Impact of HIF-1α and HIF-2α on proliferation and migration of human pulmonary artery fibroblasts in hypoxia

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Abstract

Proliferation of adventitial fibroblasts of small intrapulmonary arteries (FB_{PA}) has been disclosed as an early event in the development of pulmonary hypertension and cor pulmonale in response to hypoxia. We investigated the role of hypoxia-inducible transcription factors (HIF) in human FB_{PA} exposed to hypoxia. Primary cultures of FB_{PA} displayed a strong mitogenic response to 24h hypoxia, whereas the rate of apoptosis was significantly suppressed. In addition, the migration of FB_{PA} was strongly increased under hypoxic conditions, but not the expression of α-smooth muscle actin. Hypoxia induced a marked upregulation (protein level) of both HIF-1α and HIF-2α, alongside with nuclear translocation of these transcription factors . Specific inhibition of either HIF-1α or HIF-2α was achieved by RNA interference technology, as proven by HIF-1α- and HIF-2α mRNA and protein analysis and expression analysis of HIF downstream target genes. Employing this approach, the hypoxia induced proliferative response of the FB_{PA} was found to be solely HIF-2α dependent, whereas the migratory response was significantly reduced by both HIF-1α and HIF-2α interference. In conclusion, HIF upregulation is essential for hypoxic cellular responses in human pulmonary artery adventitial fibroblasts such as proliferation and migration, mimicking the pulmonary hypertensive phenotype in vivo. Differential HIF subtype dependency was noted, with HIF-2α playing a predominant role, which may offer for future intervention strategies

Key words: adventitia, α-smooth-muscle actin, remodeling, RNA interference

Introduction

Prolonged alveolar hypoxia, as occurs e.g. at high altitude, in chronic obstructive pulmonary disease and in various restrictive lung diseases, results in a remodeling of the pulmonary vasculature, which is characterized by proliferative changes in the intima, media and adventitia of the pulmonary artery. These changes may cause chronic pulmonary arterial hypertension and subsequent cor pulmonale.

Several findings indicate that the adventitial layer of the pulmonary arteries is centrally involved in the hypoxia-driven remodeling process: 1) pulmonary artery fibroblasts (FB_{PA}) were found to show a strong proliferative response to hypoxia, exceeding that observed in endothelial cells and vascular smooth muscle cells (1-5), 2) major adventitial changes have been observed in the lung vessels of young infants dying of high-altitude induced pulmonary hypertension, reminiscent of changes in persistent pulmonary hypertension of the newborn (6, 7), 3) in animal models of hypoxia induced pulmonary hypertension, the earliest and most prominent structural changes are found in the adventitial compartment of the vessel wall, thereby contributing to vessel stiffening and initiation of remodeling of the medial and intimal layer (1-3, 8), and 4) in fibroblasts from the remodeled pulmonary artery of rats under chronic hypoxia, but not in systemic artery fibroblasts of these animals, different mitogen-activated protein kinases were found to be strongly upregulated (9, 10).

The molecular mechanisms of hypoxia sensing and signaling underlying this basic regulatory mechanism in FB_{PA} are not understood. However, evidence exist that HIF-factors are involved (11, 12). Evidence from analysis of genetic altered mice suggests hypoxia-inducible transcription factors (HIF) to be mainly involved in hypoxic adaptive processes in the vasculature of lungs. In heterozygous HIF-1α knockout mice, hypoxia induced vascular remodeling is significantly impaired (13). Also, HIF-2α heterozygous deficient mice did not

develop pulmonary hypertension upon prolonged hypoxia (14). However, the responsible changes in cellular function triggered by HIF are not described.

Thus, the aim of this study was to analyze the role of hypoxia-inducible transcription factors in adaptive responses of isolated human FB_{PA} to hypoxia. To this end, we employed RNA interference technology for specific inhibition of HIF-1a and HIF-2a (15, 16). Our study revealed that hypoxia dependent proliferation and migration of FB_{PA} are HIF-1 and/or HIF-2 dependent, whereas α-SMA expression is not primarily dependent on hypoxia or HIF.

Methods

Preparation of pulmonary artery fibroblasts (FB_{PA}):

Human cell preparations were established from excess lung tissue originating from human donor lungs employed for transplantation. This protocol was approved by the Justus-Liebig University Ethics committee. Cells were isolated by careful dissection of parenchymal connective tissue as described (17).

Counting of cell number

For assessment of the cell number, the medium was removed and the cells resuspended in 1000 µl trypsin/EDTA at room temperature. Counting was performed using a Neubauer hemocytometer (Merck). Three wells were counted for each condition.

Measurement of proliferation

Proliferation was assessed by incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) into the DNA of replicating cells using a commercially available colorimetric immunoassay according to the recommended protocol of the company (Cell Proliferation ELISA, Boehringer Mannheim, FRG).

For ^{3}H-thymidine incorporation (1.5 µCi/100 µl) cells were incubated in serum-free medium for 24h. After removal of this mixture and washing of the cell, a 5% trichloric acid solution was added for removal of free 3H-thymidine and cells incubated for further 30 min. Finally cells were lysed in 0.5 N NaOH and and genomic incorporated ^{3}H measured in a scintillation counter.

Measurement of cell migration

Migration assay was performed using a modified boyden chamber with a transwell pore size of 8µm. Cells were trypsinized, counted and seeded equally. Counting of migrated cells was done after fixation and trypan blue staining under light microscope.

Detection of apoptosis and cell cycle analyses

After trypsinization for cell detachment, the fibroblasts were incubated in 50% FBS for 15 min to restore membrane integrity and then centrifuged for 5 min at 1200 rpm. Detached cells were stored by retention of culture medium and recovered by centrifugation. Apoptotic cells were detected by assaying annexin V binding by flowcytometry (commercially available test, provided by Boehringer Mannheim, Germany). In order to exclude necrotic cells, we double-stained the cells with 5µg/ml propidium iodide (PI) in PBS. Analysis of cell cycle was performed by using 1 X 10^6 cells with serum-depletion for 24 hrs in 21% O_2. After 24 hrs of hypoxia (1%O_2), cells were quickly washed in ice-cold PBS before trypsinization. Cells were fixed with 75% ethanol and afterwards digested with DNase-free RNAse in PBS containing 5µg/ml PI for DNA-staining for 45 min at 37 °C. Propidium iodide and forward light scattering were detected by using a flow cytometer of the type FACSCalibur (Beckton Dickinson USA) equipped with Cellquest software.

Western-blot analysis

Cells were lysed in Laemmli buffer (Nupage, LDS Sample, Invitrogen) including 2% mercaptoethanol and proteins run on a sodiumdodecyl-sulfate polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane (Millipore) by electro-blotting. Protein transfer was confirmed by Ponceau S staining. Detection of HIF-1α was performed using a 1:1000 dilution of a monoclonal mouse HIF-1α antibody from BD Clontech. For detection of

HIF-2α an anti human HIF-2α antibody (monoclonal mouse, dilution 1:2000, Novus Biologicals) was used. For α-SMA a monoclonal anti-α-SMA antibody (Sigma/Aldrich 1:3000 dilution) was used and for ß-actin a mouse monoclonal antibody (Abcam, 1:10000 dilution) was used. Specific immunoreactive signals were detected by peroxidase based chemiluminescence (ECL, Amersham, Buckinghamshire, England) employing a secondary anti mouse antibody (Abcam).

Immuncytochemistry

FB_{PA} were grown on chamber slides and treated as indicated. Cells were fixed immediately in acetone and methanol (1:1). The fixed cells were incubated overnight at 4°C with phosphate-buffered saline containing an anti human HIF-1α antibody (monoclonal mouse, dilution 1:100. BD Biosciences, Heidelberg, Germany) and an anti human HIF-2α antibody (monoclonal mouse, dilution 1:100, Novus Biologicals) diluted 1:100. Fluorescence microscopy was performed after incubation with FITC-conjugated rabbit anti-mouse IgG antibodies (DAKO, Denmark) diluted 1:100 in phosphate-buffered saline.

siRNA for HIF-1a and HIF-2a

RNA interference and siRNA preparation was performed as described previously for HIF-1α (15) and HIF-2α (16). Effects of siRNA for HIF-1α (si-HIF-1α) or HIF-2α (si-HIF-2α) were compared with those of a random siRNA sequence (15). Transfection of siRNA was carried out at a final concentration of 40 nmol/l employing Oligofectamine^{TM} (Invitrogen) according to the protocol of the company. After an incubation period of 24 hr in 21% O_2, the cells were exposed to hypoxia or incubated in 21% O_2 for additional 24 hrs.

RNA-Extraction and RT-PCR

RNA extraction and realtime PCR was performed as described (15, 18). The HIF-1α, PGK, VEGF and PBGD primers were described previously (15, 18). The primers for HIF-2α, and for KDR (VEGF-receptor II) were as follows: HIF-2α-forward TCG TGA GAA CCT GAG TCT CAA A), reverse: ATC CGG TAC TGG CCA CTT ACT A) KDR-forward (ATT GGA GTG CAA ACC GGT AG), reverse (GCC TAG AAG ACT GGC TCC CT).

RESULTS

Initially, we analyzed cultured human FB_{PA} with respect to proliferation, apoptosis, cell migration and the expression of α-SMA, as these features are considered to be relevant for hypoxic induced pulmonary artery vascular remodeling. A strong increase of proliferation was observed when culturing the cells in hypoxic condition, as assessed by BrdU incorporation and counting of cell number (Fig. 1A). When analyzing the cell cycle, enhanced transition from the G_1 into the S-phase was measured under hypoxic conditions (Fig.1 A). In parallel, the apoptosis rate of FB_{PA} was significantly reduced in hypoxia, when compared to normoxic FB_{PA}, as measured by annexin V staining (Fig. 1B). To exclude necrotic cells, double-staining with propidium iodide was performed in these experiments. Focusing on cell migration, a strong time-dependent induction of cellular migration was noticed in hypoxia and found to be of a similar magnitude as caused by angiotensin (Fig. 1 C). Considering α-SMA content by Western-blot analysis (Fig. 2A top), no major changes were observed in hypoxia. These studies were performed at different cell densities, which influence cellular proliferation (Fig. 2B) and may affect α-SMA expression (19). In these samples, we also measured HIF-1α as a typical marker for hypoxic induction as well as cytoplasmic β-actin as a stable loading control (Fig.2 A bottom). On the other hand, addition of transforming growth factor β (TGF-β), a known stimulus of α-SMA expression (20), provoked the typical increase of α-SMA in FB_{PA}. Thus, we conclude that α-SMA is apparently not under direct control of HIF-1 in these cells.

Since the aim of this study was to analyze the differential role of hypoxia-inducible factors in the adaptive response of adventitial fibroblasts to hypoxia, we next analyzed HIF-1α and HIF-2α expression by Western-Blot analysis (Fig. 3A). We found induction of both HIF-1α and HIF-2α protein in hypoxia. The cellular localisation of the HIF isoforms was monitored by

immunocytochemistry (Fig. 3B). HIF-1α, almost not detectable in normoxia, appeared under hypoxic condition in the nucleus. HIF-2α illustrated a cytosolic presence with weak staining around the nucleus under normoxic conditions, with strong translocation response into the nucleus occurring under hypoxia.

Furthermore, we analyzed the hypoxic induction of well known HIF dependent target genes by measurement of the respective mRNAs (Fig. 3C). These genes included vascular-endothelial-growth factor (VEGF) and PGK as common target genes of HIF-1α and HIF-2α, as well as VEGF-receptor II (KDR) a specific target gene of HIF-2α. All target genes were significantly upregulated under hypoxia.

To analyze a possible link between HIF induction and the hypoxia induced changes in FB_{PA} proliferation and migration, we established RNA interference for the inhibition of HIF-1α and HIF-2α in FB_{PA}. Transfection efficiency of siRNA employing a fluorescence labeled siRNA displayed a high efficiency as described (17). Treatment of cells with siRNA for either HIF-1α (si-HIF-1α) or HIF-2α (si-HIF-2α) showed a reduction of HIF-1α (Fig. 4A) by si-HIF-1α and of HIF-2a by si-HIF-2α (Fig. 4B). These effects were confirmed on protein level by immuncytochemistry, showing inhibition of HIF-1α (Fig. 5A) and HIF-2α (Fig. 5B) by the corresponding siRNAs. When measuring HIF-dependent target genes (Fig. 6), we found reduced levels of phospoglycerate kinase (PGK) mRNA and VEGF mRNA by treatment with either si-HIF-1α or si-HIF-2α. The HIF-2-dependent target gene KDR was only targeted by si-HIF-2α. These data prove efficient and specific inhibition of HIF-1 and HIF-2 by the respective siRNAs.

We next analyzed, if the observed induction of proliferation and migration in hypoxia depends on the activation of either HIF-1 or HIF-2. Significant inhibition of hypoxia induced FB_{PA} proliferation was observed by si-HIF-2α in hypoxia, while si-HIF-1α was without effect

on proliferation (Fig. 7a). The effect of the combination of both HIF-1α-siRNA and HIF-2α-siRNA did not surpass the effect of si-HIF-2α alone. When studying cell migration, both si-HIF-1α and si-HIF-2α were found to exert significant inhibitory effects (Fig. 7B), with some higher efficacy of the combined application of these two agents.

Discussion

This study investigated hypoxia-induced responses of human pulmonary artery adventitial fibroblasts, which are assumed to play a major role in the remodeling process of the lung vasculature under conditions of oxygen deprivation. Hypoxia induced a strong upregulation of proliferation and migration of the $FB_{PA,}$ whereas apoptosis was down-regulated, which is well in line with the phenotypic changes observed in hypoxia induced pulmonary hypertension in vivo. In contrast, the expression of α-SMA was apparently not substantially affected by the hypoxic conditions.

These findings extend previous observations in animal models (2, 3, 9) showing that the proliferative response of pulmonary artery adventitial fibroblasts to hypoxia holds also true for cells of human origin. Of note, this response profile is specific for the lung vasculature: preceding studies in hypoxic rats demonstrated strong fibroblast proliferation in pulmonary, but not in systemic vessels. The data support the view that the fibroblasts within the adventitial layer of the small intrapulmonary arteries have a central function in promoting the unique remodeling process driven by hypoxia in this vasculature.

Focusing on the underlying molecular mechanisms, expression analysis of the hypoxia-inducible transcription factor subtypes was performed. Hypoxia induced a marked upregulation (protein level) of both HIF-1α and HIF-2α, alongside with nuclear translocation of these transcription factors. HIF-1α, being not detectable in the FB_{PA} in normoxia, displayed a strong increase at the protein level in hypoxia, a typical feature of this transcription factor (21, 22). HIF-2α, which was already detectable in normoxia, was strongly upregulated in response to hypoxia, alongside with translocation from the cytoplasm to the nucleus. Specific inhibition of either HIF-1α or HIF-2α was achieved by RNA interference technology, as proven by HIF-1α- and HIF-2α mRNA and protein analysis and by expression

analysis of the common target genes VEGF, PGK as well as the specific HIF-2 target gene KDR.

Employing this approach, the hypoxia induced proliferative response of the FB_{PA} was found to be solely HIF-2α dependent, whereas the migratory response was significantly reduced by both HIF-1α and HIF-2α interference.

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The finding of HIF subtype dependency of hypoxia-induced events in adventitial fibroblasts, and the dissociation of the dependency profile of the proliferative response (sole HIF-2α dependency) and the migratory response (both HIF-1α and HIF-2α dependency) is of particular interest, as it might be employed for future differential intervention strategies. The analysis of the convergence and differences in the molecular pathways driving lung adventitial fibroblast proliferation and migration downstream of HIF induction needs to be elucidated in further studies. Regulation of cell cycle regulatory proteins by HIF-1, as recently described for endothelial cells, may represent one important mechanism (23). Also, the particular up-regulation of HIF dependent genes encoding growth factors, such as endothelin-1, platelet-derived-growth-factor, fibroblast-growth-factor, TGF-ß3 and insulin-like-growth-factor may be relevant. Several of these proteins have been implicated in the pathogenic sequel of acute and chronic pulmonary hypertension (24-31).

The HIF-2α response is of particular interest, as this hypoxia-induced transcription factor is predominantly expressed in the lung and heart vasculature, in contrast to the ubiquitously expressed HIF-1α. HIF-1α and HIF-2α have been shown to activate common target genes via the hypoxia-responsive-element site (22, 32, 33). In addition, target genes carrying specific responsive elements for HIF-2 have been described, such as KDR and endothelial nitric oxide synthase (34, 35). Interestingly, hypoxic induction of the erythropoietin gene in hepatoma and neuroblastoma cells, revealed a strong HIF-2α responsiveness as analyzed by inhibition of

HIF-2α in comparison to HIF-1α employing siRNA (36). The authors of this study suggest DNA enhancer regions beyond the HRE element of erythropoietin gene as relevant for the specificity of HIF-2α which may also dependent on other not identified nuclear factors. In another study, a deviated HRE sequence as identified in endothelin-1 and VEGF-1 receptor (Flt-1) gene did not reveal any differences in either HIF-1α or HIF-2α binding, however conferred attenuated hypoxic induction as particularly demonstrated for endothelin-1 (37). The authors also observed that the context of the HRE sequence within the regulatory unit of the VEGF-Receptor 1 (Flt-1) may contribute for hypoxic gene induction by HIF and may differ from results obtained from the isolated HRE.

Considering the literature and our data, we propose the hypothesis that HIF-1α and HIF-2α act in a cell specific manner on common and specific targets. We suggest that the differences in HIF-1α and HIF-2α targets may contribute to the differential effects of these factors on cell proliferation and migration. HIF-1α and HIF-2α targets may comprise the target gene level, dependent on the respective DNA binding elements or on directly interacting proteins which still have to be identified.

Our data are in line with analysis of mice partially deficient for HIF-1α or HIF-2α (12-14). Both heterozygote knockout mice showed a delayed onset of pulmonary hypertension development under hypoxia, as measured by the right to left heart ratio, right ventricular pressure, and the degree of adventitial muscularization. In the HIF-2α deficient mice this was suggested to be related to decreased induction of endothelin-1 and catecholamines in response to hypoxia. These mice were largely protected against pulmonary hypertension and right ventricular dysfunction during hypoxia.

In this study, we could not find any hypoxic induction of α-SMA in the human FB_{PA}. α-SMA is a marker for differentiation towards a more contractile phenotype and has been observed to be upregulated in several fibrotic conditions which are linked to local hypoxia. In one study performed in adventitial fibroblasts of bovine origin, a strong induction of α-SMA was observed in Western-blot and immuncytochemistry (19). The increase appeared to be mediated on transcriptional level since reporter studies employing a rat α-SMA promoter construct showed strong induction of reporter activity in hypoxia. This induction was shown to be independent of the cellular proliferation status. A most likely explanation of the divergence in response to hypoxia between rat/bovine and human with respect to α-SMA are differences in the regulatory regions of the α-SMA gene (38). The homology of the corresponding 5'regulatory upstream regions is only 76%. Functionally proven HRE elements of the α-SMA gene from human or other species have not been described. On the other hand, increase of α-SMA was observed in human renal fibroblasts in hypoxic condition (39). Thus, the regulation of α-SMA in fibroblasts appears to be complex and may differ between species and different cell types.

Taken together, we have shown that HIF-1 and HIF-2 are essential for hypoxic cellular responses such as proliferation and migration in human pulmonary artery adventitial fibroblasts. Differential HIF subtype dependency was noted, with HIF-2α playing a predominant role, which may offer for future intervention strategies.

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Figure legends

Figure 1)

A) Proliferation of human FB_{PA} under conditions of normoxia (N) versus hypoxia (1% oxygen concentration) (H) or in normoxia in the presence of 10% fetal-calf-serum (FCS) as a control stimulus for proliferation over 24 h was assessed by BrdU incorporation into cellular DNA (left). Additionally, counting of cells (middle) and cell cycle analysis (left) were performed. Significant difference compared to normoxic control (mean, SEM, n = 5, *p<0.05, Man Whitney test). B) Left: Measurement of apoptosis by flow-cytometric annexin-V detection after culturing cells in normoxia (N) or hypoxia (H) for 24h. The apoptotic rate of the normoxic cells was normalized to 100%. Right: a representative FACS scan analysis of apoptosis by fluorescent annexin V staining. To differentiate necrotic from apoptotic cells, double-staining with propidium iodide (PI) for detection of DNA content was performed. The x-axis displays annexin V fluorescence and the y-axis PI fluorescence. Only PI negative and annexin V positive cells were considered (lower right squares). C) Left: Measurement of cellular migration of FB_{PA} under hypoxic versus normoxic conditions (24h) employing a modified boyden chamber. As a positive control, normoxic cells were treated with angiotensin (AngII) which is known to stimulate migration. Significant difference from normoxic control cells (mean, SEM, n=5, *p<0.05, Man Whitney test). Right: kinetics of migration after 10, 24 and 48 h (mean, SEM, n = 5).

Figure 2)

A) Top: Western-blot analysis of α-SMA and HIF-1α in FB_{PA} cultured for 24h in normoxia (N) or hypoxia (H). As a loading control, Western-blot analysis for cytoplasmic ß-actin is included. No hypoxic induction of α-SMA was observed, while HIF-1α was strongly induced. Bottom: BrdU incorporation of cells being 50% confluent or 90% confluent. B) Western-blot analysis showing induction of α-SMA in FB_{PA} by TGF-ß and in comparison to non-stimulated smooth muscle cells (SMC_{PA}) cells.

Figure 3)

A) Western-blot analysis of HIF-1α and HIF-2α in FB_{PA}, being cultured in normoxia (N) or hypoxia (H) for 24 h. B) Immuncytochemistry for HIF-1α and HIF-2α after culturing FB_{PA} for 24h in normoxia (N) or hypoxia (H). C) Relative quantification of HIF-1 and HIF-2 dependent target expression by realtime PCR. Concentrations of mRNAs encoding PGK, VEGF, and KDR, related to the house keeping gene PBGD, are given. Significant difference from normoxic control cells (mean, SEM, n=5, *$p<0.05$, Man Whitney test).

Fig. 4)

Impact of siRNA targeting HIF-1α (siHIF-1α) or HIF-2α (siHIF-1α) on HIF-1α and HIF-2α mRNA. Quantification of HIF-1α and HIF-2α mRNA was performed by realtime PCR in relation to the house keeping mRNA PBGD. Cells were treated with either siRNA targeting HIF-1α (siHIF-1α) or siRNA targeting HIF-2α and compared to cells treated with a random siRNA sequence (si-control). Significant difference compared to random siRNA (mean, SEM, n=5, *$p<0.05$, Man Whitney test).

Fig. 5)

A) Immuncytochemistry for HIF-1α after culturing cells for 24h in normoxia (N) or hypoxia (H). Cells were treated either with a random siRNA (si-control) or with siRNA targeting HIF-1α (si-HIF-1α). B) Immuncytochemistry for HIF-2α after culturing cells for 24h in normoxia (N) or hypoxia (H). Cells were treated either with a random siRNA (si-control) or with siRNA targeting HIF-2α (si-HIF-2α).

Fig. 6)

Impact of siRNA targeting HIF-1α (siHIF-1α) or HIF-2α on target gene expression. Quantification of HIF-1α and HIF-2α dependent target expression was performed by realtime PCR. mRNAs encoding the HIF-1 and HIF-2 dependent target genes PGK and VEGF, or the solely HIF-2 dependent KDR gene, were quantified in relation to the house keeping mRNA PBGD. Cells were treated with either siRNA targeting HIF-1α (siHIF-1α) or siRNA targeting HIF-2α (siHIF-2α) and compared to cells treated with a random siRNA sequence (si-control). Significant difference compared to random siRNA (mean, SEM, n=5, *$p<0.05$, Man Whitney test).

Fig. 7)

Measurement of proliferation (top) and migration (bottom) in FB_{PA} treated with specific siRNAs. Cells were treated with either siRNA targeting HIF-1α (siHIF-1α) or siRNA targeting HIF-2α (si-HIF-2α) or both together and compared to cells treated with a random siRNA sequence (si-control). Significant difference compared to si-control (mean, SEM, n=5, *$p<0.05$, *$p<0.01$, Man Whitney test).

Figure 1

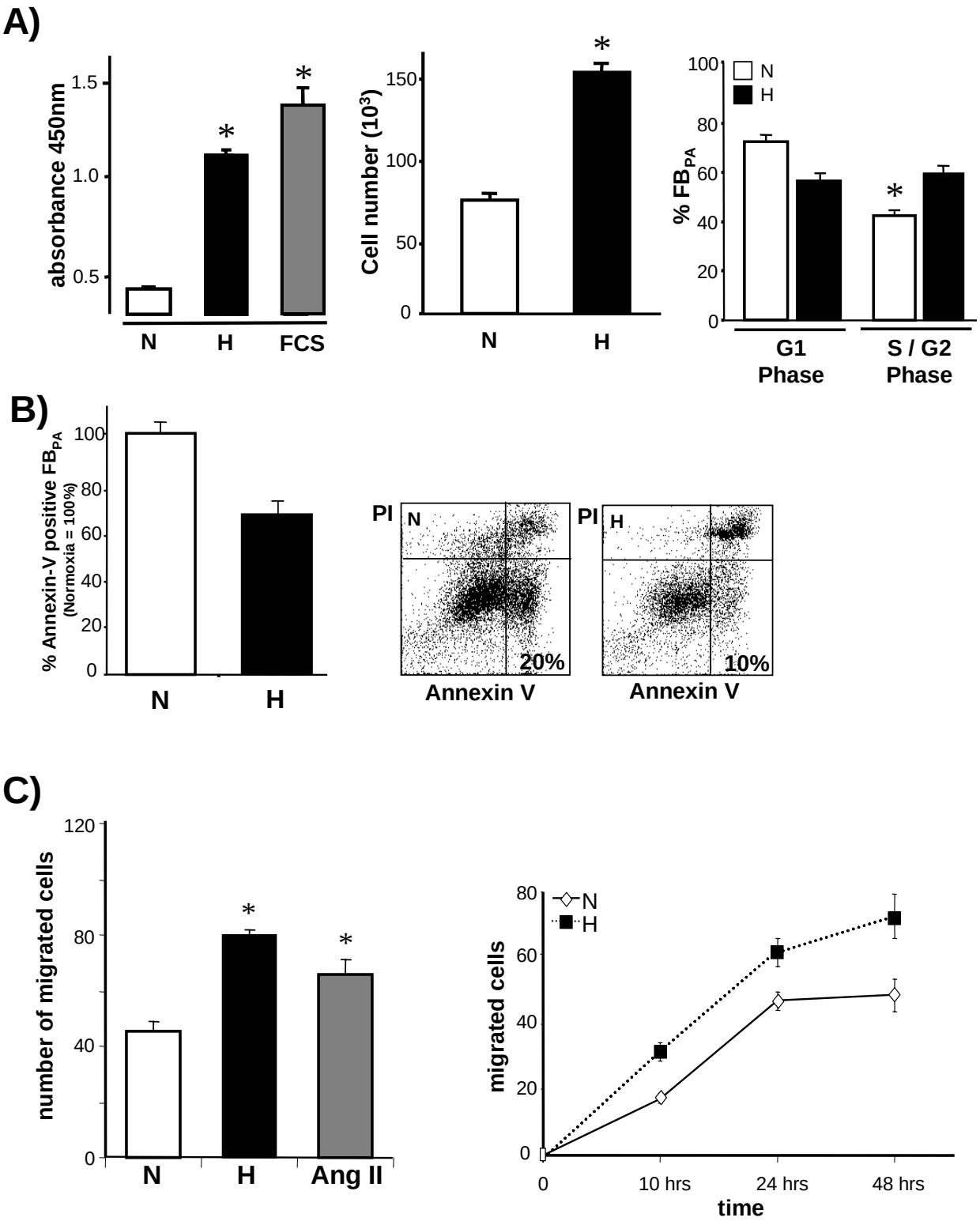
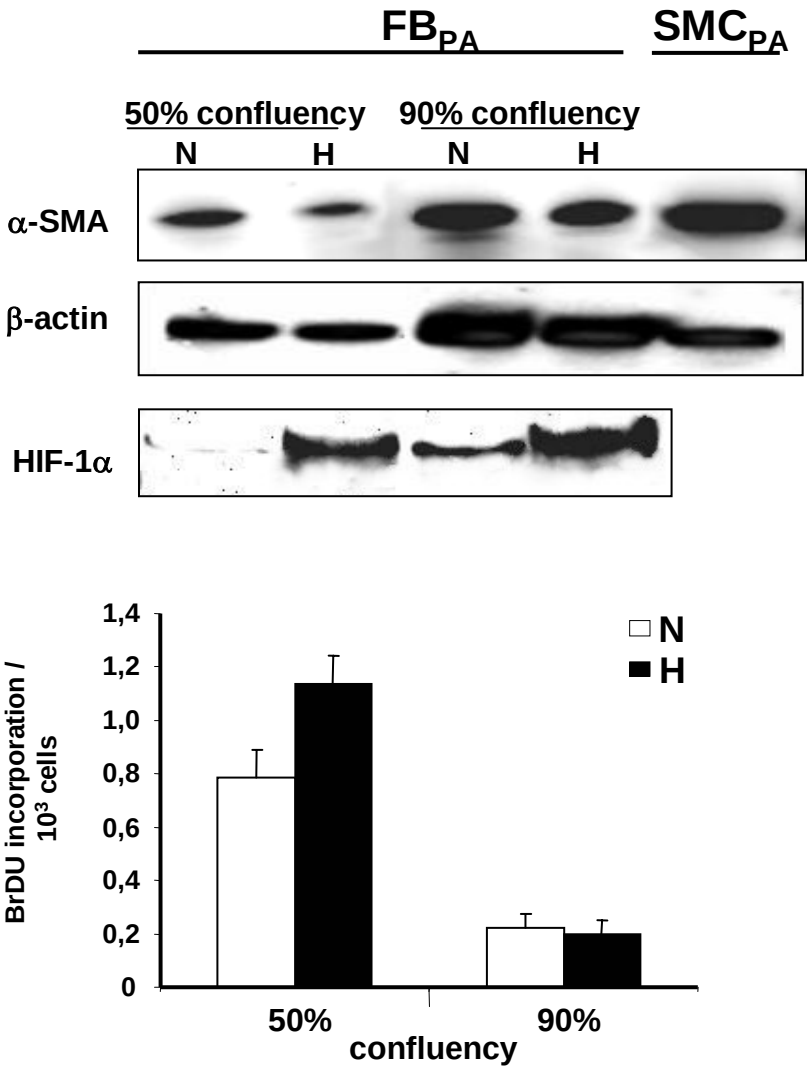

A)
absorbance 450nm
1.5
1.0
0.5
N
H
FCS
Cell number (10³)
150
100
50
0
N
H
% FB_PA
100
80
60
40
20
0
N
H
G1 Phase
S / G2 Phase
B)
% Annexin-V positive FB_PA (Normoxia = 100%)
100
80
60
40
20
0
N
H
PI
N
20%
Annexin V
PI
H
10%
Annexin V
C)
number of migrated cells
120
80
40
0
N
H
Ang II
migrated cells
80
60
40
20
0
N
H
0
10 hrs
24 hrs
48 hrs
time

Figure 2

A)


FB$_{PA}$
SMC$_{PA}$
50% confluency
90% confluency
N
H
N
H
α-SMA
β-actin
HIF-1α
BrDU incorporation / 10^3 cells
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
N
H
50%
90%
confluency

B)

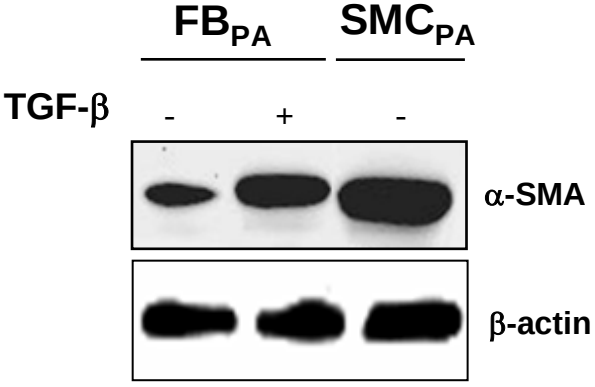

FB$_{PA}$
SMC$_{PA}$
TGF-β
-
+
-
α-SMA
β-actin

Figure 3

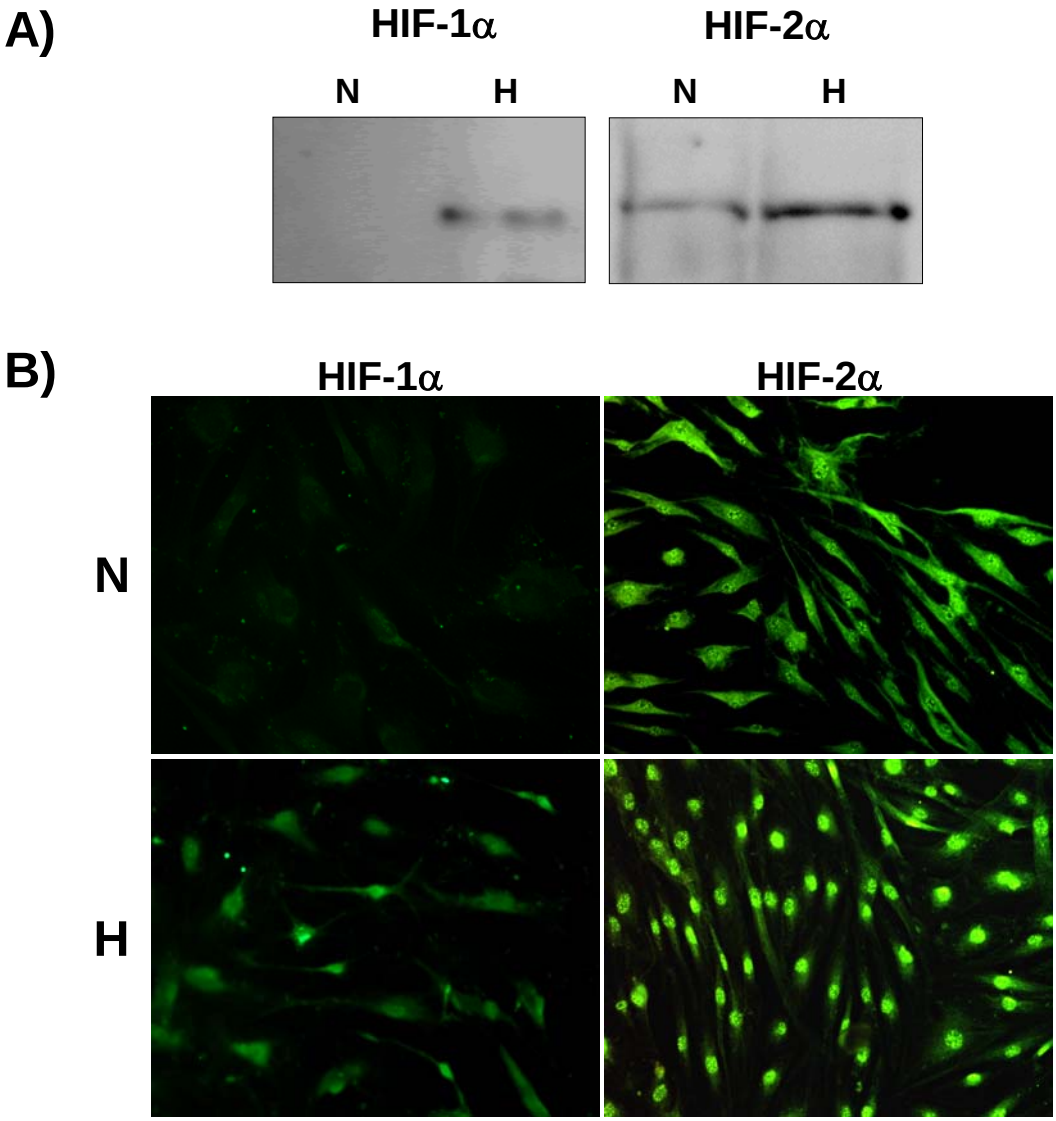

A)
HIF-1α
HIF-2α
N
H
N
H
B)
HIF-1α
HIF-2α
N
H
C)
N
H
target gene mRNA / PDGB mRNA
1,6
1,2
0,8
0,4
0,0
*
*
*
PGK
VEGF
KDR

Figure 4

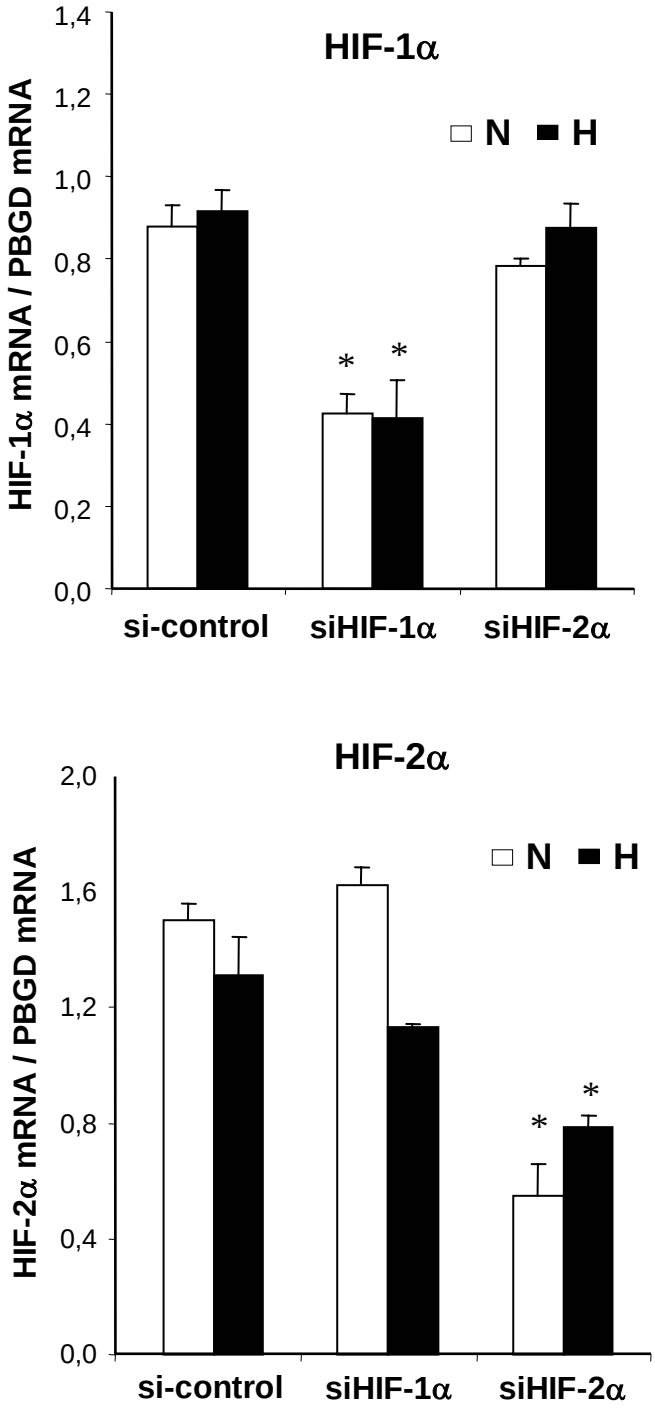

HIF-1α
N H
HIF-1α mRNA / PBGD mRNA
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
*
si-control
siHIF-1α
siHIF-2α
HIF-2α
N H
HIF-2α mRNA / PBGD mRNA
2,0
1,6
1,2
0,8
0,4
0,0
*
*
si-control
siHIF-1α
siHIF-2α

Figure 5

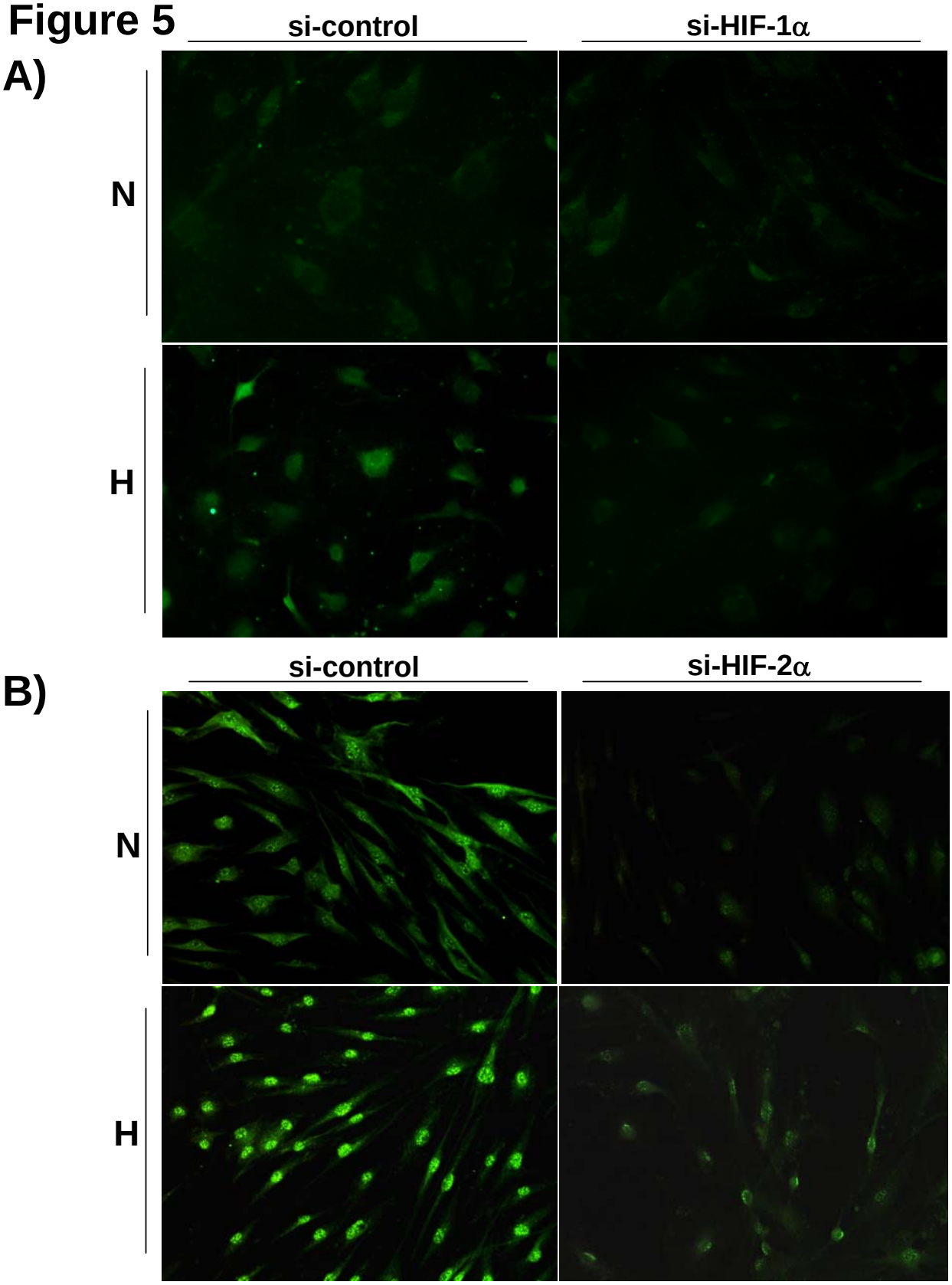
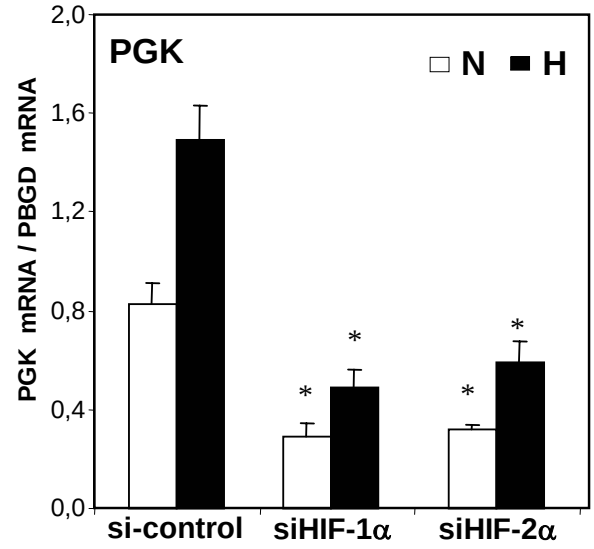
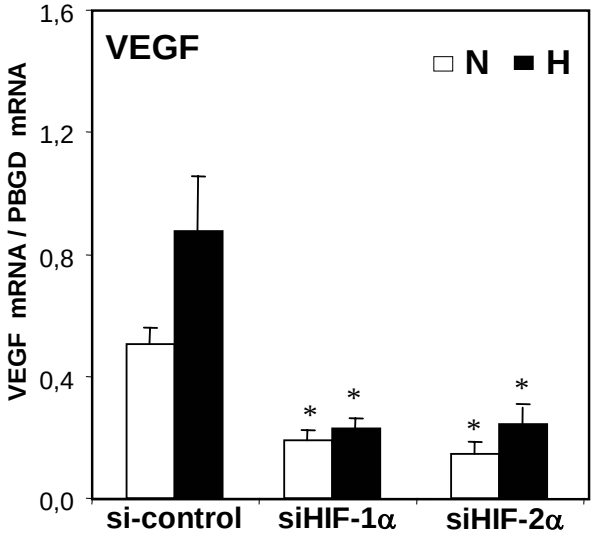


Figure 6


PGK
N
H
PGK mRNA / PBGD mRNA
2,0
1,6
1,2
0,8
0,4
0,0
si-control
siHIF-1α
siHIF-2α


VEGF
N
H
VEGF mRNA / PBGD mRNA
1,6
1,2
0,8
0,4
0,0
si-control
siHIF-1α
siHIF-2α

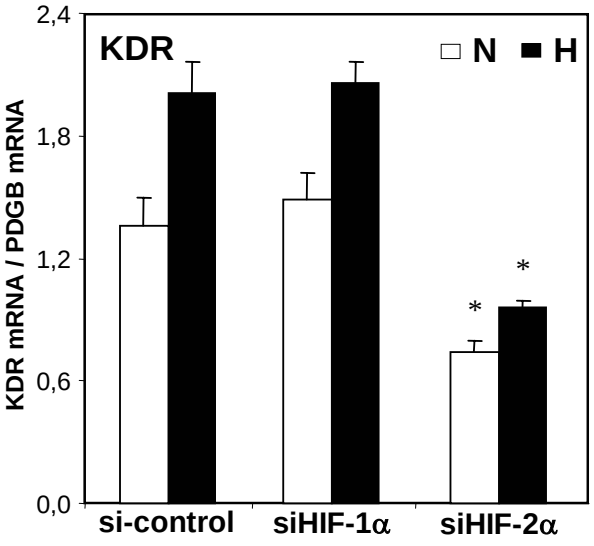
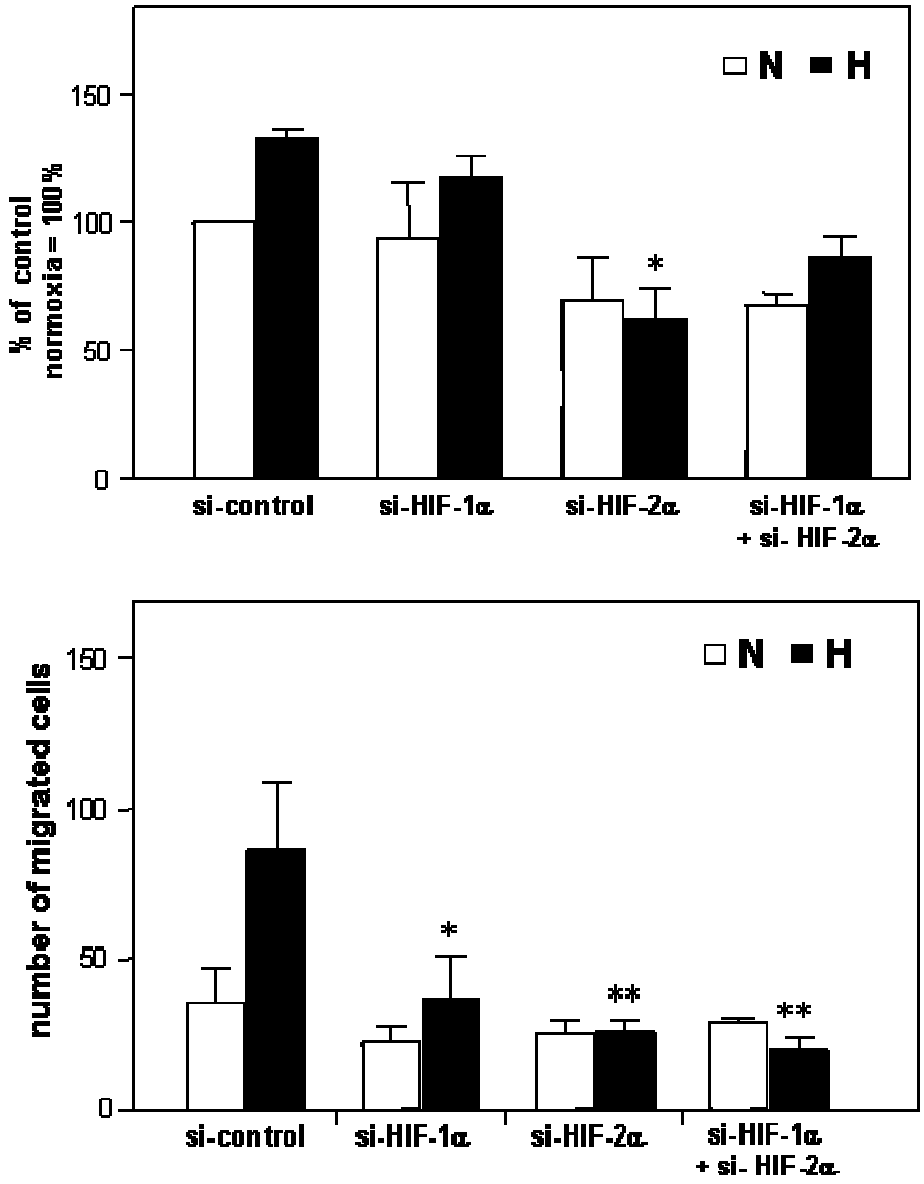

KDR
N
H
KDR mRNA / PDGB mRNA
2,4
1,8
1,2
0,6
0,0
si-control
siHIF-1α
siHIF-2α

Figure 7


N
H
% of control
normoxia = 100%
150
100
50
0
*
si-control
si-HIF-1α
si-HIF-2α
si-HIF-1α
+ si- HIF-2α
number of migrated cells
150
100
50
0
*
**
**
si-control
si-HIF-1α
si-HIF-2α
si-HIF-1α
+ si- HIF-2α