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ARTICLES

Hypoxia-Inducible Factor 1α Modulates Adhesion, Migration, and FAK Phosphorylation in Vascular Smooth Muscle Cells

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Abstract Hypoxia promotes angiogenesis by modulating the transcriptional regulator hypoxia-inducible factor 1α (HIF-1α). HIF-1α is a master regulator of the hypoxic response, and its proangiogenic activities include, but are not limited to, regulation of vascular endothelial growth factor (VEGF). The remodeling of the vasculature during angiogenesis requires an initial destabilization step, which facilitates endothelial sprouting, followed by vessel growth, and restabilization through investment of smooth muscle cells. The complex dynamics of hypoxia-induced angiogenesis prompted us to investigate what aspects of this multi-step process are regulated by HIF-1α. To do so, we analyzed the molecular properties of aortic and coronary artery smooth muscle cells in response to forced expression of HIF-1α, and by treatment with cobalt chloride, which mimics hypoxia. Our results demonstrate that HIF-1α causes a marked reduction in the ability of smooth muscle cells to migrate and adhere to extracellular matrices. Analysis of focal adhesion proteins showed no significant difference in expression or localization of vinculin or focal adhesion kinase (FAK). However, investigation of FAK phosphorylation, a critical mediator of adhesion and migration, revealed tyrosine phosphorylation of FAK is diminished in the presence of HIF-1α and cobalt chloride. These results indicate that during hypoxia-induced vessel remodeling, HIF-1α functions to dampen adhesion and migration of smooth muscle cells by modulating FAK activity. We suggest that HIF-1α expression in smooth muscle cells may augment vessel sprouting by loosening smooth muscle cell attachments to the basement membrane and endothelial cells. J. Cell. Biochem. 96: 971–985, 2005.

Key words: hypoxia-inducible factor 1 (HIF-1); smooth muscle cells; adhesion; migration; hypoxia; focal adhesion kinase (FAK)

Cells in a hypoxic environment undergo adaptive changes to cope with oxygen deprivation. Within the vasculature, hypoxia promotes angiogenesis, which serves to alleviate inadequate oxygen levels by sprouting new blood vessels [Carmeliet and Jain, 2000; Pugh and Ratcliffe, 2003; Semenza, 2003]. In coronary artery disease, for example, vascular occlusion gives rise to hypoxia, leading to cardiac ischemia [White et al., 1992; Banai et al., 1994; Semenza, 2003]. Under these conditions, collateral blood vessels form to circumvent the occlusion and carry oxygen-rich blood to the ischemic heart. The neovascularization that occurs within the ischemic heart is vital. In patients with myocardial infarction, the degree of coronary collateralization correlates with myocardial viability [Sabia et al., 1992].

A key cellular mediator of the hypoxic response is hypoxia-inducible factor 1 (HIF-1) [Semenza, 2000, 2002; Maxwell and Salnikow, 2004]. HIF-1 is a heterodimeric transcription factor composed of a hypoxia-regulated α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β/ARNT). HIF-1α is stabilized in oxygen-deprived cells and dimerizes with HIF-1β to activate transcription of an array of genes required for oxygen homeostasis. Equally significant to the protective function of HIF-1 in oxygen-deprived cells is its ability to promote angiogenesis [Semenza, 2002; Pugh and Ratcliffe, 2003; Yamakawa et al., 2003]. In hypoxic conditions, such as tumor growth and

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myocardial ischemia, HIF-1 has been implicated in governing blood vessel growth, primarily through regulation of vascular endothelial growth factor (VEGF) [Forsythe et al., 1996; Semenza, 2003]. Yet, VEGF alone cannot recapitulate normal angiogenesis, which suggests that additional factors mediating vessel growth are directly activated by HIF-1 under these circumstances [Detmar et al., 1998; Larcher et al., 1998; Lee et al., 2000; Xia et al., 2003]. Indeed, studies have demonstrated that HIF-1 can promote the expression of multiple genes implicated in angiogenesis, including the angiopoietins and integrins [Yamakawa et al., 2003; Ceradini et al., 2004; Kong et al., 2004].

Although it is known that HIF-1α is stabilized in oxygen-deprived vascular cells and can promote angiogenesis, the consequence of its expression within these cells remains largely unexplored. The sprouting of new blood vessels relies on destabilization of the existing vessel to permit outgrowth of new branches [Carmeliet, 2000]. Endothelial cells form an initial immature tube, and through a process of maturation, the new vessel is stabilized by coverage with smooth muscle cells [Benjamin et al., 1998; Carmeliet, 2000]. While HIF-1-regulated expression of VEGF is key to endothelial cell tube formation, the role of HIF-1 in the smooth muscle cells surrounding the endothelium has not been investigated. Therefore, to gain a better understanding of HIF-1's function in hypoxia-induced vascular remodeling, we sought to investigate the direct effect of HIF-1α in vascular smooth muscle cells.

In this study, we analyzed the molecular properties of coronary artery and aortic smooth muscle cells in response to forced expression of HIF-1α and cobalt chloride ($CoCl_2$), which mimics hypoxia by stabilizing the HIF-1α subunit [Kanaya and Kamitani, 2003; Yuan et al., 2003; Maxwell and Salnikow, 2004]. Both HIF-1α overexpression and $CoCl_2$ treatment caused cultured smooth muscle cells to become less adhesive and attenuated their migration. In contrast, there was no effect on proliferation or the expression of differentiation-specific smooth muscle markers. The decline in migration and adhesion suggested a disruption in focal adhesions. Although there was no obvious difference in number and size of focal contacts between experimental and control groups, a more in depth analysis revealed that tyrosine phosphorylation of focal adhesion kinase (FAK) was reduced in the HIF-1α-expressing and $CoCl_2$-treated cells. These data represent the first direct evidence linking HIF-1α to the regulation of migration, adhesion, and FAK phosphorylation in smooth muscle cells.

MATERIALS AND METHODS

Cell Culture

Primary cultures of bovine coronary artery smooth muscle cells (BCASMCs) and bovine aortic smooth muscle cells (BASMCs) were purchased from Cambrex (BioWhittaker). Cells were grown in D-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and maintained in humidified 5% CO_2 at 37°C. For studies performed in low serum, cells were cultured in 0.5% FBS 24 h prior to initiation of experiments. All experiments were performed using cells between passage four and eight. Where indicated, cells were supplemented with 200 μM $CoCl_2$ (Sigma), 5 ng/ml PDGF-BB (Peprotech), or 5 ng/ml TGF-β_1 (Peprotech).

Construction of HIF-1α Adenovirus Vector and Viral Infection

The human *HIF-1α* cDNA (a gift from Dr. Greg Semenza, Johns Hopkins University) was cloned into the pAdTrack shuttle vector [He et al., 1998] in front of the CMV promoter using KpnI and EcoRI restriction sites. The adenovirus plasmids containing GFP alone (AdGFP) and GFP together with HIF-1α (AdHIF-1α) were transfected into HEK293 cells, and the viral particles were amplified and purified as described [He et al., 1998]. For smooth muscle infections, viral particles were diluted in a minimum volume of Opti-MEM (Invitrogen) required to cover cells, and following a 2-h incubation were supplemented with normal growth media to attain a standard culture volume. The following day, the virus-containing media was replaced with fresh media, and infection efficiency was evaluated by GFP expression. AdGFP and AdHIF-1α viral particles were titrated to achieve an infection efficiency in which 90%–100% of the cells were GFP positive (Fig. 1A). Expression of HIF-1α was confirmed by Western blotting (Fig. 1B).

Immunoblotting

Protein extract isolation and Western blot analyses were performed as described [Harlow,

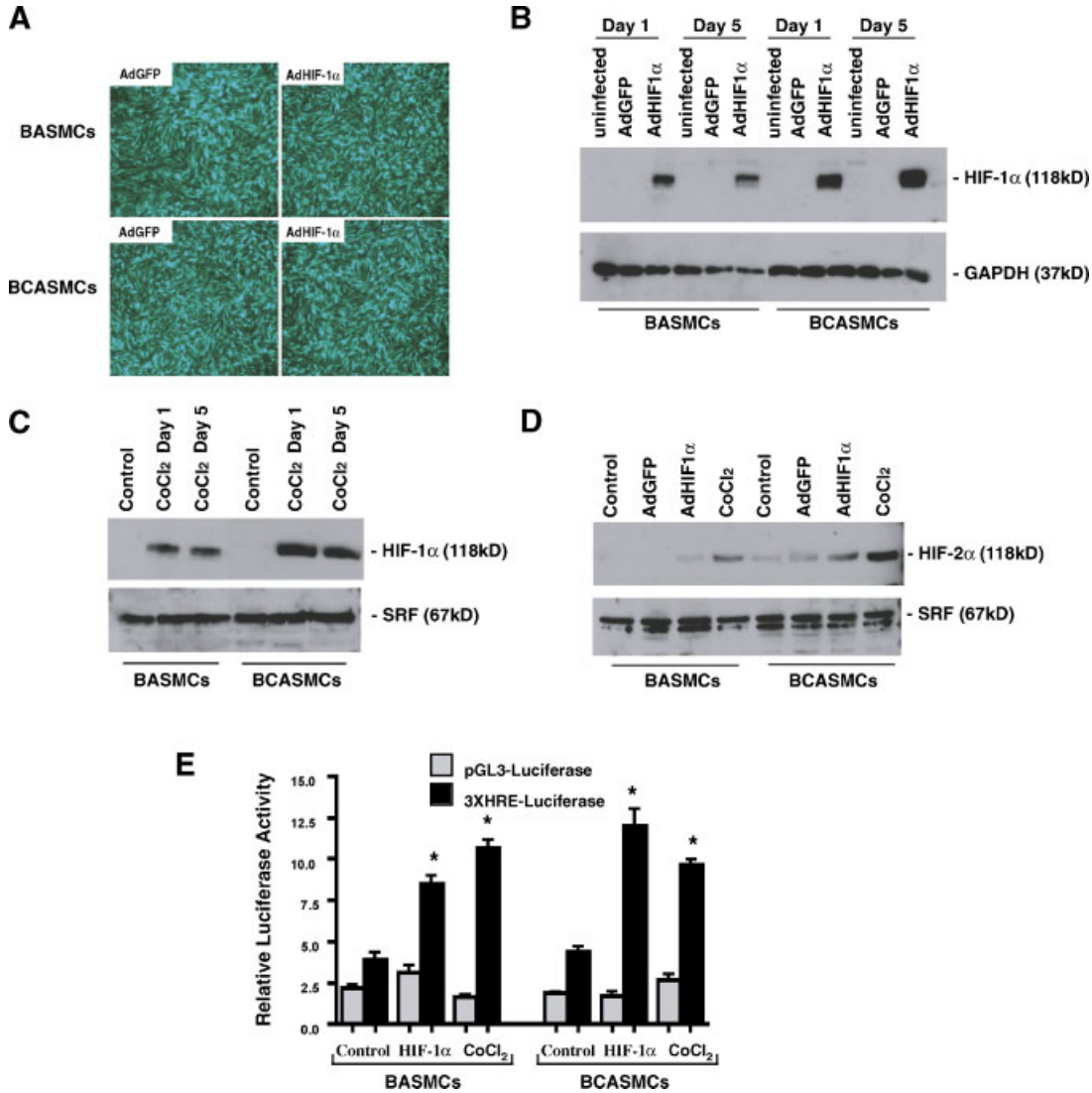

Fig. 1. Expression of HIF-1α in smooth muscle cells. (**A**) Adenovirus harboring a GFP expression cassette (as control) or GFP together with a HIF-1α expression cassette were used to infect primary cultures of smooth muscle cells. In both bovine aortic smooth muscle cells (BASMCs) and bovine coronary artery smooth muscle cells (BCASMCs) infection resulted in approximately 90% of the cells expressing GFP. (**B**) Western blot using extracts derived from infected cells demonstrates expression of the HIF-1α protein from the AdHIF-1α-containing adenovirus. No HIF-1α expression was detected in the uninfected and GFP-alone infected extracts. GAPDH was used to demonstrate equivalent sample loading. (**C**) Western blot of nuclear extracts shows endogenous HIF-1α expression is induced in BASMCs and BCASMCs by addition of 200 μM $CoCl_2$. Serum response factor (SRF) was used as a loading control. (**D**) Analysis of HIF-2α by Western blot demonstrates that cellular levels of HIF-2α protein are increased by HIF-1α overexpression and $CoCl_2$ treatment. (**E**) Transcriptional activity of HIF-1 was examined by utilizing a HIF-1 response element reporter construct (3XHRE-luciferase). Basal 3XHRE-luciferase activity was measured and compared to cells cotransfected with HIF-1α cDNA, and stimulated with $CoCl_2$. For non-specific activity, a luciferase construct devoid of HRE sequences (pGL3-luciferase) was used as a control. Data represents mean ± SEM ($n = 3$). *$P < 0.05$, statistically significant difference from respective control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1999]. Protein samples were run on 10% SDS–PAGE gels, transferred to Immobilin-NC (Amersham), and subjected to consecutive incubations with primary and secondary antibodies. Proteins were detected by enhanced chemiluminescence (ECL), using horseradish peroxidase-conjugated (HRP) secondary antibodies (Amersham). Primary antibodies used: HIF-1α (1:1,000) (Santa Cruz), smooth muscle α-actin (1:10,000) (Sigma), smooth muscle myosin heavy chain (1:2,000) (Biomedical Technologies, Inc.), calponin (1:1,000) (DAKO), vinculin (1:10,000) (Sigma), GAPDH (1:1,000) (Novus biologicals), FAK (1:1,000) (Upstate), HIF-2α

(1:1,000) (Novus biologicals), c-Met (1:500) (R&D systems), SRF (1:1,000) (Santa Cruz). Secondary antibodies were: sheep anti-mouse Ig-HRP (1:8,000), donkey anti-goat (1:5,000), and donkey anti-rabbit Ig-HRP (1:8,000) (Amersham).

Transfection and Luciferase Assays

To measure the transcriptional activity of HIF-1α in BASMCs and BCASMCs, three copies of a HIF-1 response element (HRE) [Semenza and Wang, 1992] were cloned into pGL3 promoter luciferase vector (Promega) using BglII linkers. HRE sequence—Upper: GATCTGCATACGTGGGCTCCA Lower: GATCTGGAGCCCACGTATGCA. Cells were transiently transfected at 90% confluence using Lipofectamine 2000 (Invitrogen) and were harvested 48 h after the start of transfection. One microgram of the pGL3-luciferase or pGL3-3XHRE-luciferase reporter, and 0.5 μg of the HIF-1α expression construct were transfected. To normalize for transfection efficiency, 0.2 μg of CMV-β-galactosidase (LacZ) was cotransfected, and luciferase activities were normalized based on equivalent amounts of LacZ activity. Luciferase assays were performed as described [Ausubel, 1995] and quantified using a Turner Diagnostics luminometer. LacZ activity was measured as described [Neville and Hauschka, 1998]. Experiments were repeated three times, and LacZ and luciferase assays were measured in duplicate. Statistical significance was determined using a student's *t*-test. Values were considered significant at $P < 0.05$.

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated by M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instructions. For each 25 μl PCR reaction, 2 μl of cDNA was added to 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$, 200 μM dNTPs, 15 μM of each primer, and 1 U Taq DNA polymerase (Invitrogen). VEGF primers were designed to amplify a 508-bp fragment of mRNA [Tscheudschilsuren et al., 2002]. Primer sequences: VEGF sense -5′-TACCTTCACCATGCAAG, VEGF antisense -5′-CACATCTGCAAGTACGTTCG. GAPDH primers (BD-Clontech) were used as an internal control for RNA integrity. GAPDH sequences: 5′-ACCACAGTCCATGCCATCAC, 5′-TCCACCACCCTGTTGCTGTA. PCR products were quantified using a Kodak Gel Logic 100 imaging system to measure band intensity. Quantity of VEGF was normalized to GAPDH levels and student's *t*-tests were performed using three independent experiments to determine *P*-values.

Proliferation Assays

Proliferation studies were carried out on cells grown in standard culture media supplemented with 0.5% (low serum) or 10% (high serum) FBS. Cell numbers were measured using a methylene blue absorbance assay [Oliver et al., 1989] and were counted manually using a hemocytometer (Hausser Scientific). Both methods gave equivalent results. For the methylene blue assay, cells were infected with adenovirus (AdGFP or AdHIF-1α) or treated with $CoCl_2$, and 24 h later were trypsinized, counted and plated into 96-well plates for 0, 24, and 48-h time points. Proliferation assays were repeated three times, and statistical significance was determined by a student's *t*-test using GraphPad Prism software.

Wound Healing Migration Assay

For wound assays, cells were plated in a 24-well dish and infected with AdGFP or AdHIF-1α at 80% confluence. Thirty-six hours following infection and 12 h after $CoCl_2$ addition, the monolayer was wounded with a plastic pipet tip to create an acellular line within the culture dish. Cells were washed in PBS and fresh media was added, containing 10% (high serum) or 0.5% (low serum) FBS. Cells were cultured for an additional 24 h to monitor migration. Hydroxyurea (2 mM) (Sigma) was added to indicated cultures 4 h before wounding. Pictures were captured immediately after wounding and 24 h later using a Leica DMIL inverted microscope and Leica 320 digital camera.

Cell Attachment Assay

BASMCs and BCASMCs were grown in 35-mm culture dishes and infected with adenovirus as described above. Forty-eight hour-post-infection, cells were trypsinized, counted, and used for attachment assays. $CoCl_2$ was added 24 h prior to the start of the assay. Attachment assays were performed as described [Xu et al., 1998], with minor modifications. Briefly, 96-well culture plates were coated overnight at 4°C with 10 μg/ml of bovine serum albumin (BSA), collagen type-I, collagen type-III, collagen type-IV, elastin, laminin, or fibronectin (Sigma).

Wells were rinsed three times with PBS, and 20,000 cells (2.0×10^5 cells/ml) were placed in each well and allowed to adhere for 45 min. Plates were rinsed three times in PBS to remove nonadherent cells, and attached cells were fixed with 4% formaldehyde for 30 min. After fixation, cells were rinsed three times with PBS and stained with 1% Toluidine Blue for 1 h. Plates were washed to remove excess dye, and the remaining blue dye was solubilized in 2% SDS. Absorbance was measured at 620 nm. Assays were carried out four times, and statistical analysis was performed using GraphPad Prism software.

Immunofluorescence

For immunofluorescence, cells were plated on glass coverslips, fixed in 4% formaldehyde for 10 min, and permeabilized in 0.2% Triton-X-100 for 5 min. Antibody incubations were carried out as described [Spector and Leinwand, 1998]. Primary antibodies used: phosphotyrosine (1:200) (Cell Signaling Technologies), vinculin (1:1,000) (Sigma), and FAK (1:100) (Upstate). Fluorescently tagged secondary antibodies were: Alexa-Fluor 594 goat anti-mouse (1:300), and Alexa-Fluor 594 goat anti-rabbit (1:300) (Molecular Probes). Coverslips were mounted in Prolong Gold anti-fade reagent (Molecular Probes), and cells were visualized using a Leica DM5000B microscope with a 60× Apochromat objective.

FAK Immunoprecipitations

Protein extracts were isolated by scraping cells into a cell lysis buffer (50 mM Tris-HCl pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, pepstatin; 1 mM Na_3VO_4, 1 mM NaF). For each sample condition, 4 μg of FAK antibody (Upstate) was preincubated with 30 μl of Protein A/G agarose beads (Santa Cruz) for 2 h. Anti-FAK-bound agarose beads were washed, recovered, and added to 500 μg of protein extracts from each condition. Immunoprecipitations were incubated overnight, after which beads were pelleted, washed three times, and resuspended in SDS–PAGE loading buffer. All incubations and washes were performed at 4°C in the cell lysis buffer. Each sample (20%) was separated on a 10% SDS–PAGE gel, transferred to Immobilin-NC (Millipore), and incubated with primary and secondary antibodies to detect FAK (1:1,000) and phosphorylated tyrosine (1:1,000) (Upstate clone 4G10).

RESULTS

Expression and Activity of HIF-1α in Aortic and Coronary Artery Smooth Muscle Cells

Our goal was to determine the role of HIF-1α in smooth muscle cells, with the intention of understanding the direct effect of HIF-1α in conditions of hypoxia-induced neovascularization. For these analyses, we used primary cultures of bovine smooth muscle cells derived from the aorta and coronary artery. Small diameter coronary arteries are prone to collateralization under hypoxic conditions [White et al., 1992], while the large vessel of the aorta is never subject to this same environment. We reasoned that by comparing and contrasting cells from these unique vascular beds, we might uncover differences in the hypoxic response of these smooth muscle subpopulations.

To assess the effect of HIF-1α on smooth muscle cells, we generated an adenovirus expressing the human *HIF-1α* cDNA under the control of the CMV promoter. The AdEasy adenovirus system [He et al., 1998] harbors the green fluorescent protein (GFP) driven by CMV, which allows for easy determination of viral infection. Infection of primary cultures of BASMCs and BCASMCs with adenovirus containing HIF-1α and GFP (AdHIF-1α), or GFP alone (AdGFP) resulted in approximately 90% of the cells expressing GFP (Fig. 1A). GFP expression was maintained throughout the duration of our experiments, which were conducted between 2 and 5 days post-infection. To verify expression of the HIF-1α protein, we performed Western blot analysis on extracts isolated from infected cells. In uninfected smooth muscle cells or those infected with AdGFP, we failed to detect the HIF-1α protein. However, in cells infected with AdHIF-1α, the human HIF-1α protein was abundantly expressed 24 h after infection, and continued to be expressed after 5 days (Fig. 1B). We additionally treated cells with $CoCl_2$, which mimics hypoxic conditions by stabilizing the HIF-1α subunit [Kanaya and Kamitani, 2003; Yuan et al., 2003]. Western blots using nuclear extracts showed an increase in endogenous HIF-1α protein in cells treated with $CoCl_2$ for 1 and 5 days (Fig. 1C). These results demonstrate that the HIF-1α protein is not present at

detectable levels in aortic and coronary artery smooth muscle cells, however infection with a HIF-1α-containing adenovirus or stimulation with $CoCl_2$ resulted in robust expression of the HIF-1α protein. These data are consistent with previous reports in which HIF-1α was only present in smooth muscle cells exposed to $CoCl_2$ or hypoxia [Richard et al., 2000; Page et al., 2002; Rose et al., 2002]. Whereas cobalt prevents HIF-1 degradation by disrupting the interaction with the von Hippel–Lindau protein (pVHL) [Kanaya and Kamitani, 2003; Yuan et al., 2003], overexpression of HIF-1α likely overwhelms the pVHL-mediated degradation pathway leading to an increase in HIF-1 protein. If this is the case, then one would expect other proteins that succumb to degradation via the pVHL pathway to also be increased. Indeed, amounts of the HIF-2α protein were greater in cells not only treated with $CoCl_2$, but also overexpressing HIF-1α (Fig. 1D). Thus, these data indicate that strategies to stabilize HIF-1α have additional consequences, such as increasing HIF-2α, which may contribute to downstream effects.

We next measured the activity of HIF-1α in our cultured smooth muscle cells, by utilizing a multimerized HIF-1 response element (HRE) [Semenza and Wang, 1992] fused to a luciferase reporter. Transfection of BASMCs and BCASMCs resulted in an increase in luciferase activity in response to cotransfection with the HIF-1α cDNA or by $CoCl_2$ stimulation (Fig. 1E). HRE-driven reporter activity increased between twofold and threefold in the presence of HIF-1α and $CoCl_2$ treatment, whereas the control luciferase reporter lacking the HRE sequences was not significantly activated. These data confirm that HIF-1 becomes transcriptionally active by introduction of an exogenous HIF-1α subunit, or by stabilization of endogenous HIF-1α with $CoCl_2$. Given these results, we proceeded to investigate the functional consequences of HIF-1α expression.

HIF1α Does Not Alter Smooth Muscle Proliferation

Several studies have implicated HIF-1 in promoting cell cycle exit [Gardner et al., 2001; Goda et al., 2003], while others have linked HIF-1 to supporting cell proliferation [Rose et al., 2002; Tang et al., 2004]. This discrepancy has lead to the notion that HIF-1's activities are cell-type dependent. The proliferation of smooth muscle cells is an integral part of vascular remodeling, and thus we speculated that HIF-1α might alter the proliferation of these cells. To test this, we measured the proliferation indices of smooth muscle cells overexpressing HIF-1α and exposed to $CoCl_2$, and compared them to control cells. Surprisingly, there was no significant difference in the rate of cell division measured over a 48-h time course (Fig. 2). BASMCs and BCASMCs exhibited similar proliferation rates, yet in both cases there was no difference among uninfected cells, cells treated with $CoCl_2$ or those infected with control AdGFP and AdHIF-1α. Proliferation assays were conducted in the presence of high serum (10%) (Fig. 2) and low serum (0.5%) (data not shown), with similar results. These data demonstrate that expression of HIF-1α does not alter the proliferation of smooth muscle cells. Thus, unlike previous findings in which HIF-1α has been implicated in cell cycle withdrawal or progression, our data directly show that replication of aortic and coronary artery smooth muscle cells remains unaffected.

HIF-1α and Smooth Muscle Modulation

A hallmark of smooth muscle cells is their ability to modulate between different phenotypes in response to changes in environmental conditions [Owens et al., 2004]. For example, in response to vascular injury, smooth muscle cells take on proliferative and migratory phenotypes, which generally coincide with a downregulation of differentiation-specific genes [Andres, 1998; Owens et al., 2004]. Our data showed that HIF-1α overexpression and $CoCl_2$ treatment have no effect on smooth muscle cell proliferation. We next assessed the phenotype of smooth muscle cells by examining the expression of differentiation-specific markers. In the presence of AdHIF-1α or $CoCl_2$, levels of smooth muscle α-actin, smooth muscle myosin heavy chain, and calponin did not change in comparison to untreated and AdGFP controls (Fig. 3A). This was the case in both high and low serum conditions. To confirm that our cultured cells were capable of typical modulatory behavior, we treated them with growth factors known to alter smooth muscle-specific gene expression [Owens et al., 2004]. As predicted, PDGF-BB caused a reduction, and TGF-β_1 augmented the expression of smooth muscle differentiation markers in both BASMCs and BCASMCs (Fig. 3B). These results indicate that HIF-1α expression and

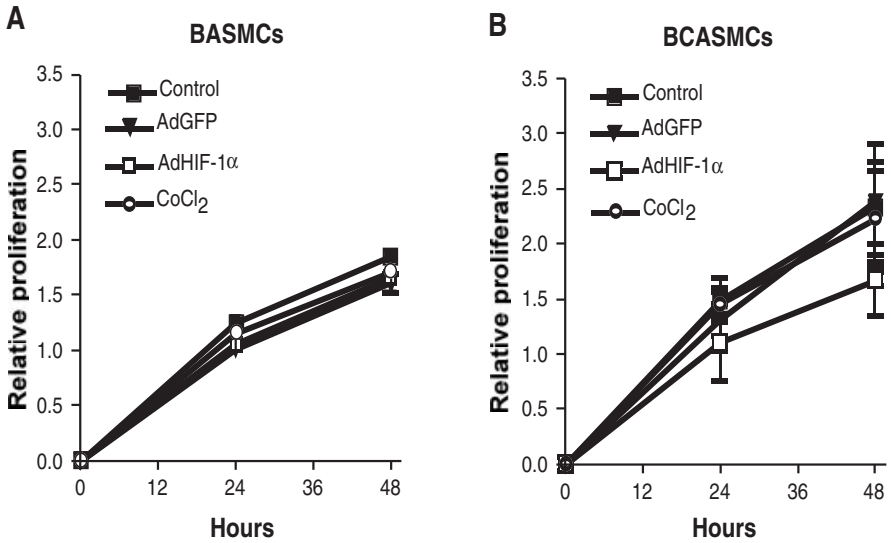

Fig. 2. Proliferation of smooth muscle cells. Proliferation rates were monitored over a 48-h time course to evaluate the effect of HIF-1α expression and $CoCl_2$ on smooth muscle cell division. There was no significant difference in proliferation indices of cells from each of the four conditions. Untreated cells (control), AdGFP-infected, AdHIF-1α-infected, and $CoCl_2$ treated cells showed comparable rates of proliferation in both BASMCs (**A**) and BCASMCs (**B**). Error bars represent ± SEM from three experiments performed in triplicate.

$CoCl_2$ do not appreciably alter the expression of smooth muscle differentiation genes. Taken together, our findings suggest that during hypoxia-induced angiogenesis, HIF-1 does not function to modify characteristic smooth muscle phenotypes associated with vascular remodeling.

The lack of smooth muscle modulation was surprising given that HIF-1 functions as a transcriptional activator. To determine if HIF-1α could activate known target genes in our smooth muscle cell cultures, we analyzed the expression of VEGF by RT-PCR. An increase in VEGF expression was detectable in low serum conditions (0.5%), within 24 h of HIF-1α infection or $CoCl_2$ treatment, but was absent in the presence of 10% serum (Fig. 4A,B). These results are consistent with previous data that reported hypoxia-dependent activation of VEGF was inhibited by serum [D'Angelo et al., 2000]. We additionally examined expression of the Met protooncogene, which is also known to be induced by hypoxia, and is upregulated in pVHL-deficient cells [Koochekpour et al., 1999; Oh et al., 2002; Pennacchietti et al., 2003]. Expression of Met protein was not increased in the HIF-1α-expressing and $CoCl_2$-treated cells in high or low serum conditions (Fig. 4C). Thus, these results show that overexpression of the HIF-1α subunit and $CoCl_2$ in smooth muscle cells can induce the hypoxia-related gene VEGF, but not the Met protooncogene.

HIF-1α and $CoCl_2$ Attenuate Smooth Muscle Cell Migration

The inability of HIF-1α to modulate proliferation and differentiation-specific gene expression in aortic and coronary artery smooth muscle cells suggests that it serves a more specialized function within these cells. HIF-1α mediates neovascularization by inducing endothelial cell sprouting, which relies on destabilization of the existing vessel to initiate formation of a new sprout [Carmeliet, 2000]. Given the need for a localized breakdown of the vessel wall, we hypothesized that HIF-1α might change the cell surface properties of smooth muscle cells. To test this, we first analyzed the ability of aortic and coronary artery smooth muscle cells to migrate using a wound assay, in which a confluent layer of smooth muscle cells was scratched with a pipet tip and allowed to migrate into the wounded area. In the presence of AdHIF-1α or $CoCl_2$, migration was dramatically attenuated in both BASMCs and BCASMCs (Fig. 5). Over a 24-h period, migration of cells treated with $CoCl_2$ was essentially abolished, while in the presence of AdHIF-1α some migration was observed. Analysis of GFP expression in the AdHIF-1α-infected cultures

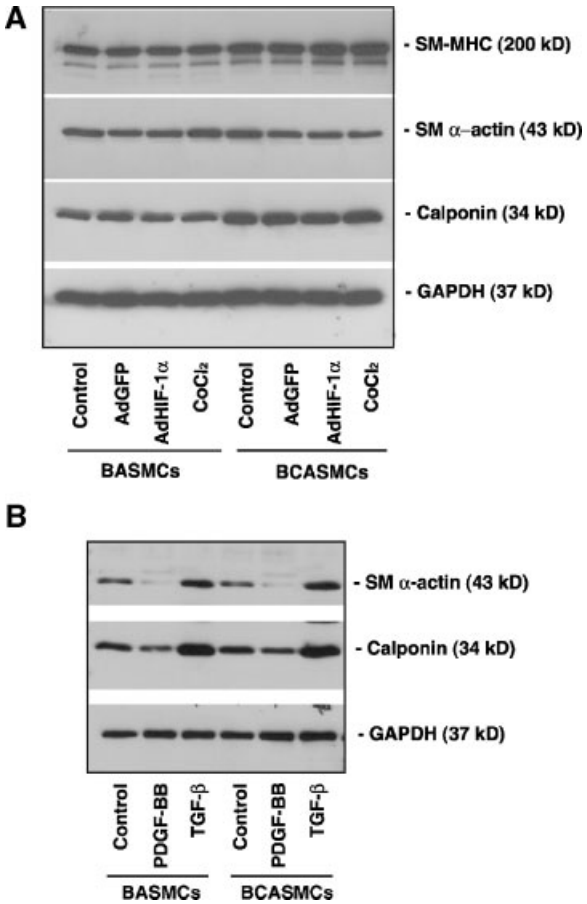

Fig. 3. Smooth muscle modulation. Western blots were used to evaluate changes in the expression of smooth muscle-specific proteins. (**A**) In the presence of HIF-1α and $CoCl_2$, the levels of differentiation-specific genes remained unchanged in BASMCs and BCASMCs, compared to untreated control and AdGFP. This is in marked contrast to the modulation achieved by growth factors PDGF-BB and TGF-β_1. (**B**) PDGF-BB diminishes and TGF-β_1 enhances expression of calponin and smooth muscle α-actin. GAPDH was used as a loading control.

revealed that the majority of cells within the wounded area did not express GFP, indicating these cells were not expressing HIF-1α (data not shown). The diminished migration of these cells cannot be attributed to a decrease in proliferation, as our results showed no significant difference in cell-cycle progression between the experimental and control groups (Fig. 2). We performed migration assays on cells cultured in 10% serum (Fig. 5) and in low serum (0.5%) (data not shown) with similar results. We further examined migration in the presence of hydroxyurea, which inhibits DNA synthesis and prevents cell division [Yarbro, 1992; Bundy et al., 1999]. Consistent with the previous results, HIF-1α-expressing and $CoCl_2$-treated cells exhibited reduced migration. These experiments demonstrate that HIF-1α, whether overexpressed or induced by $CoCl_2$, reduces the migratory abilities of smooth muscle cells.

Adhesion of Smooth Muscle Cells is Diminished by HIF-1α and $CoCl_2$

The decrease in smooth muscle cell migration suggested that HIF-1α was in some way altering the focal contacts that are necessary for attachment and propulsion. In an attempt to uncover the basis of this HIF-1α-dependent defect, we evaluated adhesion of these cells to extracellular matrices that are normally found in the basement membrane of the vascular wall [Moiseeva, 2001; Bou-Gharios et al., 2004]. We performed cell attachment assays to quantify adhesion to collagen I, collagen III, collagen IV, fibronectin, elastin and laminin, and found that cells expressing HIF-1α or in the presence of $CoCl_2$ were less adherent to each of the substrates (Fig. 6). The average reduction in adhesion ranged from 27% less in BASMCs to 38% less in BCASMCs compared to the two control groups (uninfected and AdGFP), which were statistically indistinguishable. Cell adhesion was not significantly different among the matrices, which precluded us from pinpointing a class of integrins that might be explicitly affected by HIF-1α and $CoCl_2$. Further, these data suggest that the defect in adhesion is due to a more general mechanism governing cell attachment. To our knowledge, these results represent the first report describing a direct effect of HIF-1α on adhesion and migration of smooth muscle cells. In the context of hypoxia-induced angiogenesis, we believe these findings have important implications regarding the mechanisms by which HIF-1 promotes blood vessel formation.

FAK Phosphorylation is Decreased by HIF-1α and $CoCl_2$

Cell attachment and migration are dependent upon integrin engagement and tyrosine phosphorylation of multiple proteins, which cluster at focal adhesion contacts [Panetti, 2002; Webb et al., 2002]. Proteins within the focal contacts serve as structural anchors and mediators of signal transduction. The decline in migration and adhesion induced by HIF-1α suggested that it might be altering the composition of these

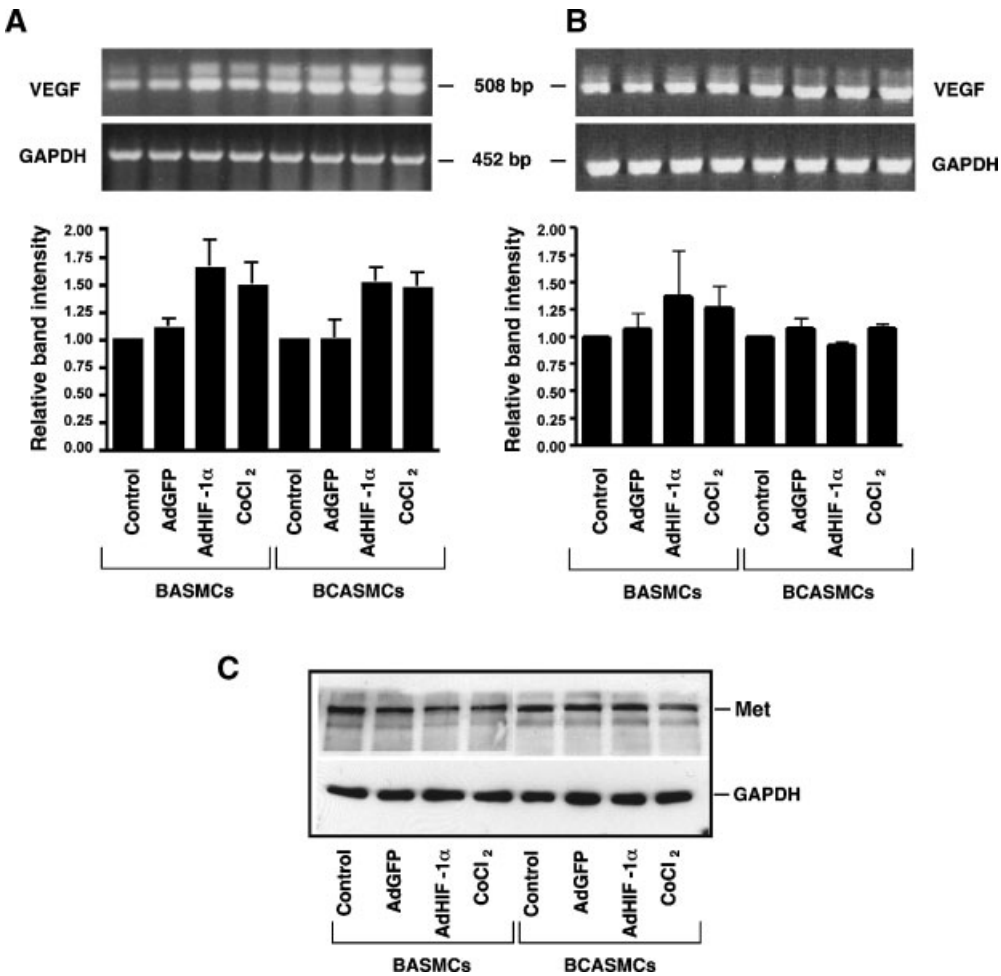

Fig. 4. **A**, **B**: RT-PCR to detect VEGF transcripts. To evaluate expression of VEGF in smooth muscle cells, semi-quantitative RT-PCR was performed. An increase in VEGF was observed in low serum conditions (0.5%) at 24 h post-infection with HIF-1α or $CoCl_2$ addition (A), but was not detected in high serum conditions (B). Graphs below picture reflect the average band intensity of three independent experiments (±SEM) normalized to GAPDH. (**C**) Western blot to detect the Met protooncogene demonstrates that Met is not increased in BASMCs and BCASMCs overexpressing HIF-1α or treated with $CoCl_2$. GAPDH served as a loading control.

focal adhesions. To examine this, we immunostained cells to detect proteins that localize to the focal contacts. Localization of the protein vinculin within the control groups (untreated and AdGFP), and experimental groups (AdHIF-1α, and $CoCl_2$-treated) showed no detectable differences in the number, size, or location of the focal attachments (Fig. 7). We additionally stained cells with an antibody that recognizes FAK, and similarly observed no difference in the cell attachment sites (data not shown). Western blot analysis confirmed that the expression levels of FAK and vinculin were not different between control and experimental samples (Fig. 8A). Although the analysis of focal adhesion proteins was in no way exhaustive, these experiments show that focal contacts in the presence of HIF-1α and $CoCl_2$ are quantitatively and structurally indistinguishable from controls.

The decrease in migration and adhesion of smooth muscle cells expressing HIF-1α or treated with $CoCl_2$ suggested focal adhesions were disrupted, however no obvious differences were observed in these structures. Because tyrosine phosphorylation plays a critical role in the regulation of cell motility and adhesion [Panetti, 2002], we sought to investigate the phosphorylation state of regulatory proteins found within sites of attachment. A key mediator of tyrosine phosphorylation at focal adhesions is FAK, which itself becomes phosphorylated during integrin-mediated binding of cells to the extracellular matrix [Gerthoffer and

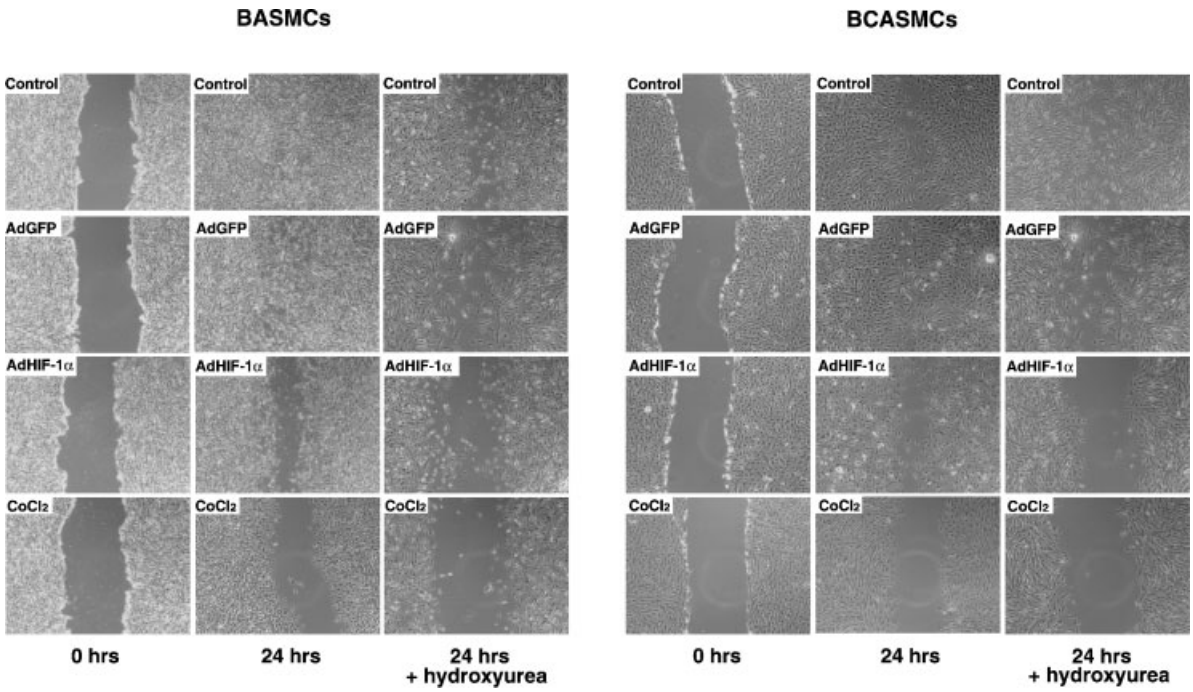

Fig. 5. Migration of smooth muscle cells is attenuated by HIF-1α and $CoCl_2$. Wound assays were used to evaluate the migration of cells overexpressing HIF-1α and exposed to $CoCl_2$. Cell monolayers were scraped with a pipet tip and monitored for migration into the wounded area. Pictures were captured at the time of initial wound (0 h) and 24 h later (24 h). In untreated cells (control) and those infected with AdGFP, the wounded area was completely filled in after 24 h. In contrast, cells infected with AdHIF-1α or treated with $CoCl_2$, the wounded area remained largely devoid of cells. In the presence of 2 mM hydroxyurea, which blocks cell division, similar results were observed, despite an overall slower rate of migration.

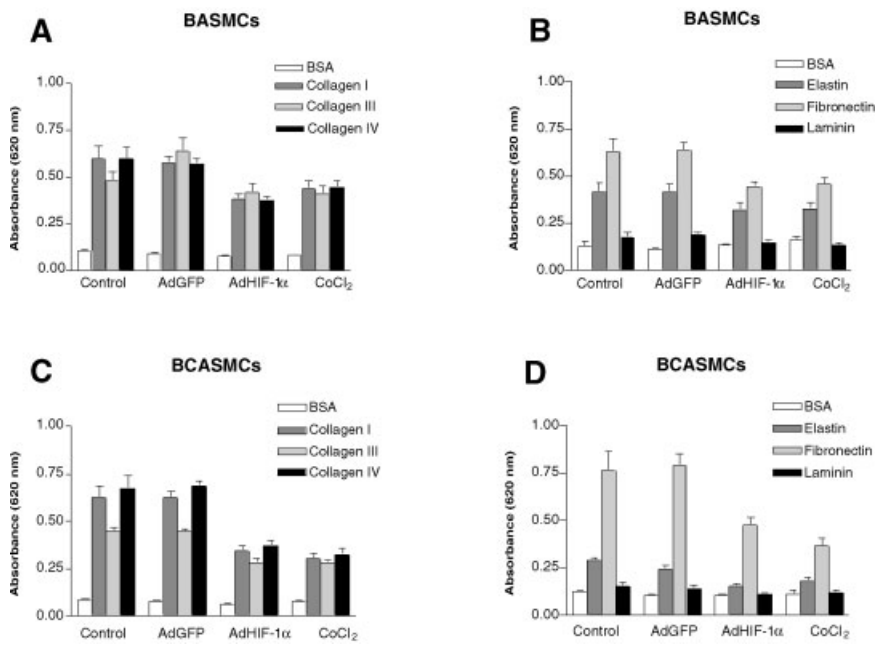

Fig. 6. HIF-1α and $CoCl_2$ diminish adhesion of smooth muscle cells to extracellular matrices. Attachment assays were performed to quantify cell adhesion to collagen I, collagen III, and collagen IV (**A**, **C**), and elastin, fibronectin, and laminin (**B**, **D**). In the presence of AdHIF-1α and $CoCl_2$, adhesion was comparably reduced in BASMCs (A, B) and BCASMCs (C, D). The average decline of adhesion observed in BASMCs was 27% less, and BCASMCs were 38% less adhesive. BSA served as a control for non-specific binding. Bars represent the average absorbance (±SEM) from four independent assays.

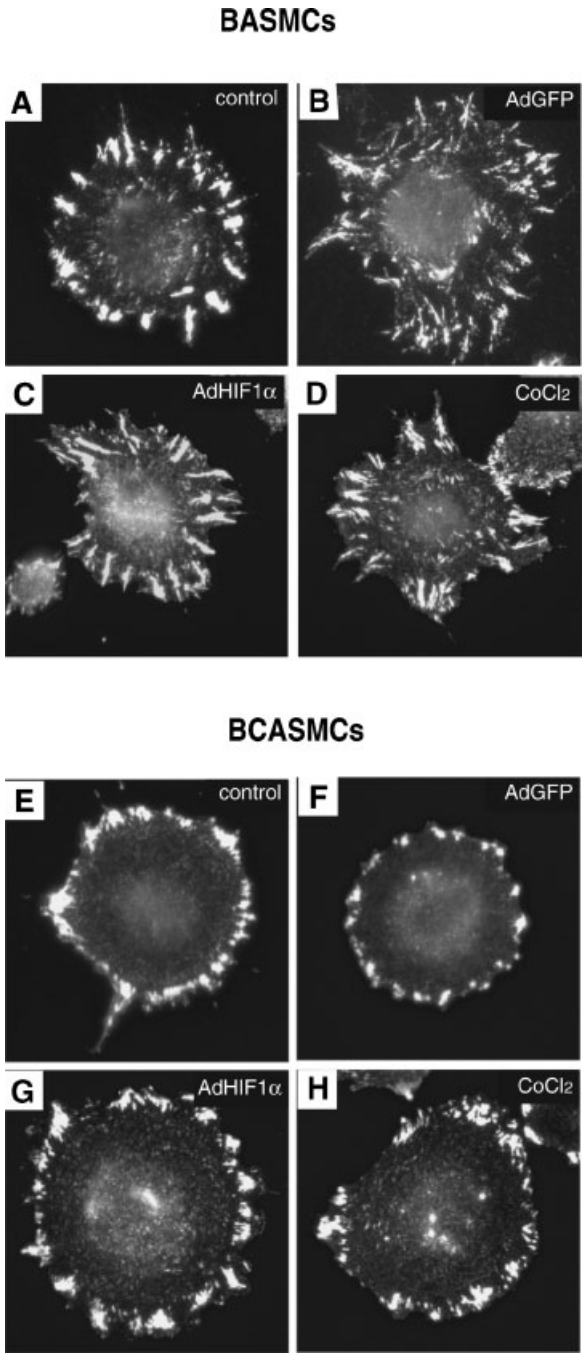

Fig. 7. Focal adhesions are structurally similar in the presence of HIF-1α and $CoCl_2$. Smooth muscle cells were plated on 10 μg/ml fibronectin and allowed to attach for 60 min prior to fixing for immunostaining. BASMCs (**A**–**D**) and BCASMCs (**E**–**H**) were immunostained to visualize focal adhesions using a vinculin antibody. Focal contacts in experimental (AdHIF-1α and $CoCl_2$) and control (untreated and AdGFP) groups were indistinguishable in the expression and localization of the vinculin protein. (A, E) untreated control, (B, F) AdGFP, (C, G) AdHIF-1α, (D, H) $CoCl_2$.

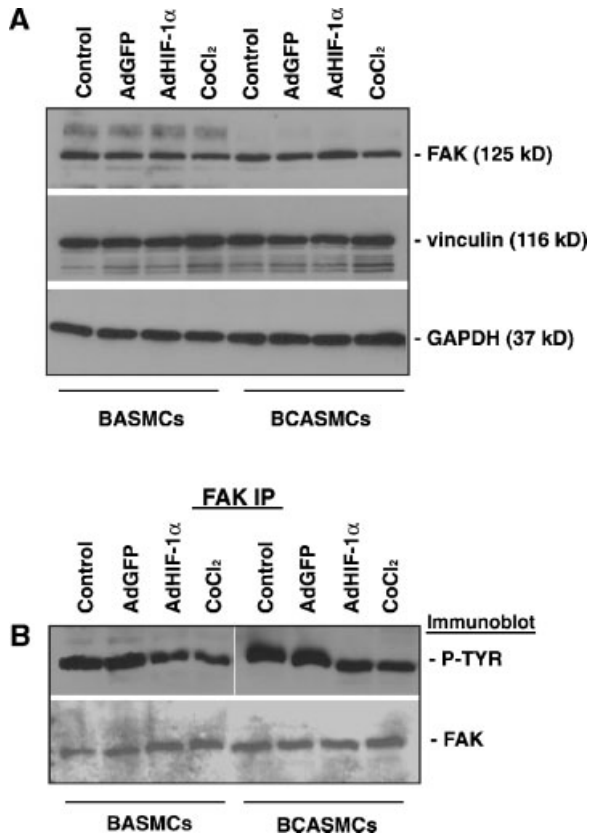

Fig. 8. HIF-1α and $CoCl_2$ decrease FAK phosphorylation in smooth muscle cells. (**A**) Western blot to detect FAK and vinculin reveals that expression of these focal adhesion proteins is not altered in the presence of AdHIF-1α and $CoCl_2$ in comparison to untreated (control) and AdGFP. GAPDH was used as a standard to demonstrate equivalent protein. (**B**) Immunopreciptiation of FAK to evaluate tyrosine phosphorylation. FAK was immunoprecipitated from cell extracts and immunoblotted for phosphotyrosine (P-TYR). The amount of tyrosine phosphorylated FAK was reduced in BASMCs and BCASMCs. FAK immunoblot demonstrates equal amounts of immunoprecipitation protein.

Gunst, 2001; Schlaepfer and Mitra, 2004]. To measure FAK phosphorylation, we immunoprecipitated FAK from smooth muscle cell extracts and performed an immunoblot with an antibody to detect phosphorylated tyrosine. Our data revealed that tyrosine phosphorylation of FAK was diminished in comparison to control samples (Fig. 8B). Both BASMCs and BCASMCs exhibited a reduction in FAK phosphorylation when overexpressing HIF-1α or when exposed to $CoCl_2$. These findings indicate that HIF-1α expression modulates the phosphorylation of FAK in bovine aortic and coronary artery smooth muscle cells, and suggest a mechanism through which HIF-1 governs adhesion and migration. These results represent the first direct link between HIF-1α expression and

modulation of adhesion, migration and FAK phosphorylation in smooth muscle cells.

DISCUSSION

HIF-1 is a major player in the induction of angiogenesis under hypoxic conditions, such as those associated with tumor growth and ischemic cardiovascular disease [Pugh and Ratcliffe, 2003; Semenza, 2003; Paul et al., 2004]. Although it is established that HIF-1α is activated by hypoxia in cells of the vasculature, the effect HIF-1 has on vascular remodeling remains largely unexplored. We investigated the role of HIF-1 in vascular smooth muscle cells to gain a better understanding of its direct function in angiogenesis. To do so, we examined the cellular properties of cultured aortic and coronary artery smooth muscle cells in response to overexpression of HIF-1α and by stabilization of the HIF-1α subunit with $CoCl_2$ treatment. In all experiments, the expression of HIF-1α and stimulation by $CoCl_2$ gave similar results, which served to substantiate our findings by demonstrating that the source of HIF-1α, whether exogenous or endogenous, was not an influential factor in the results. Both treatments also resulted in an increase in HIF-2α expression. While $CoCl_2$ acts to stabilize the HIF-2α subunit, overexpression of HIF-1α presumably serves to stabilize HIF-2α by overwhelming the degradation pathway. Thus, although results are focused on the role of HIF-1α, we cannot exclusively rule out that HIF-2α and/or pVHL deficiency may be a determining factor in these findings.

We predicted that coronary artery smooth muscle cells, which are prone to collateralization due to hypoxia [White et al., 1992], would have a more acute response to the effects of HIF-1α, yet we saw no dramatic difference in comparison to aortic smooth muscle cells. The most significant difference observed was in the adhesion assays in which the aortic smooth muscle cells were 27% less adhesive in the presence of increased HIF-1 levels, whereas the coronary smooth muscle cells exhibited a 38% decrease in adhesion. These data imply that coronary artery smooth muscle cells may be more responsive to a hypoxic signal, but fall short of providing convincing evidence. Possibly smooth muscle populations are not greatly different in their response to hypoxia, however we cannot dismiss the possibility that culture conditions might mask genuine differences that exist between these two subpopulations.

Smooth muscle is composed of dynamic cells that have the capacity to modulate between proliferative and differentiated states during vascular remodeling [Andres, 1998; Owens et al., 2004]. Because of their propensity to change phenotype, we evaluated whether HIF-1α could modulate proliferation or expression of smooth muscle differentiation genes. To our surprise, we found that HIF-1α and $CoCl_2$ had no effect on governing these smooth muscle properties. Previous studies have reported that HIF-1α expression is induced in smooth muscle cells by non-hypoxic stimuli [Richard et al., 2000; Gorlach et al., 2001; Page et al., 2002], including PDGF, thrombin, and angiotensin, all of which are known to the promote proliferation of smooth muscle cells [Casscells, 1991; Schieffer et al., 1997]. The significance of HIF-1α activation by these factors is unclear, however our data suggest that HIF-1α is not a mitigating factor in the proliferation promoted by these factors. Instead, our data support the conclusion that HIF-1α has no direct effect on modulating proliferation or differentiation-specific protein expression during vascular remodeling.

Studies of hypoxia-dependent migration and adhesion have largely been limited to circulating blood cells, in which adhesion and transendothelial migration have been shown to be increased in response to hypoxia [Friedman et al., 1998; Sultana et al., 1999; Weis et al., 2002; Ceradini et al., 2004]. In our experiments, we directly showed that attachment and migration of smooth muscle cells was attenuated by HIF-1α and $CoCl_2$. The decrease in adhesion and migration suggested that focal adhesions were altered in response to HIF-1α. A similar defect in migration was observed in fibroblasts derived from FAK null mice [Ilic et al., 1995]. However, in contrast to FAK null cells, we did not find observable differences in the number or size of the focal contacts, as assessed by vinculin staining. Therefore, the HIF-1α-dependent differences in adhesion and migration are likely due to alterations in signaling. Focal adhesion assembly and turnover are modulated by tyrosine phosphorylation, and a central component to the phosphorylation cascade is FAK [Panetti, 2002; Schlaepfer and Mitra, 2004]. Indeed, our findings showed that FAK phosphorylation is inhibited by HIF-1α and $CoCl_2$, confirming that signaling within these focal adhesions is

altered. These data establish a direct connection between HIF-1α expression and the regulation of adhesion, migration and FAK phosphorylation in vascular smooth muscle cells. Given that HIF-1 is a transcription factor, its ability to attenuate FAK phosphorylation must be through the activation of a FAK modulator. Prototypical FAK activators are integrins [Schlaepfer and Mitra, 2004], however from our adhesion data we could not distinguish a class of integrins that were specifically affected based on selective binding to extracellular matrices. Additional proteins within the focal adhesion scaffold can also modulate FAK activity [Panetti, 2002; Schlaepfer and Mitra, 2004]. Thus, the expression of one or more of these mediators may function to suppress FAK phosphorylation. VEGF and Met are two factors known to be induced by hypoxia [Forsythe et al., 1996; Pennacchietti et al., 2003], and have additionally been linked to FAK activation [Beviglia and Kramer, 1999; Eliceiri et al., 2002; Maulik et al., 2002; Le Boeuf et al., 2004]. Interestingly, while VEGF transcripts were upregulated in low serum conditions, neither VEGF nor Met exhibited increased expression in smooth muscle cells cultured in high serum. The ability of HIF-1α and $CoCl_2$ to cause a decrease in migration, adhesion, and FAK phosphorylation was the same whether cells were cultured in high or low serum. Consequently, although serum concentration may have an effect on HIF-1α-induced gene expression, it did not influence the cell attachment processes that we examined. Furthermore, the absence of robust activation of VEGF and Met suggests that smooth muscle cells might be wired to respond to hypoxia much differently than endothelial or cancer cells. Undoubtedly, the events that occur between HIF-1 transactivation and the phosphorylation of FAK are of considerable interest for future studies.

What is the significance of these findings with respect to clarifying the role of HIF-1 in hypoxia? Under hypoxic conditions, such as myocardial ischemia, HIF-1 acts as a primary responder by initiating angiogenesis though the induction of VEGF, which promotes endothelial cell sprouting [Semenza, 2003]. In the early phases of angiogenesis, the vessel wall becomes destabilized to permit emergence of the nascent vessel, which is dependent upon the loosening of the surrounding smooth muscle cells [Carmeliet, 2000]. If the role of HIF-1 is to initiate angiogenesis, then decreasing vascular smooth muscle cell attachments seems a likely strategy to facilitate the breakdown of the intact vessel. After the new vessel is formed, additional angiogenic factors may trigger smooth muscle cell proliferation and enhance migration to contribute to vessel maturation. Despite the uncertainty of HIF-1α's precise function in hypoxia-induced angiogenesis, our data provides evidence for a mechanism in which HIF-1α governs smooth muscle cell attachment and propulsion by modulating focal adhesion activity through FAK phosphorylation.

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REFERENCES

Andres V. 1998. Control of vascular smooth muscle cell growth and its implication in atherosclerosis and restenosis (review). Int J Mol Med 2:81–89.

Ausubel FM. 1995. Current protocols in molecular biology: Laboratory manual John Wiley and Sons, Inc.

Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. 1994. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: Implications for coronary angiogenesis. Cardiovasc Res 28:1176–1179.

Benjamin LE, Hemo I, Keshet E. 1998. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125: 1591–1598.

Beviglia L, Kramer RH. 1999. HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells. Int J Cancer 83:640–649.

Bou-Gharios G, Ponticos M, Rajkumar V, Abraham D. 2004. Extra-cellular matrix in vascular networks. Cell Prolif 37:207–220.

Bundy R, Marczin N, Chester AH, Yacoub M. 1999. Differential regulation of DNA synthesis by nitric oxide and hydroxyurea in vascular smooth muscle cells. Am J Physiol 277:H1799–H807.

Carmeliet P. 2000. Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395.

Carmeliet P, Jain RK. 2000. Angiogenesis in cancer and other diseases. Nature 407:249–257.

Casscells W. 1991. Smooth muscle cell growth factors. Prog Growth Factor Res 3:177–206.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD,

Levine JP, Gurtner GC. 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10:858–864.

D'Angelo G, Ladoux A, Frelin C. 2000. Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. Biochem Biophys Res Commun 267:334–338.

Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, Fukumura D, Monsky W, Claffey KP, Jain RK. 1998. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. J Invest Dermatol 111:1–6.

Eliceiri BP, Puente XS, Hood JD, Stupack DG, Schlaepfer DD, Huang XZ, Sheppard D, Cheresh DA. 2002. Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. J Cell Biol 157:149–160.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604–4613.

Friedman GB, Taylor CT, Parkos CA, Colgan SP. 1998. Epithelial permeability induced by neutrophil transmigration is potentiated by hypoxia: Role of intracellular cAMP. J Cell Physiol 176:76–84.

Gardner LB, Li Q, Park MS, Flanagan WM, Semenza GL, Dang CV. 2001. Hypoxia inhibits G1/S transition through regulation of p27 expression. J Biol Chem 276:7919–7926.

Gerthoffer WT, Gunst SJ. 2001. Invited review: Focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. J Appl Physiol 91:963–972.

Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS. 2003. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol 23:359–369.

Gorlach A, Diebold I, Schini-Kerth VB, Berchner-Pfannschmidt U, Roth U, Brandes RP, Kietzmann T, Busse R. 2001. Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. Circ Res 89:47–54.

Harlow E, Lane D. 1999. Using antibodies: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. 1998. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 95:2509–2514.

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377:539–544.

Kanaya K, Kamitani T. 2003. pVHL-independent ubiquitination of HIF1alpha and its stabilization by cobalt ion. Biochem Biophys Res Commun 306:750–755.

Kong T, Eltzschig HK, Karhausen J, Colgan SP, Shelley CS. 2004. Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of beta2 integrin gene expression. Proc Natl Acad Sci USA 101:10440–10445.

Koochekpour S, Jeffers M, Wang PH, Gong C, Taylor GA, Roessler LM, Stearman R, Vasselli JR, Stetler-Stevenson WG, Kaelin WG, Jr., Linehan WM, Klausner RD, Gnarra JR, Vande Woude GF. 1999. The von Hippel-Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. Mol Cell Biol 19:5902–5912.

Larcher F, Murillas R, Bolontrade M, Conti CJ, Jorcano JL. 1998. VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. Oncogene 17:303–311.

Le Boeuf F, Houle F, Huot J. 2004. Regulation of vascular endothelial growth factor receptor 2-mediated phosphorylation of focal adhesion kinase by heat shock protein 90 and Src kinase activities. J Biol Chem 279:39175–39185.

Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. 2000. VEGF gene delivery to myocardium: Deleterious effects of unregulated expression. Circulation 102:898–901.

Maulik G, Kijima T, Ma PC, Ghosh SK, Lin J, Shapiro GI, Schaefer E, Tibaldi E, Johnson BE, Salgia R. 2002. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res 8:620–627.

Maxwell P, Salnikow K. 2004. HIF-1: An oxygen and metal responsive transcription factor. Cancer Biol Ther 3: 29–35.

Moiseeva EP. 2001. Adhesion receptors of vascular smooth muscle cells and their functions. Cardiovasc Res 52:372–386.

Neville C, Rosenthal N, Hauschka S. 1998. DNA transfection of cultured muscle cells: "Methods in cell biology." San Diego: Academic Press.

Oh RR, Park JY, Lee JH, Shin MS, Kim HS, Lee SK, Kim YS, Lee SH, Lee SN, Yang YM, Yoo NJ, Lee JY, Park WS. 2002. Expression of HGF/SF and Met protein is associated with genetic alterations of VHL gene in primary renal cell carcinomas. Apmis 110:229–238.

Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ. 1989. A rapid and convenient assay for counting cells cultured in microwell plates: Application for assessment of growth factors. J Cell Sci 92(Pt 3):513–518.

Owens GK, Kumar MS, Wamhoff BR. 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84:767–801.

Page EL, Robitaille GA, Pouyssegur J, Richard DE. 2002. Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. J Biol Chem 277:48403–48409.

Panetti TS. 2002. Tyrosine phosphorylation of paxillin, FAK, and p130CAS: Effects on cell spreading and migration. Front Biosci 7:d143–d150.

Paul SA, Simons JW, Mabjeesh NJ. 2004. HIF at the crossroads between ischemia and carcinogenesis. J Cell Physiol 200:20–30.

Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. 2003. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3:347–361.

Pugh CW, Ratcliffe PJ. 2003. Regulation of angiogenesis by hypoxia: Role of the HIF system. Nat Med 9:677–684.

Richard DE, Berra E, Pouyssegur J. 2000. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. J Biol Chem 275:26765–26771.

Rose F, Grimminger F, Appel J, Heller M, Pies V, Weissmann N, Fink L, Schmidt S, Krick S, Camenisch G, Gassmann M, Seeger W, Hanze J. 2002. Hypoxic pulmonary artery fibroblasts trigger proliferation of vascular smooth muscle cells: Role of hypoxia-inducible transcription factors. FASEB J 16:1660–1661.

Sabia PJ, Powers ER, Ragosta M, Sarembock IJ, Burwell LR, Kaul S. 1992. An association between collateral blood flow and myocardial viability in patients with recent myocardial infarction. N Engl J Med 327:1825–1831.

Schieffer B, Drexler H, Ling BN, Marrero MB. 1997. G protein-coupled receptors control vascular smooth muscle cell proliferation via pp60c-src and p21ras. Am J Physiol 272:C2019–C2030.

Schlaepfer DD, Mitra SK. 2004. Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev 14:92–101.

Semenza GL. 2000. HIF-1 and human disease: One highly involved factor. Genes Dev 14:1983–1991.

Semenza GL. 2002. HIF-1 and tumor progression: Pathophysiology and therapeutics. Trends Mol Med 8:S62–S67.

Semenza GL. 2003. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med 54:17–28.

Semenza GL, Wang GL. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12:5447–5454.

Spector D, Goldman R, Leinwand L. 1998. Cells: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sultana C, Shen Y, Johnson C, Kalra VK. 1999. Cobalt chloride-induced signaling in endothelium leading to the augmented adherence of sickle red blood cells and transendothelial migration of monocyte-like HL-60 cells is blocked by PAF-receptor antagonist. J Cell Physiol 179:67–78.

Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS. 2004. Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell 6: 485–495.

Tscheudschilsuren G, Aust G, Nieber K, Schilling N, Spanel-Borowski K. 2002. Microvascular endothelial cells differ in basal and hypoxia-regulated expression of angiogenic factors and their receptors. Microvasc Res 63:243–251.

Webb DJ, Parsons JT, Horwitz AF. 2002. Adhesion assembly, disassembly and turnover in migrating cells—over and over and over again. Nat Cell Biol 4:E97–E100.

Weis M, Schlichting CL, Engleman EG, Cooke JP. 2002. Endothelial determinants of dendritic cell adhesion and migration: New implications for vascular diseases. Arterioscler Thromb Vasc Biol 22:1817–1823.

White FC, Carroll SM, Magnet A, Bloor CM. 1992. Coronary collateral development in swine after coronary artery occlusion. Circ Res 71:1490–1500.

Xia YP, Li B, Hylton D, Detmar M, Yancopoulos GD, Rudge JS. 2003. Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. Blood 102:161–168.

Xu W, Baribault H, Adamson ED. 1998. Vinculin knockout results in heart and brain defects during embryonic development. Development 125:327–337.

Yamakawa M, Liu LX, Date T, Belanger AJ, Vincent KA, Akita GY, Kuriyama T, Cheng SH, Gregory RJ, Jiang C. 2003. Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. Circ Res 93:664–673.

Yarbro JW. 1992. Mechanism of action of hydroxyurea. Semin Oncol 19:1–10.

Yuan Y, Hilliard G, Ferguson T, Millhorn DE. 2003. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem 278:15911–15916.