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Epigenetic control of hypoxia inducible factor-1α-dependent expression of placental growth factor in hypoxic conditions

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Keywords: hypoxia, hypoxia inducible factor (HIF), chromatin, histone modification, DNA methylation, angiogenesis, VEGF family, placental growth factor (PlGF)

Abbreviations: HIF-1α, hypoxia inducible factor-1α; PlGF, Placental Growth Factor; VEGF, vascular endothelial growth factor; HRE, hypoxia responsive element; EC, endothelial cell; VEGFR-1, VEGF Receptor-1; MTF-1, metal responsive transcription factor 1; NF-κB, nuclear factor κB; ChIP, chromatin immunoprecipitation; HUVEC, human umbilical vein endothelial cell

Hypoxia plays a crucial role in the angiogenic switch, modulating a large set of genes mainly through the activation of hypoxia-inducible factor (HIF) transcriptional complex. Endothelial cells play a central role in new vessels formation and express placental growth factor (PlGF), a member of vascular endothelial growth factor (VEGF) family, mainly involved in pathological angiogenesis. Despite several observations suggest a hypoxia-mediated positive modulation of PlGF, the molecular mechanism governing this regulation has not been fully elucidated. We decided to investigate if epigenetic modifications are involved in hypoxia-induced PlGF expression. We report that PlGF expression was induced in cultured human and mouse endothelial cells exposed to hypoxia (1% O_2), although DNA methylation at the *Plgf* CpG-island remains unchanged. Remarkably, robust hyperacetylation of histones H3 and H4 was observed in the second intron of *Plgf*, where hypoxia responsive elements (HREs), never described before, are located. HIF-1α, but not HIF-2α, binds to identified HREs. Noteworthy, only HIF-1α silencing fully inhibited PlGF upregulation. These results formally demonstrate a direct involvement of HIF-1α in the upregulation of PlGF expression in hypoxia through chromatin remodeling of HREs sites. Therefore, PlGF may be considered one of the putative targets of anti-HIF therapeutic applications.

Introduction

Placental growth factor (PlGF), the second member of vascular endothelial growth factor (VEGF) family discovered,[1] is mainly involved in pathological angiogenesis,[2,3] a complex biological phenomenon associated to many multifactorial diseases, such as cancer, atherosclerosis, arthritis, diabetic retinopathy and age-related macular degeneration.[4,5]

Low oxygen tension is one of the major stimuli responsible for angiogenic switch, a time-restricted event during tumor progression where the balance between pro- and anti-angiogenic factors tilts toward a pro-angiogenic outcome, resulting in the transition from dormant avascularized hyperplasia to outgrowing vascularized tumor.[6] The main response of cells to hypoxia is represented by the activation of hypoxia inducible factor (HIF) transcriptional complex, which modulates the expression of a large set of genes through the binding of the hypoxia responsive element (HRE) located in their promoters[7,8] or along the gene body.[9,10] Several genes upregulated by hypoxia encode for proteins having as target endothelial cells (ECs), whose proliferation, migration and differentiation is essential for new vessel formation.[11] The EC itself produces several factors involved in angiogenesis, such as the pro-angiogenic members of the VEGF family and related receptors, which play relevant autocrine and paracrine functions with a central role in the mechanisms underlying new vessels formation.[12-14]

Among the pro-angiogenic members of the VEGF family and related receptors, it has long been known that, in hypoxic condition, HIF is directly involved in the increase of transcription of VEGF-A and VEGF Receptor-1 (VEGFR-1).[15,16] Despite the strict biochemical and functional relationship between VEGFR-1, PlGF, and VEGF-A,[17-20] a direct involvement of HIF in the modulation of PlGF transcription has not been demonstrated. Furthermore, even though the analysis of promoter/enhancer region of mouse and human *Plgf* showed the presence of putative HREs, their functionality has never been demonstrated.[21-23]

Nevertheless, some reports indicate a hypoxia-induced positive modulation of *Plgf* transcription through the involvement of

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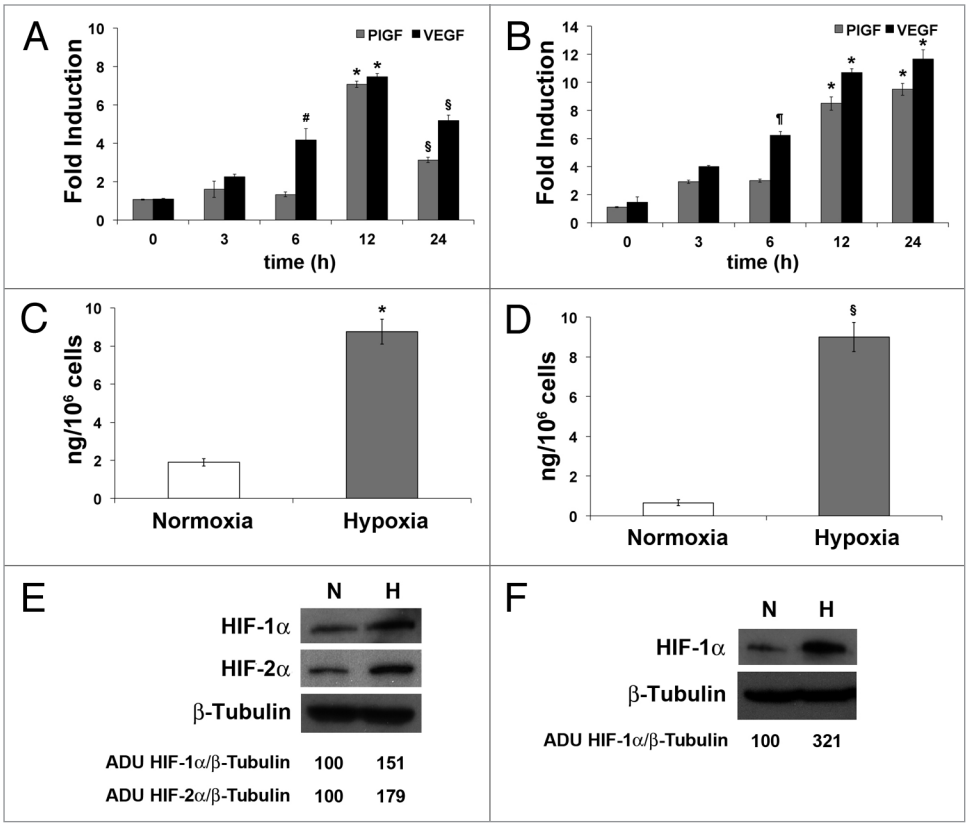

Figure 1. Overexpression of PlGF mRNA and protein in ECs exposed to low oxygen tension. Time dependent PlGF and VEGF-A mRNA expression determined by qRT-PCR in HUVEC (**A**) and mouse H5V (**B**) ECs exposed to 1% O_2. PlGF and VEGF mRNA levels in hypoxic conditions were compared with the normoxic levels and normalized against RPL32, in human cells, and Rpl13a, in mouse cells. Data are expressed as fold induction compared with normoxic condition and represent the mean ± SEM of two independent experiments performed in triplicate. *$P < 0.0005$; §$P < 0.005$; ¶$P < 0.001$; #$P < 0.05$ vs normoxic condition. Determination of PlGF concentration in the culture medium of HUVEC (**C**) and H5V (**D**) exposed to 1% O_2 for 24 h, as assessed by sandwich ELISA. As control, cells cultured in normoxic condition were used. Data represent the mean ± SEM of two independent experiments performed in triplicate. *$P = 0.0013$ and §$P = 0.0008$ vs normoxic condition. Western blot analysis of human HIF-1α and HIF-2α (**E**) and of mouse HIF-1α (**F**) performed on HUVEC and H5V protein extracts, after cells exposure to 1% O_2 for 24 h. Densitometry analyses are reported as percentage of arbitrary densitometry units (ADU) of the ratio of HIF-1α or HIF-2α and β-Tubulin, assigning the value of 100 to the relative ratio obtained in normoxic condition.

metal responsive transcription factor 1 (MTF-1) in immortalized/H-Ras-transformed mouse embryonic fibroblasts (mEFs),[21] and of nuclear factor κB (NF-κB), in human embryonic kidney 293 (HEK-293) cells.[24] Surprisingly, no PlGF upregulation by hypoxia has been observed in human aortic and human umbilical vein endothelial cells.[24] However, overexpression of HIF-1α in human endothelial cells[25] or in mouse primary cardiac and vascular cells[26] positively affects PlGF expression. In vivo, PlGF upregulation occurs in cardiomyocytes and neovessels in the model of myocardial infarct.[2,27] Recently, its expression has been reported in human colorectal carcinomas[28] and in pediatric medulloblastomas.[29] Overall, these data suggest a possible involvement of HIF-1α in the modulation of PlGF expression even if a direct functional link between HIF activity and PlGF expression has never been demonstrated.

Due to the central role of ECs in angiogenic switch, we decided to investigate whether PlGF expression is effectively modulated by hypoxia via HIF in human and mouse ECs. Studies previously accomplished excluded a direct role of the HRE located in *Plgf* promoter. We decided to investigate if this modulation could be mediated by additional intragenic HREs, taking into account that alteration of chromatin structure has an important role in the response to hypoxia.[30]

In this frame, CpG methylation and histone H3 and H4 acetylation have been examined in order to identify whether epigenetic changes occur for hypoxia-induced *Plgf* transcriptional regulation. The data here presented highlight for the first time a direct functional link between HIF-1α and PlGF overexpression in hypoxic condition.

Results

Hypoxia increases PlGF expression in HUVEC and H5V cells

To evaluate the impact of hypoxia on PlGF expression, HUVEC or H5V[31] cells were exposed to 1% O_2. At 3, 6, 12 and 24 h, RNA was extracted to quantify the expression of PlGF and, as control, of VEGF-A by qRT-PCR. In HUVECs, no change of PlGF was detected until 6 h, while the VEGF mRNA was already increased at 3 h, compared with normoxic condition (at 6

Table 1. Time-dependent differential modulation of human PlGF isoforms by hypoxia

Hours	PlGF -1	PlGF-2	PlGF-3	PlGF-4
0	1	1	1	1
3	0.96 ± 0.04	1.43 ± 0.12	0.90 ± 0.02	1.18 ± 0.20
6	0.71 ± 0.12	1.10 ± 0.13	1.14 ± 0.22	1.21 ± 0.24
12	**3.86 ± 0.09***	**2.73 ± 0.18***	1.18 ± 0.27	1.00 ± 0.03
24	**1.76 ± 0.28§**	**1.71 ± 0.20§**	0.97 ± 0.03	1.07 ± 0.07

Data are expressed as fold induction compared with normoxic condition and represent the mean ± SEM of two independent experiments performed in triplicate. In bold the values indicating the upregulation of PlGF-1 and PlGF-2 after 12 and 24 h of exposure to 1% O2. *$P < 0.005$ and §$P < 0.05$.

h, $P < 0.05$). At 12 h a significant increase (~6-folds, $P < 0.0005$) of PlGF mRNA was observed and maintained up to 24 h (~3.5-fold, $P < 0.005$). The mRNA level of VEGF-A raised until 12 h (~8-fold increase, $P < 0.0005$), and at 24 h the upregulation is still evident (~5.5-fold, $P < 0.005$) (**Fig. 1A**). In mouse endothelial cells, the increase of PlGF showed a trend similar to that of VEGF-A, with a peak of expression at 12 h, as in human endothelial cells (~7.6- and ~9.6-fold increase compared with normoxic condition, respectively, $P < 0.0005$). These levels of expression were maintained also at 24 h (~8.6- and ~10.5-fold increase, respectively, $P < 0.0005$) (**Fig. 1B**).

In order to verify whether a protein increase corresponded to the PlGF mRNA overexpression, ELISA assays were performed to quantify human and mouse PlGF secreted in the culture medium. An increase of ~4.6- ($P = 0.0013$) and ~11.5-fold ($P = 0.0008$) of PlGF protein was detectable after 24 h of hypoxia in HUVEC and H5V medium culture, respectively, compared with normoxic condition (**Fig. 1C and D**). The effectiveness of hypoxia condition was confirmed by the increase of HIF-1α in both cell lines, as well as of HIF-2α in HUVECs, as assessed by western blot analysis (**Fig. 1E and F**).

These data clearly indicate that hypoxic condition induces the upregulation of mRNA and protein of human and mouse PlGF.

Since four isoforms of human PlGF have been described (PlGF 1 to 4),[1] differently from mouse in which a single PlGF form corresponding to human PlGF-2 has been identified, qRT-PCR was performed to evaluate whether hypoxia could differentially modulate human PlGF isoforms.[32] The low oxygen tension significantly upregulated the two main isoforms PlGF-1 and PlGF-2, with a preference for the soluble PlGF-1 isoform, starting from 12 h of exposure to 1% O_2 (**Table 1**).

Hypoxia does not change DNA methylation status at human *Plgf* promoter

In order to investigate a possible role of DNA methylation in hypoxia-induced PlGF upregulation, we compared the cytosine methylation profile of *Plgf* promoter region under hypoxic and normoxic conditions. A CpG island overlapping the exon 1 (nucleotides -388/+156) was identified on human *Plgf* gene (**Fig. 2A**). Genomic DNA extracted from HUVECs grown in normoxic or in hypoxic conditions was subjected to bisulfite sequencing.[33] The analysis of 20 clones for each condition showed general low CpG site methylation in both samples, without any significant change in hypoxia compared with normoxic condition (**Fig. 2B**).

Hypoxia determines *Plgf* histone acetylation changes

To establish whether hypoxia might induce changes in the chromatin structure of the *Plgf* gene, we measured histone H3 and histone H4 acetylation levels, marks of permissive chromatin. Ten different regions spanning *Plgf* gene were analyzed by ChIP assay (**Fig. 3A**; **Table 2**). Under hypoxic condition, we found an increase of H3 and H4 acetylation in *Plgf* promoter region 6, as assessed by qRT-PCR ($P < 0.05$ vs normoxic control) (**Fig. 3A and B**). No increase in histone acetylation was observed in promoter regions 4 and 5, where putative HREs, already reported by other authors, are present (not shown).[21,34] Surprisingly, a strong enrichment of H3 and H4 acetylation was instead observed in region 7, located in the second *Plgf* intron ($P < 0.005$ for histone H3 and $P < 0.01$ for histone H4, vs normoxic control) (**Fig. 3B**). The sequence analysis of this region evidenced the presence of three previously unknown HRE elements centered in position +2324, +2407 and +2422 (**Fig. 3A**). The first one is also flanked by an additional consensus sequence frequently associated with functional HRE sites (**Table 3**).[9,35]

A comparative analysis among different species revealed the conservation of one or more HREs in the same area of the second intron of rhesus, dog and mouse *Plgf* gene. In all species, at least one HRE resulted flanked by the additional consensus sequence (**Table 3**). Interestingly, also in the second intron of *Vegf-a* gene, a HRE was identified centered at position +5184.

HIF-1α, but not HIF-2α, binds to the HREs on the human *Plgf* second intron

ChIP analyses using anti-HIF-1α and anti-HIF-2α antibodies were performed to establish whether HIFs are involved in the binding of HREs located in the second intron of *Plgf*. We also investigated two *Plgf* promoter regions where two known putative HRE elements are located (regions 4 and 5, **Fig. 3A**; **Table 2**). As positive control, the canonical *Vegf-a* promoter area including an active HRE was analyzed (V-CP), whereas a region lacking HRE sites (V-NC) was amplified as negative control (**Table 2**).[15,36] Moreover we also analyzed the *Vegf-a* second intron region where, as in the *Plgf* gene, a HRE is located (V-2-Int).

A direct binding of HIF-1α to the H3 and H4 hyperacetylated region 7 of *Plgf* second intron was observed, as well as to the *Vegf-a* promoter area ($P < 0.005$ vs normoxic controls) (**Fig. 3C**). Conversely, the two putative HREs located in the *Plgf* promoter regions 4 and 5 not differentially H3 and H4 acetylated, were not recognized by HIF-1α and/or HIF-2α. Interestingly, while both HIF-1α and HIF-2α were able to bind the HRE in V-PC region ($P < 0.01$ and 0.05 vs normoxic control),[37] HIF-2α did not interact with *Plgf* second intron HRE (**Fig. 3C**).

Finally, the HRE located in the second intron of *Vegf-a* was not recognized by HIFs, indicating that the involvement of this intragenic site is a peculiarity of *Plgf* gene.

Silencing of HIF-1α abrogates human PlGF overexpression in hypoxic condition

To demonstrate a direct functional link between HIF-1α and PlGF overexpression in hypoxic condition, we knocked down

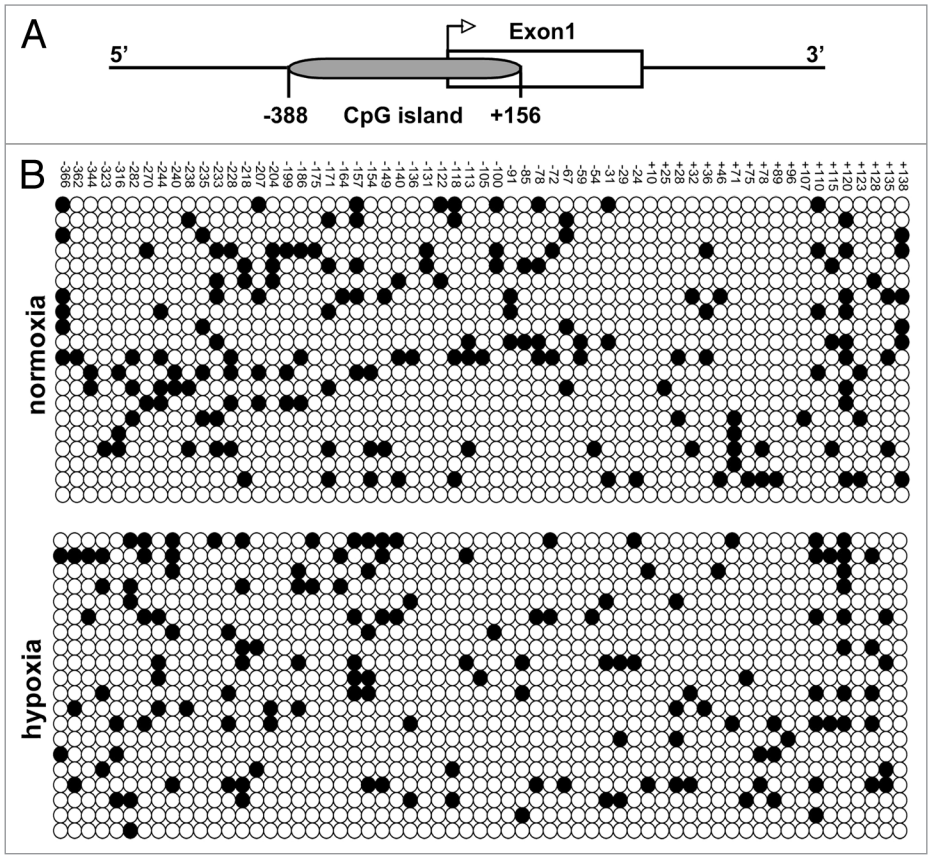

Figure 2. Methylation status of human *Plgf* promoter in normoxic and hypoxic conditions. (**A**) Schematic representation of the CpG island located in human *Plgf* promoter at position -388/+156 with respect to the transcription start site. (**B**) Sequence analysis of bisulfite-treated genomic DNA from HUVECs exposed (bottom) or not (top) to hypoxia for 9 h. Twenty clones for each group have been analyzed. Each row indicates a single clone, whereas each column represents a single CpG site. White circles indicate unmethylated cytosines, whereas black circles represent the methylated ones.

HIF-1α by introducing specific siRNA in HUVECs. As control, HUVECs transfected with siRNA for HIF-2α or non-targeting (NT) siRNA, as well as mock transfected cells, were used. After confirming by western blot analysis that HIF-1α or HIF-2α were efficiently silenced (**Fig. 4C**), we observed the abrogation of hypoxia-mediated PlGF overexpression only in cells transfected with specific siRNA for HIF-1α, whereas silencing of HIF-2α or transfection of NT-siRNA were ineffective (**Fig. 4A and B**).

These data clearly demonstrate the direct role of HIF-1α in the modulation of PlGF expression under hypoxic condition.

HIF-1α also modulates the hypoxia-induced overexpression of mouse *Plgf* gene

To assess whether hypoxia-mediated PlGF upregulation is conserved in mouse, we examined histone acetylation and HIF-1α binding in murine *Plgf* gene by ChIP analysis. For mouse *Plgf* gene, a total of six regions were analyzed to monitor the level of H3 and H4 acetylation, covering all putative HRE sequences detected along the entire gene (**Fig. 5A**; **Table 2**). The positive (V-PC) and negative (V-NC) controls were represented by regions of mouse *Vegf-a* promoter containing or not functional HREs.[38] An increase in histone acetylation was observed in the promoter region 13, and again only in the region of second intron (region 14) where a HRE, similar to the first one observed in human *Plgf* second intron, is located (**Fig. 5B**; **Table 3**). ChIP analysis performed with anti-HIF-1α confirmed a direct binding exclusively to the HRE in the second intron (**Fig. 5C**).

Finally, silencing of HIF-1α in H5V cells, confirmed by western blot analysis (**Fig. 5F**), fully prevent the hypoxia-induced upregulation of mouse PlGF mRNA and protein, as assessed by qRT-PCR and ELISA, whereas the NT-siRNA was ineffective (**Fig. 5D and E**).

Discussion

PlGF is redundant for developmental and physiological processes but plays an important role in different pathological contexts in which angiogenesis and inflammation are involved through endothelial stimulation and bone marrow-derived cells recruitment and activation.[39-41] Moreover, since PlGF is able to act on different cell types, thanks to the broad expression of its specific receptor VEGFR-1,[19,42] the list of biological processes in which its pleiotropic activities are involved is still growing.[1,3]

Considering this scenario, the control of PlGF expression levels is of great interest, as confirmed also by many preclinical models that clearly showed how PlGF deregulation resulted directly correlated with pathological conditions. For these reasons, we decided to investigate the molecular mechanism governing the modulation of PlGF by oxygen tension. We

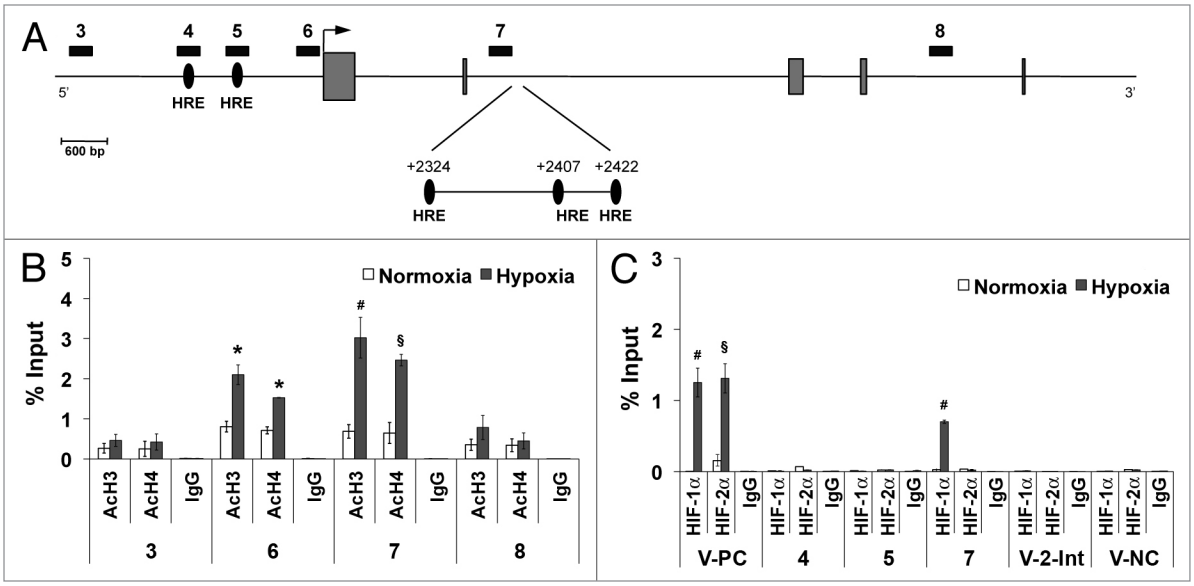

Figure 3. Histone acetylation enrichment and HIF-1α binding to human *Plgf* second intron. (**A**) Schematic representation of part of human *Plgf* gene. Regions analyzed by ChIP analysis are indicated by numerated black rectangles. Gray boxes represent exons. An arrow indicates the transcription start site. Black ovals indicate the putative HRE located in the promoter and in the second intron, identified by numbers indicating the central bases (G) of HRE core consensus sequence, with respect to the transcription start site. ChIP analysis for H3 (AcH3) and H4 (AcH4) acetylation (**B**) or for HIF-1α or HIF-2α (**C**), performed starting from chromatin sample from HUVEC exposed to hypoxia for 9 h. As control, species matched IgG were used. Data obtained by qRT-PCR are expressed as enrichment of chromatin-associated DNA fragments immunoprecipitated by specific antibody compared with input (% Input) and represent the mean ± SEM of two independent experiments performed in triplicate. $^{*}P < 0.05$; $^{§}P < 0.01$ and $^{\#}P < 0.005$ vs normoxic controls. Numbers indicate the amplified regions of *Plgf* gene, as reported in (**A**). V-PC and V-NC represent *Vegf-a* positive and negative controls, respectively, containing or not active HRE. V-2-Int, indicates *Vegf-a* second intron region containing a putative HRE.

focused our interest on ECs because of their central role in new vessels formation.

Despite several reports indicate that hypoxia, as well as the overexpression of HIF-1α, are responsible in vitro and in vivo for a positive modulation of PlGF,[2,25-29] the direct involvement of the HIF transcriptional complex has not been evidenced with classical transcriptional studies on *Plgf* promoter.[21,24,34] Considering that it has recently been reported that epigenetic regulation of chromatin structure plays an important role in defining the response to hypoxia,[30] we decided to investigate the role of epigenetic modifications, such as DNA methylation and histone acetylation, in the modulation of PlGF expression under hypoxic stimuli.

First, we assessed that exposure to 1% O_2 of human and mouse ECs is sufficient to unambiguously induce an increase of PlGF mRNA and protein. Then, we verified whether hypoxia might influence the DNA methylation status of the *Plgf* promoter. Detailed CpG methylation profile evidenced hypomethylation at *Plgf* promoter without significant changes between normoxic and hypoxic conditions. Previous reports indicated that low CpG methylation level at the HREs binding sites is required to allow gene transcriptional induction.[43,44] However, our findings suggest a role for specific histone modifications rather than DNA methylation changes in the hypoxia-mediated PlGF induction. To date, only one previous study has investigated *Plgf* promoter methylation.[45] Interestingly, this study reported that hypermethylation of the *Plgf* promoter is associated with PlGF downregulation in human lung and colon carcinoma tissues, as well as in correspondent cancer cell lines in normoxic conditions, suggesting that the methylation of the *Plgf* promoter may change in different cell and tissue contexts.

We found that alteration of chromatin structure may influence the modulation of PlGF expression under hypoxic condition. Analyzing the H3 and H4 acetylation along the *Plgf* gene we observed an enrichment of histone acetylation in the second intron, in addition to the expected increase in the promoter. Moreover, in human *Plgf* second intron, three putative HREs never described before were found close to the region showing histone acetylation increase. The first one, centered at position +2324 with respect to the transcriptional start site, is also followed by a second consensus sequence often associated to the active HRE (**Table 3**).[9,35] We then confirmed the evolutionary conservation of one or more HREs together with the second consensus sequence in the second intron of *Plgf* among different species, thus indicating a functional role of these regions in PlGF regulation. Consistently, in hypoxic conditions, HIF-1α exclusively interacts with the second intron of human and mouse *Plgf* genes.

Moreover, we evaluated whether an involvement of HIF-2α occurred because, differently from HIF-1α that is ubiquitously expressed, HIF-2α is expressed in a restricted number of cell types among which ECs are included.[46,47] No direct binding of HIF-2α to the second *Plgf* intron, or other regions of the gene, was detected.

The absence of an active role of HIF-2α was confirmed by silencing experiments. Indeed, the specific silencing of HIF-2α

Table 2. Regions of *Plgf* and *Vegf-a* genes analyzed by ChIP

Target	Amplicon #	Region	HRE
hPlgf	1	-9730/-9570	
	2	-4903/-4747	
	3	-3561/-3419	
	4	-1702/-1554	putative (-1654)
	5	-1168/-1022	putative (-1047)
	6	-350/-176	
	7	**+2208/+2282**	putative (+2324, +2407, +2422)
	8	+8199/+8305	
	9	+13445/+13578	
	10	+15976/+16108	
hVegf	**V-PC**	**-1005/-868**	**active (-978)**
	V-NC	-1762/-1364	absent
	V-2-Int	+5161/+5391	
mPlgf	11	-1945/-1812	putative (-3100)
	12	-1001/-889	
	13	-314/-202	
	14	**+1740/+1841**	putative (+1767, +2168)
	15	+3892/+4011	putative (+4030)
	16	+6584/+6708	putative (+6593)
mVegf	**V-PC**	**-944/-831**	**active (-899)**
	V-NC	-1903/-2040	absent

The numbers in the region column indicated the area of genes analyzed with respect to transcription start site. In bold are indicated regions in which known and discovered active HRE are located. Numbers in HRE column refer to center position of HREs respect to transcription start site. V-PC, VEGF-A positive control; V-NC, VEGF-A negative control, V-2-Int, region of the second intron of Vegf-a gene where a putative HRE is located.

did not affect the hypoxia-mediated upregulation of PlGF. Conversely, HIF-1α silencing fully abrogates upregulation of PlGF mRNA and protein in both EC lines analyzed, demonstrating for the first time its direct role in this biological process, at least in ECs.

The involvement of MTF-1 in the hypoxic modulation of PlGF expression has been demonstrated in H-ras transformed mEFs; however, the same cells transformed with SV40 large T antigen were unresponsive.[21] Moreover, the activity of NF-κB on PlGF has been reported in HEK-293 cells overexpressing NF-κB p65.[24] It is important to note that both H-ras and NF-κB positively modulate HIF-1α expression.[48-50] Therefore, the upregulation of PlGF in these two peculiar cellular contexts was probably due, at least in part, to the direct activation of HIF-1α. Nonetheless, a strict collaboration between these three modulators of gene expression is probably required for a fully modulation of PlGF in hypoxic conditions.

Our data corroborate also the view that increased level of PlGF might contribute to the tumor escape strategy that follows anti-angiogenic therapy targeting VEGF-A or RTKs, included VEGFR-2.[51-54] Indeed, it is well known that these therapies

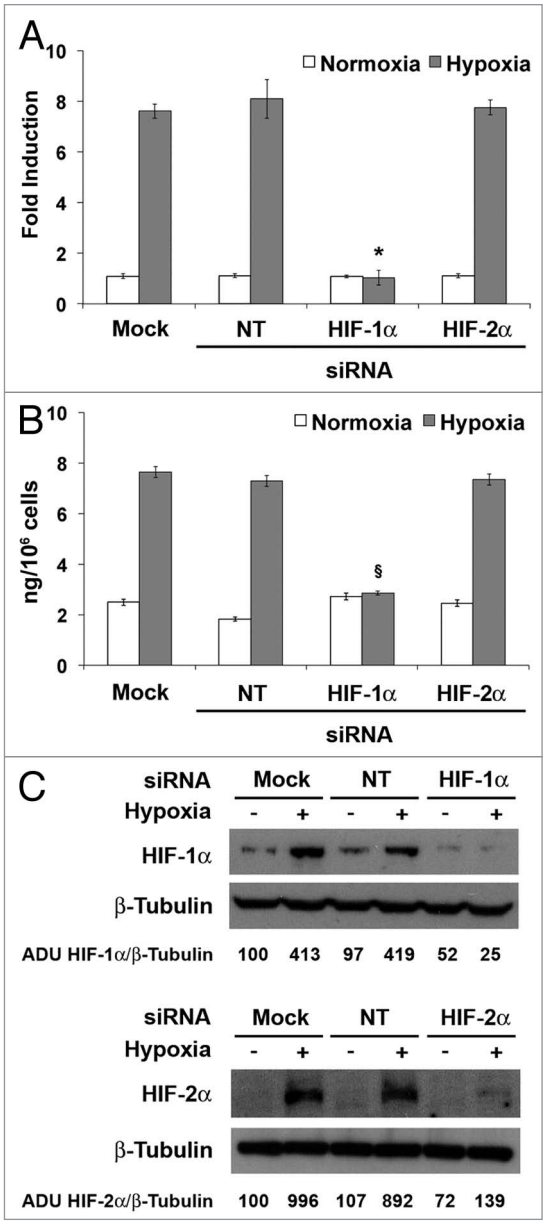

Figure 4. Knock down of HIF-1α, but not that of HIF-2α, inhibits hypoxia-induced human PlGF expression in HUVECs. (**A**) qRT-PCR analysis of human PlGF mRNA after silencing of HIF-1α or HIF-2α in HUVECs exposed to 1% O_2 for 9 h and determination of PlGF concentration by sandwich ELISA (**B**) in HUVEC culture medium harvested after silencing of HIF-1α or HIF-2α and 24 h of exposure to 1% O_2. As control, cells transfected with non-targeting (NT) siRNA and mock-transfected cells were used. Data obtained by qRT-PCR are expressed as fold induction and represent the mean ± SEM of two independent experiments performed in triplicate. Data obtained by ELISAs represent the mean ± SEM of two independent experiments performed in triplicate. $^{*}P < 0.0005$ and $^{§}P < 0.001$ vs controls and HIF-2α in hypoxia. (**C**) Western blot analysis of human HIF-1α (up) and HIF-2α (down) performed on HUVEC protein extracts, after cells exposure to 1% O_2 for 24 h. β-Tubulin detection on the same filter was used for normalization. Densitometry analyses are reported as percentage of arbitrary densitometry units (ADU) of the ratio of HIF-1α or HIF-2α and β-Tubulin, assigning the value of 100 to the relative ratio obtained in mock-transfected cells in normoxic condition.

Table 3. HREs located in the second intron of PlGF genes

Species	HRE +2324	HRE +2407	HRE +2422
Human	aaGACGTGCa aagtggcCAC ACACc	acaCGCGTGa Tag	atcTgCGTGC Tgg
Rhesus	aaGACGTGCa gagcggcCAC ACgCc	acaCGCGTGa Tag	atcTgCaTGC Tgg
Dog	ggGACGTGCa gcaaagcCAC ACgCc	acaCtCGTGa Tag	ctcCgaaaGC Tgg
Mouse	gaGACaTGGa ggatggcCAC AtACc	acaCAgaTaa tag	ttctT—GGCctgg

The positions of HREs are referred to human gene and indicate the central position of common core of putative HRE consensus, respect to the transcription start site. HRE consensus DNA sequence (G/T/C) A/G CGTG (C/G) (T/G/C) and the additional sequence CACACA G/C often associated with functional HRE sites are in uppercase and underlined (underlined lowercase indicates mismatched bases). In rhesus all three putative HRE are conserved (position +1965, +2048, +2063), in dog only the first two (position +1970, +2054). In mouse only the first one (+2168) is conserved but an additional HRE (agGACGTGaTcg) centered at position +1767 has been observed.

induce an increase of the hypoxic status of the tumor that, as confirmed by our data, positively affects PlGF expression. On the other hand, it has been reported that in the peritumor area of human hepatocellular carcinoma, the level of PlGF was significantly increased and correlated with augmented levels of HIF-1α.[55] Finally, since PlGF also positively modulates HIF-1α expression in ECs,[56] a positive loop may be established to maintain high levels of PlGF expression when required.

In conclusion, our data demonstrate that epigenetic changes, such as histone acetylation, are involved in the modulation of PlGF expression under hypoxic conditions in ECs, possibly by determining the exposition of a HRE located in the second intron of *Plgf* specifically recognized by HIF-1α. There is growing evidence that formation of chromatin loops mediated by transcription factors allows the interaction between regions far on linear DNA (i.e., promoter and gene body), thus providing an efficient control of gene expression.[57] In addition, the transcription process appears to be compartmentalized in factories occupying distinct loci in the nuclear space and genes are thought to be looped out from chromosomes territories toward these loci.[58] In line with these findings, it is reasonable to hypothesize that HIF-1α binding mediates a spatial association of the transcriptional start site and the regulatory site in the second intron. Further studies will be necessary to investigate these aspects and to confirm the three-dimensional chromatin structure of *Plgf* regulatory regions.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs, Clonetics) were cultured in endothelial basal medium (EBM-2) supplemented with endothelial growth factors (EGM-2 bullet kit, Cambrex). HUVECs at passages 4–7 were used for all the experiments. Murine-immortalized heart microvascular endothelial cell line (H5V)[31] was cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and standard concentration of antibiotics (Euroclone). For exposure to hypoxia, sub-confluent cells were placed in an appropriate incubator at 1% oxygen concentration. As control, sub-confluent cells were cultured in normoxic condition.

RNA preparation and quantitative real-time-PCR (qRT-PCR)

RNA was isolated using Trizol reagent (Invitrogen). The first strand of cDNA was obtained by reverse-transcription using Quantitect RT Kit (Qiagen). qRT-PCR was performed using SYBR green quantitative PCR on CFX96TM Real Time PCR Detection Systems (BioRad). The annealing temperatures were 58 °C for human gene and 62 °C for mouse gene. The primers were: hPlGF upper (559) ATGTTCAGCC CATCCTGTGT; lower (759) CTTCATCTTC TCCCGCAGAG - hVEGF-A upper (1130) AGGGCAGAAT CATCACGAAG; lower (1357) ATCCGCATAA TCTGCATGGT - hRPL-32 upper (324) AGTTCCTGGT CCACAACGTC; lower (519) TGCACATGAG CTGCCTACTC - mPlGF upper (325) GCTGGTCATG AAGCTGTTC; lower (454) ACCCCACACT TCGTTGAAAG - mVEGF-A upper (642) CAGGCTGCTG TAACGATGAA; lower (781) GCATTCACAT CTGCTGTGCT - mRpl13a upper (345) CCCTCCACCC TATGACAAGA; lower (565) CTGCCTGTTT CCGTAACCTC. The numbers identify the first nucleotide (5′ position) of upper primers or the last one (3′ position) of lower primers with respect to the transcription start site. The PlGF and VEGF expression levels in hypoxic condition were calculated with respect to the normoxic level and normalized against RPL32, in human cells, and Rpl13a, in mouse cells. Human PlGF isoforms (PlGF 1 to 4) were detected using specific primers as previously described.[32]

Western blot analysis

Western blot analyses were performed. with antibodies against HIF-1α (1:200, Santa Cruz Biotechnology), HIF-2α (1:500, Novus Biologicals) and β-Tubulin (1:1000, Santa Cruz Biotechnology) using standard protocols. Densitometry analyses were performed using ImageQuant 5.2 software (Molecular Dynamics). Values of arbitrary densitometry units (%) were calculated as ratio of HIF-1α or HIF-2α respect to β-Tubulin, assigning the value of 100 to ratio obtained in normoxic condition.

ELISA assay

All the reagents used in ELISA were from R&D Systems (Minneapolis, MN). The assays were performed as described elsewhere.[20,59]

Bisulfite analysis

Genomic DNA was isolated from HUVEC exposed (9 h) or not to hypoxia, using a Purelink Genomic DNA kit (Invitrogen).

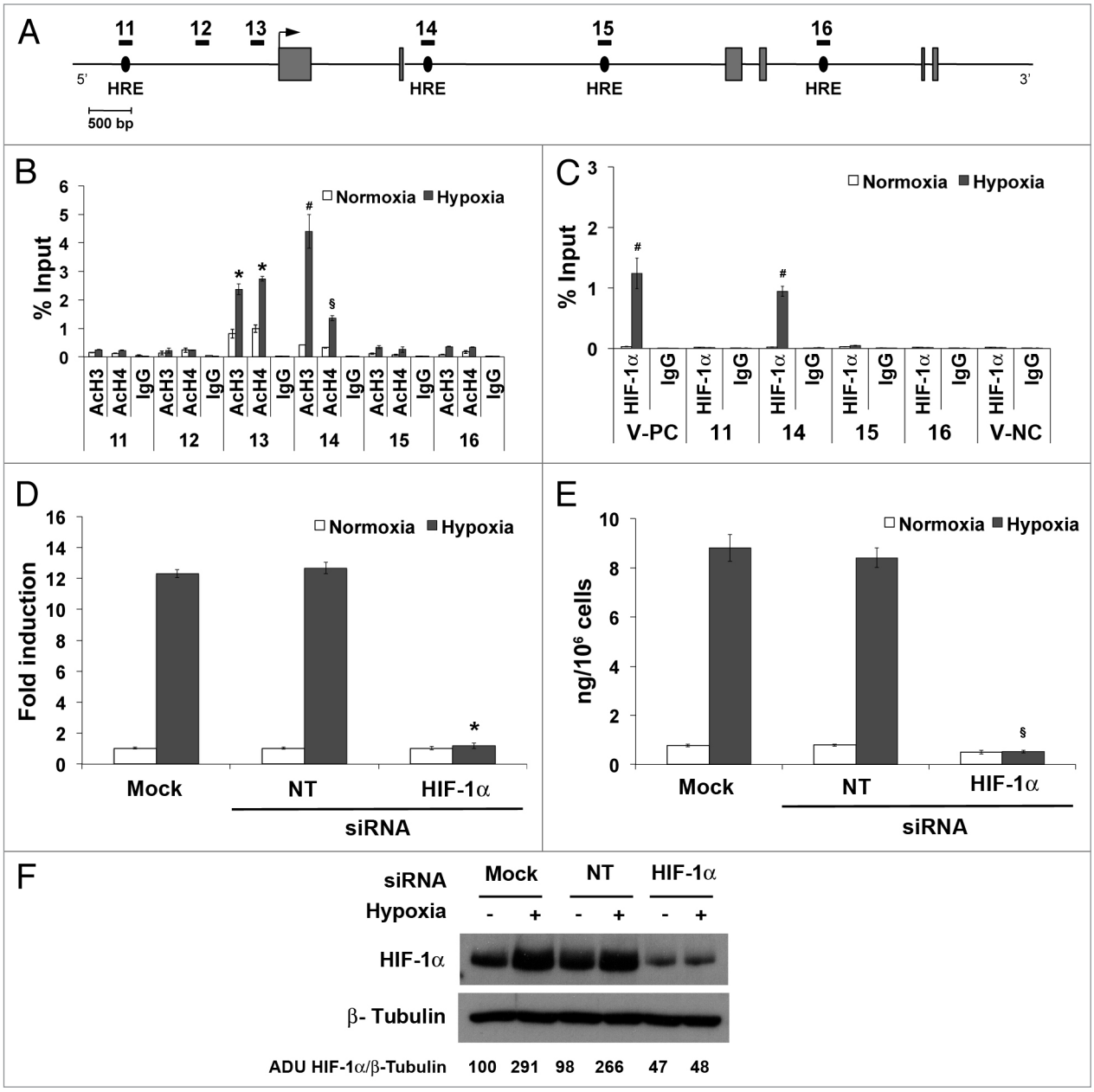

Figure 5. HIF-1α is directly involved in the hypoxia-modulation of mouse *Plgf* expression in H5V cells by HRE binding located on second intron. (**A**) Schematic representation of part of mouse *Plgf* gene. Areas analyzed by ChIP analysis are indicated by numbered black rectangles. Gray boxes represent exons. An arrow indicates the transcription start site. Black ovals indicate the putative HRE located along the gene. ChIP analysis for H3 (AcH3) and H4 (AcH4) acetylation (**B**) or for HIF-1α (**C**) performed starting from chromatin sample of H5V exposed to hypoxia for 12 h. As control, species matched IgG were used. Data obtained by qRT-PCR are expressed as enrichment of chromatin-associated DNA fragments immunoprecipitated by specific antibody compared with input (% Input) and represent the mean ± SEM of two independent experiments performed in triplicate. $^{§}P < 0.05$; $^{*}P < 0.01$ and $^{\#}P < 0.005$ vs. normoxic control. Numbers indicate the amplified regions of *Plgf* gene, as reported in (**A**). V-PC and V-NC represent *Vegf-a* positive and negative controls, respectively, containing or not active HRE. (**D**) qRT-PCR analysis of mouse PlGF mRNA after silencing of HIF-1α in H5V exposed to 1% O_2 for 12 h and determination of PlGF concentration by sandwich ELISA (**E**) in H5V culture medium harvested after silencing of HIF-1α and 24 h of exposure to 1% O_2. As control, cells transfected with non-targeting (NT) siRNA and mock-transfected cells were used. Data obtained by qRT-PCR are expressed as fold induction and represent the mean ± SEM of two independent experiments performed in triplicate. Data obtained by ELISAs represent the mean ± SEM of two independent experiments performed in triplicate. $^{*}P < 0.0001$ and $^{§}P < 0.0005$ vs NT and mock in hypoxia. (**F**) Western blot analysis of mouse HIF-1α performed on H5V protein extracts, after cells exposure to 1% O_2 for 24 h. β-Tubulin detection on the same filter was used for normalization. Densitometry analyses are reported as percentage of arbitrary densitometry units (ADU) of the ratio of HIF-1α and β-Tubulin, assigning the value of 100 to the relative ratio obtained in mock-transfected cells in normoxic condition.

1μg of genomic DNA was subjected to bisulfite modification using the Epitect Bisulfite kit (Qiagen). Nested PCR strategy was adopted to amplify the target region from bisulfite modified genomic DNA. The conditions for the 1st PCR were: 95 °C for 30s, 55 °C for 45s, and 72 °C, for 1 min - 25 cycles, while for the 2nd PCR: 95 °C for 30s, 55 °C for 45s, and 72 °C for 45s - 30 cycles. The primers used were for the 1st PCR: upper (-309) GATTTTTGGA TGTTTTTATT TTAGGTGAT; lower (+315) AAAAAAAACC ACCATACTCA TCCC and for the 2nd PCR: upper (-264) GTAGGGTTGT GGGTTTTGTG G; lower (+223) CCTCCCTCAC TACTACCCC. The numbers identify the first nucleotide (5′ position) of upper primers or the last one (3′ position) of lower primers with respect to the transcription start site. PCR products were cloned into the pCR2.1 TOPO vectors (Invitrogen). The sequence of 20 clones for each group has been performed using M13 forward and reverse primers.

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were essentially performed as previously described.[60] Briefly, HUVEC and H5V cells were exposed to hypoxia (1% O2) for 9 or 12 h, respectively. 1×10^7 cells were fixed with 1% formaldehyde. After cross-linking, chromatin was isolated and subjected to sonication, resulting in 200–1000 bp DNA fragments. After immunoprecipitation with anti-acetylated histone H3 (Upstate 06-599), and H4 (Upstate 06-866), or anti-HIF-1α (Santa Cruz Biotechnology) and anti-HIF-2α (Novus Biologicals), immunocomplexes were purified by co-precipitation with protein A-Sepharose (GE Healthcare). Species matched IgG were used as negative control. The amount of recovered DNA was determined and the quantification of chromatin-immunoprecipitated DNA fragments was performed by qRT-PCR using the primers listed in **Table S1**. The enrichment of DNA was calculated in terms of % input = $2^{-\Delta Ct} \times 100$, where ΔCt (threshold cycle) is determined by $Ct_{IP\ sample} - Ct_{Input}$ and 100 refers to the input being 1% of the chromatin amount exposed to IP.

Silencing experiments

HUVECs and H5V were plated into 6-well plates, at 3×10^5 and 5×10^5 cells/well density, respectively. 24 h later, cells were transfected with 150 nM of siRNA for human HIF-1α, human HIF-2α, or mouse HIF-1α and, as control, with non-targeting siRNA 2 (siGENOME SMART pool, Dharmacon), using nucleofection technology (Amaxa). Sixteen hours later, HUVEC and H5V cells were exposed to hypoxia (1% O_2) for 9 or 12 h, respectively, or cultured in normoxic condition for the same time. Therefore, RNA was extracted and gene expression was quantified as described before. PlGF concentration in the culture medium was evaluated after 24 h of exposure to hypoxia.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM), with P values < 0.05 considered statistically significant. Differences between groups were compared by the Student t test and two-tailed P values are reported.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/27835

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