciences, Inc., Darmastadt, Germany); and anti-PCNA and anti-HSP70 (Santa Cruz, Aachen, Germany) were used to verify the purity of nuclear fractions.

### *Quantitative real-time-PCR*

Total RNA was isolated using the TRIzol (Invitrogen) according to the manufacturer's protocol. Five μg of RNA was reversed transcribed using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen). Quantitative real-time-PCR was performed by using the LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Reaction was performed in 20 μl volume with 0.3 μM primers, by using the LightCycler-FastStart DNA Master Plus SYBR Green mix (Roche Diagnostics) from 1 μl cDNA under the following conditions: denaturation at 95 °C for 5 min; melting at 95 °C for 10 s; annealing between 58 °C for 10 s; extension at 72 °C for 10 s. Primers used were as follows: PHD2, (forward) 5′-GCACGACACCGGGAAGTT-3′, (reverse) 5′-CCAGCTTCCCGTTACAGT-3′; HIF-1α (forward) 5′-CAAGTCACCACAGGACAG-3′, (reverse) 5′-AGGGAGAAAATCAAGTCG-3′; HIF-2α (forward) 5′-TGATTTCCTGTGTGTGTTGC-3′, (reverse) 5′-CGTTACGTTGACAGGTAGGG-3′ and cyclophilin-A, (forward) 5′-TTCATCTGCACTGCCAAGAC-3′, and (reverse) 5′-TGGAGTTGTCCACAGTCAGC-3′. The number of each gene-amplified product was normalized to the number of cyclophilin-A amplified product and results were expressed as fold induction compared to the control.

### *In vitro tube formation assay*

Tube forming activity was analyzed as previously described (Spinella et al., 2009). In brief, 50 μl of Matrigel (Cultrex, basal membrane extract, Trevigen, 12.7 mg/ml) was dropped onto each well of a 96-well plate and was allowed to solidify for 1 h at 37 °C in a humidified 5% $CO_2$ incubator. LEC at 1 to 5 × $10^4$ were plated and the cells were cultured under normoxia or hypoxia. Formation of tube-like structures was photographed after 24 h using light microscopy and quantified by counting the number of tubule intersections in 10 randomly chosen fields using the Scion image analysis program. Magnification ×20.

### *Statistical analysis*

Each experiment was repeated at least three times with comparable results, unless indicated otherwise. Statistical analysis was carried out by using the Student's *t* test or one-way analysis of variance (ANOVA) as appropriate. All statistical tests were two-sided and were performed using the PRISM software (GraphPad Prism version 5.0, Software, Inc., San Diego, CA). p values were significant at $p < 0.05$.

## Results

### *ET-1 induces HIF-1α and HIF-2α stabilization through $ET_BR$*

To assess whether ET-1 regulated the expression of HIF-1α and HIF-2α protein by inhibiting its degradation, we monitored the levels of protein after protein synthesis blockade by cycloheximide (CHX), as positive control LEC were exposed to hypoxia. Cells were treated with ET-1 (100 nM) or exposed to hypoxia (1% $O_2$) for 24 h and then transferred to normoxia and cultured in the presence of CHX for the indicated times. As shown in Fig. 1A, ET-1 or hypoxia markedly increased the HIF-1α and HIF-2α protein levels. After removing ET-1 the levels of ET-1-induced HIF-1α and HIF-2α decayed after 120 min, while that

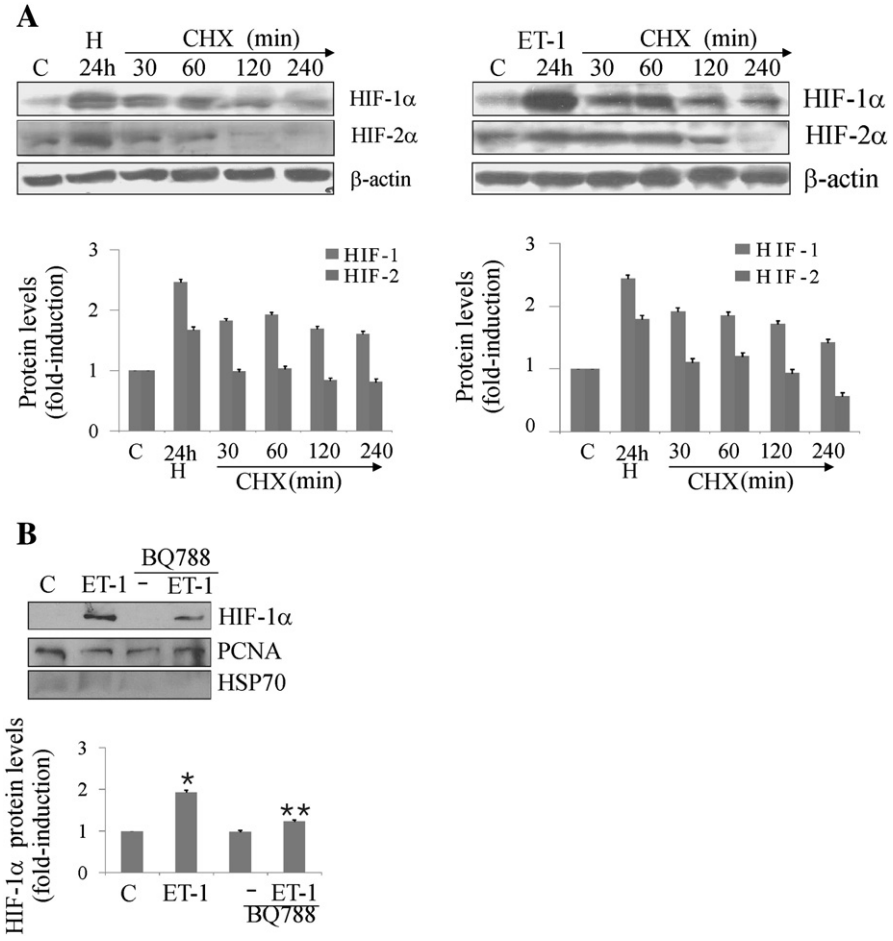


**Fig. 1.** ET-1 induces HIF-1α and HIF-2α stabilization and nuclear accumulation through $ET_BR$. (A) LEC were stimulated with ET-1 (100 nM) or hypoxia (1% $O_2$) for 24 h. After that cycloheximide (CHX) was added and cells were transferred under normoxic conditions. Total cell lysates were analyzed for HIF-1α and HIF-2α protein expression by Western blotting assay, β-actin was used as loading control. The average of HIF-1α and HIF-2α shown in the graphics corresponds to the quantification of protein bands in images, normalized to β-actin content ± SD. (B) LEC were stimulated with ET-1 (100 nM) alone or in combination with BQ788 (1 μM) and after 6 h nuclear extracts were analyzed by Western blotting for HIF-1α. Proliferating cell nuclear antigen (PCNA) antibody was used as loading control and HSP70 to verify the purity of nuclear extracts. The average of nuclear HIF-1α shown in the graphics corresponds to the quantification of protein bands in images from three independent experiments, normalized to PCNA content ± SD. *$p < 0.05$ (versus control); **$p < 0.01$ (versus ET-1).

observed after the transfer of cells from hypoxia to normoxia decayed at 60 min (Fig. 1A). Densitometry analysis showed that the accumulation of HIF-1α induced by hypoxic stimulus (Fig. 1A left panel) or ET-1 treatment (Fig. 1A right panel) promoted both HIF-1a and HIF-2a accumulations, although the levels of HIF-2α were increased to a minor extent. Next, to test whether HIF-1α accumulation resulted in an increased nuclear translocation, nuclear extracts from LEC stimulated with the selective $ET_BR$ antagonist BQ788 alone or in combination with ET-1 were analyzed by Western blotting. As shown in Fig. 1B while ET-1 induced a strong induction of HIF-1α nuclear accumulation the presence of BQ788 inhibited this effect. Densitometric analysis showed that ET-1-induced HIF-1α nuclear accumulation (2 fold versus the control) was reduced (1.2 fold compared to the control) by BQ788, demonstrating that ET-1 increases HIF-1α stabilization and nuclear translocation through $ET_BR$.

### *ET-1 induces LEC migration and cord formation via HIF-1α and HIF-2α*

Despite the large evidence that endothelial cells showed a similar response to ET-1 and to hypoxia in terms of angiogenic factor production and cord formation induction (Spinella et al., 2002; Spinella et al., 2009, Garrafa et al., 2012), little is known about the molecular mechanism that transduces these processes. Therefore we assessed whether the observed increase in HIF-1α and HIF-2α stabilization may be responsible for ET-1-dependent induction of LEC capillary morphogenesis. To this end we transfected LEC with siRNA oligonucleotides targeting HIF-1α and HIF-2α and cells were stimulated with ET-1. After 16 h mRNA was analyzed by real time PCR. Transfection led to inhibition of the corresponding mRNA expression (Fig. 2A and B) in both unstimulated and ET-1 stimulated cells. In particular HIF-1α and HIF-2α mRNA levels were inhibited by 70% and 80% respectively (Fig. 2A). In contrast, a scramble siRNA used as negative control (SRC) did not affect mRNA expression of either HIF-1α and HIF-2α. To investigate the involvement of HIF-1α and HIF-2α in the ET-1 induction of cord formation, LEC untransfected or transfected with SRC or siRNA targeting HIF-1α or HIF-2 were seeded on Matrigel and stimulated with ET-1 for 24 h. Unstimulated LEC formed few cords while under ET-1 stimulation we observed the formation of a rich network of tubular structures. Transfection of siRNA oligonucleotides targeting HIF-1α or HIF-2α significantly ($p < 0.01$) reduced cord formation induced by ET-1. Consistently, quantification analysis demonstrated a reduction of junction number in that cells silenced for HIF-1α and HIF-2α (Fig. 2C) indicating that these transcriptional factors are required for ET-1 induction of cord in LEC.

### *ET-1 induces PHD2 inhibition through $ET_BR$*

Under normoxic conditions HIF-1α and HIF-2α proteins are degraded by PHD hydroxylation occurring on their oxygen-dependent degradation domain (ODDD) encompassing the PHD-targeted prolines (Metzen et al., 2005). To further investigate the role of ET-1 on the stability of HIF-α we used a reporter plasmid expressing HIF-α-ODDD fused with luciferase

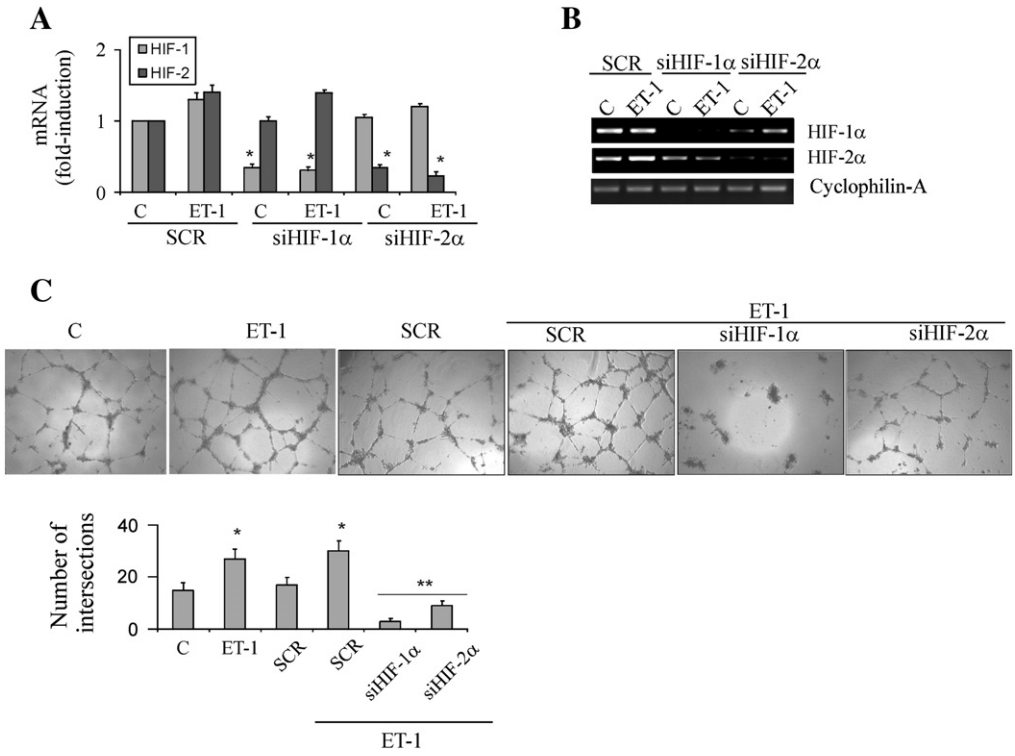


**Fig. 2.** HIF-1α and HIF-2α siRNA inhibit cord formation induced by ET-1. (A) LEC were transfected with a negative control scramble (SCR) or siRNA for HIF-1α or HIF-2α. Cells were then treated with ET-1 100 nM for 24 h and HIF-1α and HIF-2α mRNAs were analyzed by real time PCR. Results are expressed as copy numbers of HIF-1α and HIF-2α transcripts over cyclophilin-A. Bars, ± SD. *, $p < 0.05$ compared to the control. (B) PCR products for HIF-1α, HIF-2α and cyclophilin-A. (C) LEC transfected with the indicated siRNA were seeded on Matrigel in serum-free media in the presence or absence of ET-1 (100 nM) under normoxic conditions for 24 h and cord formation was examined. Quantification analysis was performed by measuring the number of intersections. Data are means ± SD. *$p < 0.05$ (versus control); **$p < 0.01$ (versus ET-1).

(CMV-Luc-ODDD). Following the transfection, LEC were stimulated for different times with ET-1 or cultured under hypoxia. As shown in Fig. 3A, luciferase-ODDD stabilization increased in a time-dependent manner after stimulation with ET-1 with maximal levels obtained at 6 h. These effects were blocked by BQ788 (Fig. 3B). We next evaluated the oxygen sensing enzymes that may be implicated in regulating HIF-α stability. To this end we focalized our attention on PHD2, and analyzed its expression at mRNA and protein levels. As found by real time PCR and Western blotting, ET-1 significantly decreased PHD2 mRNA and protein levels (Fig. 3C and D), this effect was impaired by BQ788, indicating that $ET_BR$ activation specifically inhibits PHD2. Our results provide the evidence that ET-1 controls HIF-1α and HIF-2α stability through a mechanism that involves prolyl hydroxylase-dependent degradation.

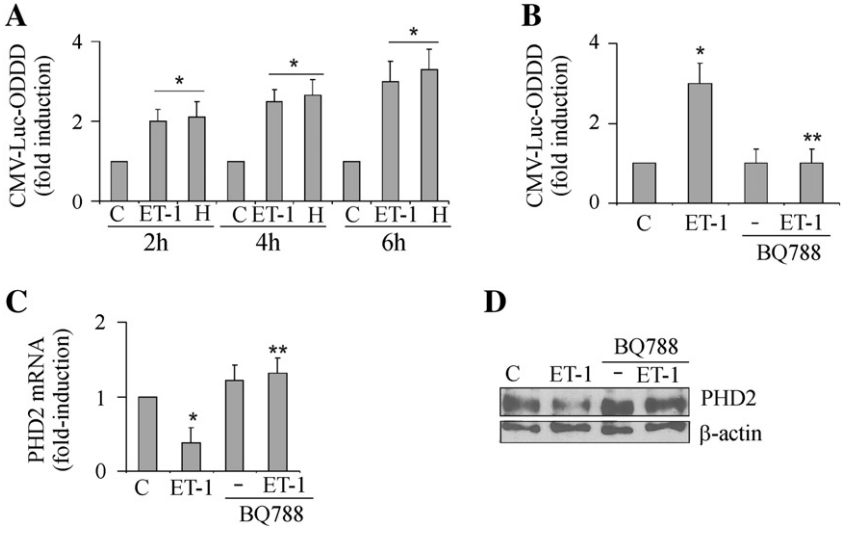


**Fig. 3.** ET-1 induces HIF-1α and HIF-2α protein stability by impairing PHD2-mediated HIF α hydroxylation. LEC were transfected with CMV-Luc-ODDD construct. After 24 h cells were treated for different times with ET-1 or hypoxia (H) (A), or pretreated with BQ788 alone or with ET-1 for 6 h (B). Luciferase activity was measured, normalized against β-galactosidase, and results were expressed as fold induction compared to the control. Bars, ± SD. *, $p < 0.01$ compared to control; **, $p < 0.001$ compared to ET-1. LEC were treated with ET-1 alone or in combination with BQ788 for 6 h and cell lysates were analyzed for PHD2 expression by real time PCR (C) and Western blotting assay (D).

## Discussion

Clinical cancer studies have shown a correlation between tumor hypoxia, HIF-1α expression, and lymphatic metastases (Schindl et al., 2002; Kurokawa et al., 2003; Katsuta et al., 2005; Schito et al., 2012; Hanna et al., 2013). Similarly, animal studies have shown that HIF-1α blockade reduces lymphatic metastasis, which suggests that HIF-1α regulates tumor-associated lymphangiogenesis and that targeting HIF-1α may represent a potential therapeutic strategy to modulate lymphangiogenesis (Yeo et al., 2003; Shin et al., 2007). PHDs function as negative regulators of HIF-1α or HIF-2α thereby suggesting that the inactivation of PHD may provide a critical mechanism in modulating HIF-α. Until now very little is known regarding the molecular regulation of PHD and on its role in controlling HIF-1α and HIF-2α in lymphangiogenesis. Here we provide a mechanism controlled by ET-1 in which the increased stability and nuclear accumulation of HIF-1α and HIF-2α are tightly regulated by a prolyl hydroxylation enzymatic reaction and are involved in ET-1-induced LEC migration and cord formation.

We previously demonstrated that in LEC ET-1, similar to hypoxia, increases the expression of HIF-1α protein (Spinella et al., 2009). In this study by examining the kinetics of ET-1-induced HIF-1α and HIF-2α protein we observed that ET-1 increased HIF-1α and HIF-2α stability. Many activators of HIF-1, including hypoxia, have been shown to induce HIF-1 protein expression by inhibiting ubiquitination and degradation (McMahon et al., 2006; Berchner-Pfannschmidt et al., 2007). Our results demonstrated that ET-1 shares mechanistic similarities with hypoxia regulating prolyl hydroxylation of HIF-α subunits. Indeed, treatment with ET-1 increased in a time-dependent manner the integrity of a construct containing the ODD domain of HIF-α protein targeted by prolyl hydroxylase enzymes and prevented its proteosomal degradation. This effect was specific as demonstrated by the use of BQ788 that inhibited the effect of ET-1. Recent studies have highlighted the importance of PHD2, the key oxygen sensor in controlling both tumor angiogenesis, vascular normalization and neovascularization (Chan and Giaccia, 2010).

Investigating the molecular mechanism that controls ET-1-reduced HIF-1α and HIF-2α hydroxylation we found that ET-1 reduced PHD2 expression. These results define the HIF-1α hydroxylase pathway as the link between ET-1 axis and the regulation of HIF-1α stabilization.

In this regard, our previous results demonstrated that the overexpression of PHD2 in tumor cells suppressed HIF-1α and HIF-2α accumulation and secretion of VEGF, while, PHD2 silencing stimulated VEGF leading to increased formation of cord-like structures in HUVEC (Spinella et al., 2010). Thus our present results in LEC further support the hypothesis that ET-1-mediated inhibition of PHD2 expression may be a conserved mechanism regulating HIF-1α in a variety of settings, including blood and lymphatic endothelial cells.

Genetic studies with endothelial cell-specific deletion of HIF-1α or HIF-2α, strongly suggest that these transcriptional factors have important non-redundant roles and are concomitantly required for the maintenance of endothelial cells (Skuli et al., 2012; Han et al., 2012). Moreover, recent studies reported that rapid degradation of HIF-1α may impair lymphangiogenesis during wound-healing assay (Zampell et al., 2012) and that HIF-2α is required for the induction of migration (Han et al., 2012) suggesting that HIF-1α and HIF-2α may play a direct role in modulating LEC behavior. Our findings demonstrated that silencing of HIF-1α and HIF-2α impairs the capacity of ET-1 to induce cell and organize vascular-like structure formation, demonstrating the key role of these transcriptional factors in LEC responses to ET-1. We previously demonstrated that ET-1 regulates VEGF-A and VEGF-C expression through HIF-1α and HIF-2α in tumor cells (Spinella et al., 2013). Moreover in LEC HIF-1α silencing impaired the capacity of ET-1 to induce VEGF (Spinella et al., 2009) and blockade of VEGF-A pathway reduces the capacity of ET-1 to promote the formation of tube-like structures on LEC (Garrafa et al., 2012). Moreover, a recent report indicated that the ET-1-induced HIF-1α stability is involved in VEGF-mediated angiogenic processes (Wu et al., 2013). In this regard, it has been demonstrated that up-regulation of ET-1 may also implicate tumor lymphangiogenesis (Cueni et al., 2010). Thus it is possible to speculate that ET-1-induced HIF-1α stabilization may represent a critical regulatory step in the initial expression of VEGF during angiogenesis and lymphangiogenesis.

The results of our study identified in $ET_BR$ a potential therapeutic target to block ET-1-induced HIF-1α stability thereby regulating LEC behavior.

## Conclusions

Similar to angiogenesis, our results demonstrate that the process of ET-1-induced lymphangiogenesis is finely tuned by HIF-1α and HIF-2α that may directly regulate LEC behavior other than regulating specific target genes, such as VEGF members. In view of the molecular mechanism that we defined the present results raise the possibility that $ET_BR$ activation contributes to lymphangiogenesis through a HIF-α transcriptional signaling controlled by PHD2-mediated HIF-1α and HIF-2α stability on LEC. Thus $ET_BR$ antagonists, which have been shown to induce concomitant anti-tumor activity and suppression of neovascularization (Spinella et al., 2009; Spinella et al., 2013), may therefore represent a targeted therapeutic approach for the prevention of tumor spreading.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

We gratefully acknowledge Mr. Aldo Lupo for his excellent technical assistance and Mrs. Maria Vincenza Sarcone for her secretarial support. Grant support: Associazione Italiana Ricerca sul Cancro (AIRC, MFAG #8910).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jvolgeores.2014.07.016.

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