Loss of HIF-1α in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis

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Summary

We deleted the hypoxia-responsive transcription factor HIF-1α in endothelial cells (EC) to determine its role during neovascularization. We found that loss of HIF-1α inhibits a number of important parameters of EC behavior during angiogenesis: these include proliferation, chemotaxis, extracellular matrix penetration, and wound healing. Most strikingly, loss of HIF-1α in EC results in a profound inhibition of blood vessel growth in solid tumors. These phenomena are all linked to a decreased level of VEGF expression and loss of autocrine response of VEGFR-2 in HIF-1α null EC. We thus show that a HIF-1α-driven, VEGF-mediated autocrine loop in EC is an essential component of solid tumor angiogenesis.

Introduction

Angiogenesis, the formation of new capillaries from existing blood vessels, is required for processes ranging from placental invasion to organogenesis during embryogenesis and from wound healing to endometrial growth in adults (Folkman and Klagsbrun, 1987). It is also an essential aspect of a number of pathologies involving new tissue growth, including the growth of solid tumors, which requires angiogenesis for expansion and metastasis (Folkman, 1996).

Angiogenesis can be stimulated by lowered physiological oxygen, or hypoxia. Hypoxia in wound healing, during cardiovascular and cerebral ischemia, and in solid tumor formation induces the increased expression of a number of factors key to mobilizing endothelial cell chemotaxis and proliferation (Pugh and Ratcliffe, 2003; Semenza, 2001). The hypoxia-inducible transcription factor-1 (HIF-1) acts to control induced expression of many, if not most, hypoxia-inducible genes, and it is clear that HIF-1 is essential for hypoxia-induced increases in glycolysis and angiogenesis in tumor cells, as well as normal tissues (Griffiths et al., 2002; Iyer et al., 1998; Ryan et al., 1998).

The vascular endothelial growth factor (VEGF) is an essential regulator of vascularization (Ferrara and Gerber, 2001). VEGF acts on endothelial cells as both a chemotactic and mitogenic factor, via endothelial cell-specific receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4) (Ferrara et al., 2003). VEGFR-2 is a major mediator of mitogenic, angiogenic, and permeability-enhancing effects of VEGF (Gille et al., 2001; Shalaby et al., 1995). The precise function of VEGFR-1 in angiogenesis is still under debate (Ferrara et al., 2003; Shibuya, 2001). VEGFR-1 has been suggested to be a negative regulator of VEGF action during early embryonic development (Fong et al., 1999) but has been indicated as a regulator of VEGF action in adult animals (Gabhann and Popel, 2004; Hiratsuka et al., 2001). VEGFR-3 is primarily expressed in lymphatic endothelial cells and is thought to be primarily a regulator of lymphatic angiogenesis (Jussila and Alitalo, 2002). The expression of VEGFR-1 and VEGFR-2 are upregulated by hypoxia; VEGFR-1 is directly upregulated by hypoxia, via a HIF binding enhancer element located in the VEGFR-1 promoter, while the upregulation of VEGFR-2 by hypoxia is mediated by posttranscriptional regulation (Gerber et al., 1997; Waltenberger et al., 1996).

These data indicate that endothelial cells have the potential to tightly regulate their hypoxic responses in coordination with changes in the oxygenation of surrounding tissues. What remains undefined is the role of specific interactions between hypoxic response, HIF-1 activation, VEGF expression, and VEGFR-2 response in vivo, particularly during tumorigenesis.

There are clearly many potential aspects of endothelial cell function that could require hypoxic response through the HIF-1

SIGNIFICANCE

Antiangiogenic therapies for the treatment of cancer have as a target the process of growth factor-induced neovascularization. This process involves the interaction of malignant cells and nontransformed endothelium. The microenvironment of the solid tumor is often hypoxic, and the hypoxia-induced changes in expression of angiogenic factors in cancer cells have been shown to be critical for the process of tumorigenesis. We show here that the hypoxic response of endothelial cells is an equally important component of tumor angiogenesis and is as well a potential novel target for cell-directed cancer therapies, due to the involvement of a hypoxia- and HIF-1α-dependent endothelial cell-specific autocrine loop.

pathway; a recent study suggested that hypoxia-inducible gene expression and migration were critically dependent on HIF-1α but not HIF-2α in EC (Sowter et al., 2003). But it is still unclear how and to what extent this pathway regulates EC in vivo and how the importance of that response stands in contrast to the hypoxic response from the cells and tissues in which the vasculature is located. This is a pressing issue in tumor formation, in that it is not clear how tissues interact with EC during hypoxia to mobilize and accomplish neovascularization.

We have specifically removed HIF-1α from endothelial cells and show here that angiogeneis in vivo is impaired by loss of HIF-1α in EC, demonstrating that HIF-1α is critical for EC function during neovascularization. We demonstrate that this impairment is a direct result of the disruption of an autocrine loop, acting through HIF-1α regulation of hypoxia-induced VEGF expression.

Results

HIF-1α is required for hypoxia-inducible gene transcription in murine endothelial cells

To determine if HIF-1α is expressed in primary EC in a hypoxia-dependent manner, EC from conditionally targeted mice were purified from lungs and then cultured under normoxic, hypoxic, and anoxic conditions; 72 hr prior to hypoxic culture, cells were infected with Cre recombinase-expressing adenovirus to induce deletion of the conditional alleles of HIF-1α; wild-type cells were infected with a control adenovirus. Nuclear and cytosolic extracts were assayed for HIF-1α protein expression: as shown in Figure 1A, only nonspecific signals and not HIF-1α protein can be detected in cytosolic extracts, and in EC, there was only a weak induction of HIF-1α protein during hypoxic culture (0.5% O_2). Much more nuclear induction of HIF-1α was observed in response to culture under anoxic conditions in wild-type cells (Figure 1A). The expression of HIF-2α protein was checked in nuclear and cytosolic extracts of EC. As shown in Figure 1A, we found that HIF-2α protein was localized primarily to the cytoplasm. HIF-2α protein expression levels in EC are not different regardless of the presence of HIF-1α. There is no detected HIF-2α protein expression in nuclear extracts of EC.

It has been shown that HIF-1α is ubiquitously transcribed but that the primary site of mRNA expression of the highly related transcription factor HIF-2α is the endothelium (Ema et al., 1997; Flamme et al., 1997; Jain et al., 1998; Tian et al., 1997). To determine the level of redundancy of HIF-1α in EC and to determine if HIF-1α alone is essential for hypoxia-induced transcription in primary endothelial cells, the expression levels of three known HIF-1 targets were assayed in these cells: the glycolytic enzyme phospoglycerate kinase *PGK*, the glucose transporter *GLUT1*, and *VEGF* following purification of endothelial cells from lungs of conditionally targeted animals (purification levels shown in Figure 1B). The mRNA expression levels were compared in primary cultures of wild-type and *HIF-1*$\alpha^{-/-}$ EC, cultured at normoxia or hypoxia. The hypoxia-inducible expression of *PGK, GLUT1*, and total *VEGF* was reduced significantly by loss of HIF-1α (Figure 1C) in a manner similar to that seen in other tissues without HIF-1α (Seagroves et al., 2001). This indicates that HIF-2α and/or other hypoxia-induced factors cannot act in such a way as to make HIF-1α completely redundant in endothelium.

Intracellular ATP levels are regulated by HIF-1α

To determine the role of HIF-1α in regulation of EC energy metabolism, we assayed ATP production by primary EC. After the EC were cultured for 24 hr under normoxia or hypoxia, whole-cell extracts were prepared to compare the levels of free ATP in the cultured cells. The amount of free ATP produced by each cell line was normalized for protein levels within each whole-cell lysate. As shown in Figure 1D, the total amounts of free ATP produced during hypoxia were dependent on the HIF-1α status of the EC. Interestingly, under hypoxic conditions, free ATP increased about 1.6-fold in wild-type EC, while in null EC the level of free ATP was slightly decreased. The reduction of cellular ATP in the HIF-1α null EC indicates that HIF-1α activity helps to maintain intracellular ATP at wild-type levels in endothelial cells under these hypoxic, albeit normoglycemic, conditions.

Conditional deletion of HIF-1α in vivo by bone marrow transplantation

We created a conditional deletion of the HIF-1α transcription factor in endothelial cells in vivo via crosses into a background of cre recombinase expression driven by the Tie2 promoter, which allows specific deletion in the endothelium (Kisanuki et al., 2001). It has been shown that the Tie2-cre transgenic mouse line facilitates cre recombinase expression in endothelial cells and in hematopoietic cells (Constien et al., 2001). Here we also found high efficiency of recombination (approximately 98%) in cells from the bone marrow isolated from Tie2 cre/HIF-1α conditionally null mice (Figure 1F, gray bar). To create endothelial cell-specific HIF-1α null mice, we performed bone marrow reconstitution to rescue the deletion of the HIF-1α gene in hematopoietic cells (Figure 1E). More than 80% of the hematopoietic cells of EC null mice were then recovered as wild-type (Figure 1F, white bar).

Defective VEGF-induced migration and proliferation in matrigel in vivo

During angiogenesis, EC differentiate and proliferate in situ within a previously avascular tissue and then coalesce to form a primitive tubular network. To analyze the effects of the conditional loss of HIF-1α function during angiogenesis in vivo, we applied a murine matrigel plug assay to HIF-1$\alpha^{+f/+f}$ (wild-type) mice and HIF-1$\alpha^{+f/+f}$ Tie2-cre (null) mice (Kisanuki et al., 2001). This is a well-established in vivo angiogenesis model; the level of angiogenesis is typically viewed by embedding and sectioning the plugs in paraffin and staining using Masson's Trichrome (Passaniti et al., 1992). Matrigel plugs containing recombinant murine VEGF at 20 ng/ml were implanted subcutaneously in the flanks of bone marrow-rescued Tie2-cre/HIF-1α conditional mice (EC null) and controls; 8 days after implantation, the matrigel plugs were removed and analyzed. The matrigel plugs removed from wild-type mice (Figure 2A) were yellowish, an initial gross indication of significant angiogenesis. Masson's trichrome staining showed that there were considerable numbers of vessels in the plugs (Figure 2C). The plugs removed from HIF-1$\alpha^{+f/+f}$ EC null mice were relatively colorless (Figure 2B). At higher magnification, it was clear that the vessels in matrigel plugs contained erythrocytes, indicating that they had formed functional capillaries (black arrows in Figures 2E and 2F). When viewed by Masson's trichrome staining, there was a 50% deduction in vessel density by Image J analysis, and in addition, most of the vessels only migrated to the edge of the

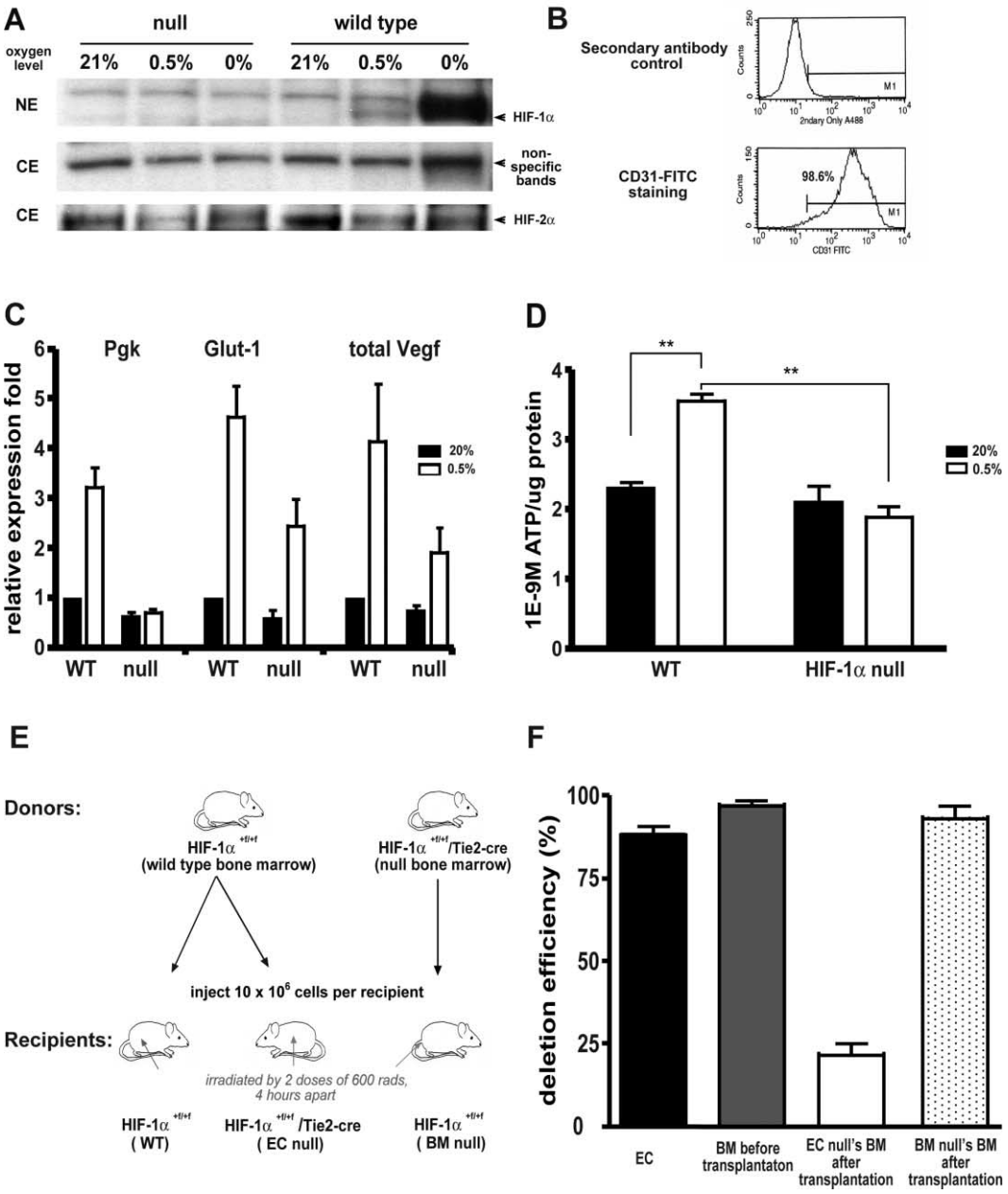

Figure 1. Microvascular endothelial cells contain functional HIF-1

A: Top: HIF-1α protein was slightly induced in the nuclear extracts of the purified wild-type EC upon exposure to 0.5% oxygen level. Under anoxic conditions, HIF-1α was induced to a greater extent. Middle: Only nonspecific bands, but not HIF-1α protein, could be detected in the cytosolic extracts of wt and null EC. Bottom: HIF-2α protein could be detected in the cytosolic extracts of wt and null mEC regardless of oxygenation. NE, nulear extracts; CE, cytosolic extracts.

B: FACScan analysis of the purity of the isolated EC: about 98.6% cells were EC by a CD31 marker.

C: Purified EC were cultured under either 20% (black bars) or 0.5% (white bars) oxygen levels for 8 hr, harvested, and expression of target genes was normalized to 18S rRNA. After normalization, the relative expression of each gene was expressed as a percentage of that observed in wild-type cells at normoxia (mean ± SD). No significant differences in gene expression were observed for any gene between HIF-1α wild-type (wt, HIF-1$\alpha^{+f/+f}$ infected with Adeno-βgal) and null cells (null, HIF-1$\alpha^{+f/+f}$ infected with Adeno-cre) cultured under 20% oxygen conditions, but under 0.5% oxygen, induction of all three mRNAs in HIF-1α null cells was decreased by at least 50%.

D: Wild-type (wt) and HIF-1α null (null) primary EC were cultured under 20% (black bars) or 0.5% (white bars) oxygen conditions for 24 hr. Then the cells were harvested and intracellular ATP concentrations were measured by means of a luciferase-based chemiluminescent assay. Statistical analysis was performed using the unpaired Student's test, **$p < 0.01$.

E: The scheme of donor and recipient groups in the bone marrow transplantation experiment. BM, bone marrow.

F: Efficiency of HIF-1α deletion in EC and bone marrow cells (BM cells). Genomic DNA was prepared from EC (black bar), BM cells (gray bar) of HIF-1$\alpha^{+f/+f}$/Tie2-cre mice before the bone marrow transplantation, BM cells (white bar) of HIF-1$\alpha^{+f/+f}$/Tie2-cre mice after transplanted with HIF-1$\alpha^{+f/+f}$ bone marrow, and BM cells (dotted bar) of HIF-1$\alpha^{+f/+f}$ mice after transplanted with HIF-1$\alpha^{+f/+f}$/Tie2-cre bone marrow.

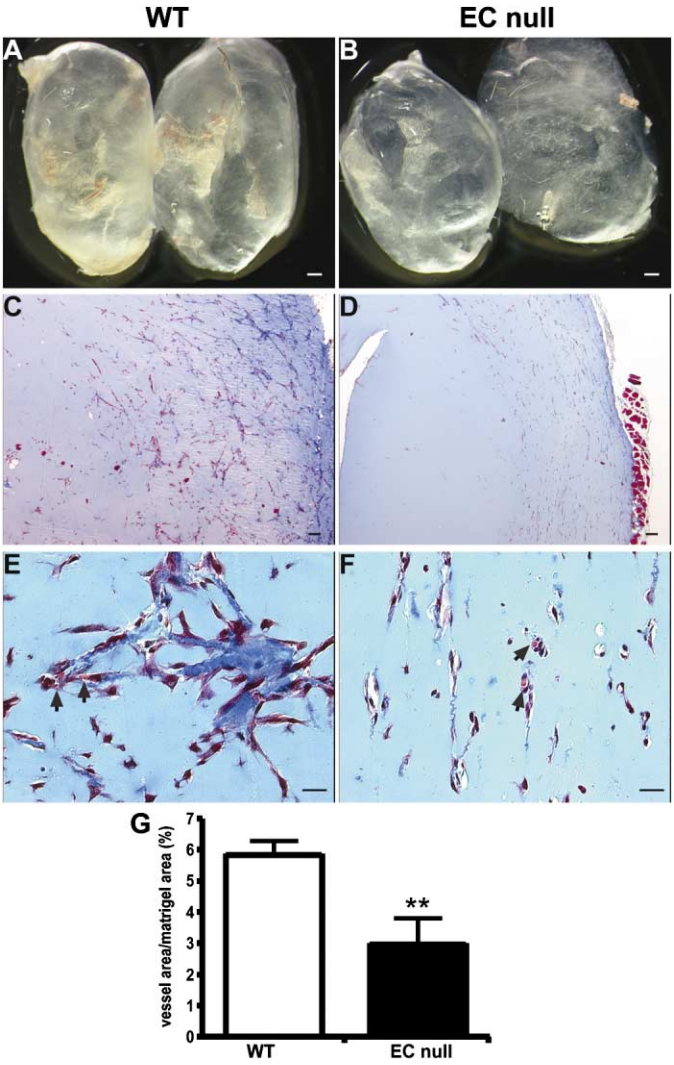

Figure 2. VEGF-induced angiogenesis in murine matrigel model is defective in HIF-1$\alpha^{+f/+f}$/Tie2-cre EC null mice

A and B: VEGF-permeated matrigel plugs from wild-type (wt) (**A**) are grossly more obviously vascularized than those from HIF-1α EC null (**B**) mice. Scale bar equals 500 μm.

C and D: Masson trichrome-stained sections of matrigel plugs. Scale bar equals 100 μm.

E and F: Sections from wt (**E**) and HIF-1α EC null (**F**) mice were individually imaged at higher power to highlight the vessel structure in the matrigel plugs. The black arrows indicate that these vessels contain erythrocytes. Scale bar equals 25 μm.

G: Mean vessel density in matrigel plugs from wt (white bar) mice and HIF-1α EC null (black bar) mice was determined with Image J software. Error bars indicate standard deviation (SD). Statistical analysis was performed using the unpaired Student's test, $^{**}p < 0.01$.

matrigel plugs (Figures 2D and 2G). These results demonstrate that loss of HIF-1α in endothelial cells disrupts VEGF-dependent signaling pathways in vivo.

HIF-1α EC null mice exhibit delayed wound healing

New microvascular network formation is a critical component of wound healing (Ferrara, 2002; Tonnesen et al., 2000). Numerous new capillaries carry oxygen and nutrients to sustain the metabolism that is needed during the healing process (Tonnesen et al., 2000). During wound healing, endothelial sprouts are induced by angiogenic factors; these sprouts then migrate into avascular zones to reestablish connection with the circulation (Tonnesen et al., 2000). EC response to hypoxia is thus likely one of the key responses in the process of wound healing. To determine the amount of hypoxia in early stages of cutaneous wound healing, the hypoxic areas of the wounds were visualized in pimonidazole-treated mice using the hypoxyprobe-1 monoclonal antibody. As can be seen in Figure 3A, there is a significant degree of hypoxia at the wound margin in experimental wounds (brown staining at wound margin).

To determine rates of wound closure, we performed three 6 mm circular punch biopsies on the back skin of each of ten HIF-1α wild-type and HIF-1α EC null littermates after recovery from bone marrow transplantation, and we measured wound closure daily over a 12-day period. As shown in Figure 3B, wound healing was significantly delayed in the HIF-1$\alpha^{+f/+f}$/Tie2-cre EC null mice compared with wild-type littermates ($^{*}p < 0.05$, $^{**}p < 0.01$ at day 3, 4, 5, 6). Histological changes in early stage wounds were examined by H&E staining. At day 6 postinjury, the epidermis of wild-type wounds deeply migrated under the fibrin clot (Figure 3C), while the leading epidermal edge of EC null wounds did not clearly invade into the fibrin clot and remained at the wound margin at this stage of healing (Figure 3D).

At very early stages of wound healing, new capillary sprouts develop from dilated vessels adjacent to the wounds and invade to the wound clot (McClain et al., 1996; Singer and Clark, 1999). To determine the amount of capillary formation in wound healing, we detected endothelial sprouting by immunostaining. At day 3 postinjury, new capillary sprouts of wild-type wounds emanated from the dilated vessels adjacent to the wounds (Figure 3E). In the wounds of HIF-1$\alpha^{+f/+f}$/Tie2-cre EC null mice, there were fewer new capillaries emanating from tissues adjacent to the wounds (Figures 3F and 3G, graph). These assays demonstrate that loss of HIF-1α in endothelial cells results in abnormal angiogenesis of wounds, which leads in turn to a delay in healing.

Reduced tumor size and severely necrotic tumors in HIF-1α EC null mice

Tumors typically initiate as small avascular masses and can develop a severely hypoxic microenvironment; this subsequently can act to help induce the angiogenesis that is required to allow further tumor growth (Folkman, 1971, 1996; Hanahan and Folkman, 1996; Thomlinson and Gray, 1955). The regulation of angiogenesis by hypoxia is an important link between vascular oxygen supply and tumor metabolic demand. We performed a standard assay of tumorigenesis to determine whether the defects in null EC would affect tumor growth. Lewis lung carcinoma cells will grow in immunocompetent C57Bl6 mice (O'Reilly et al., 1997), and so were injected subcutaneously into the HIF-1α wild-type and HIF-1$\alpha^{+f/+f}$/Tie2-cre EC null mice after bone marrow transplantation. It has been suggested that endothelial progenitor cells and bone marrow-derived myeloid cells can be involved in tumor angiogenesis (Rabbany et al., 2003; Rafii et al., 2002). Therefore, we performed the tumor experiment with the third group (BM null) of mice, in which HIF-1$\alpha^{+f/+f}$ mice were transplanted with HIF-1$\alpha^{+f/+f}$/Tie2-cre bone marrow cells (Figure 1E). As shown in Figure 1F, about 90% of HIF-1α was deleted in bone marrow in BM null mice 8 weeks after bone marrow reconstitution. A control group of nonirriadiated, non-bone mar-

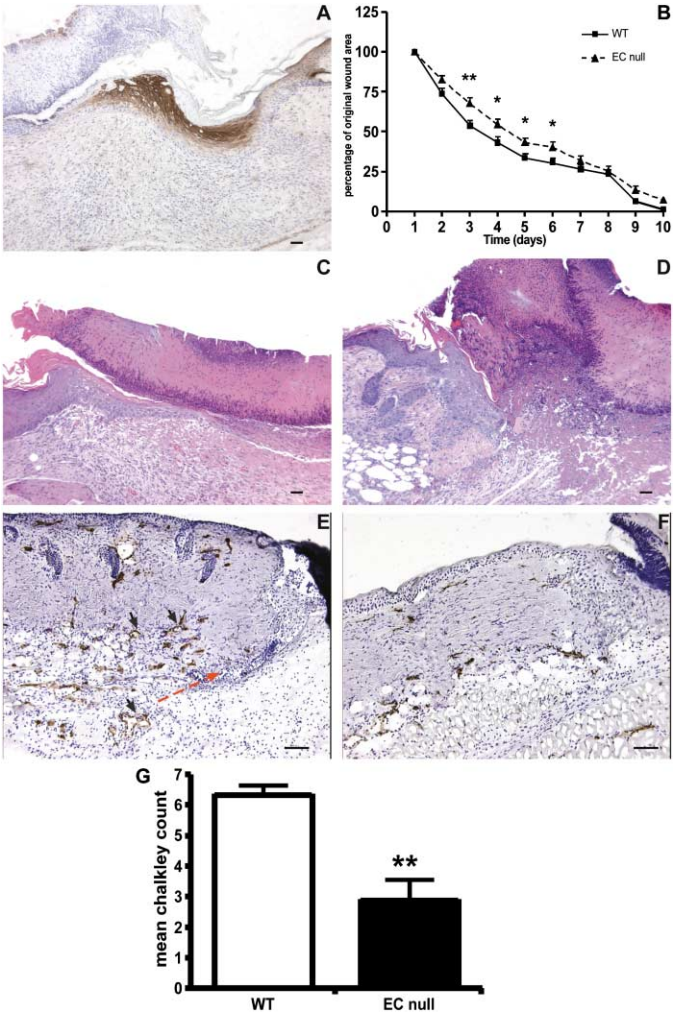

Figure 3. Wound healing is delayed in HIF-1α EC null mice

A: Hypoxyprobe-1 immunostaining was performed to the paraffin sections of wounds at day 6. Scale bar equals 50 μm. The hypoxic areas (brown color) were visualized in pimonidazole-treated mice. Counterstain is hematoxylin.

B: The measurement of the wound healing in wt (closed square) and EC null (closed triangle) mice. 8 weeks after the mice were transplanted with wild-type bone marrow, wound-healing experiments were performed. The percentage of the original wound area was calculated and the results were shown as mean value (±SEM). The wound healing of HIF-1α EC null animals was significantly delayed from the third day to the sixth day. Statistical analysis was performed using the unpaired Student's test, *$p < 0.05$, **$p < 0.01$.

C and D: Hematoxylin and eosin-stained paraffin sections of wounds at day 6. Scale bar equals 50 μm. Note the epidermal leading edge was migrating under the wound clot in the wt animals (**C**), versus the epidermal leading edge at the edge of the wound in the HIF-1α EC null animals (**D**).

E and F: CD31 immunostaining (brown color) was performed on frozen sections of wounds at day 3. Scale bar equals 50 μm. Counterstain is hematoxylin. Note the neovascularization of the wounds in wt animals (**E**) relative to preexisting dilated vessels (black arrows); new vessels have migrated toward the edge of the wound (red arrow indicates the direction of vessel migration). In HIF-1α EC null animals (**F**), there is no migration path near the wound margin.

G: Mean vessel density in wounds from wild-type (white bar) and EC null (black bar) mice in wound margin fields was determined with a chalkley eyepiece graticule.

Statistical analysis was performed using the unpaired Student's test, **$p < 0.01$.

row-transplanted HIF-1$\alpha^{+f/+f}$/Tie2-cre mice also performed the tumor experiment in parallel.

Tumors from HIF-1α EC null mice only reached 60% the weight of tumors from wild-type mice at 15 days postinjection. Tumors from BM null mice are slightly smaller than tumors from wild-type mice, but the difference is not statistically significant. Tumors from HIF-1$\alpha^{+f/+f}$/Tie2-cre mice without bone marrow reconstitution (average tumor weight is 0.44 g) are slightly smaller than the tumors from HIF-1α EC null mice (average tumor weight is 0.55 g). There is a significant difference in tumor weight between BM null mouse group and non-bone marrow-transplanted HIF-1$\alpha^{+f/+f}$/Tie2-cre mouse group (Figure 4A). The results clearly suggest that the defects observed in tumor growth are primarily due to loss of HIF-1α in EC, despite the possibility that loss of HIF-1α in bone marrow might also play a role during tumorigenesis.

In addition, the tumors removed from EC null mice showed severe central necrosis (Figure 4C), while much smaller necrotic areas were seen in the tumors removed from wild-type mice (Figure 4B). The tumors removed from non-bone marrow-transplanted HIF-1$\alpha^{+f/+f}$/Tie2-cre mice also showed the same severe central necrosis phenotype as tumors from EC null mice (N.T. and R.S.J., unpublished observation).

We next analyzed microvessel density (MVD) in these tumors. Vessels were visualized by immunostaining with anti-CD31 antibodies, and the density of the vessels was calculated by Image J software. There was a 50% reduction in tumor vessel density in the tumors removed from HIF-1$\alpha^{+f/+f}$/Tie2-cre EC null mice (Figures 4D–4F). Characterization of tumor endothelial cell proliferation in vivo was done: in Figures 4H and 4I, it can be seen that loss of HIF-1α in the endothelial cells (stained brown) results in fewer endothelial nuclei that stain positively for incorporated BrdU (blue nuclei, Figures 4H and 4I, and graph, 4G). These results demonstrate that loss of HIF-1α in endothelial cells impairs tumor angiogenesis, with a resulting severe central necrosis in the solid tumor mass, and point to a central role for endothelial cell hypoxic response during tumor angiogenesis.

HIF-1α regulates proliferation of endothelial cells during hypoxia

The hypoxia-induced mitogenic response is one of the key steps in endothelium-driven neoangiogenesis during hypoxic stress (Michiels et al., 2000; Pugh and Ratcliffe, 2003). Previous data from our laboratory demonstrated that transformed cell growth was deficient in cells lacking HIF-1α, particularly under hypoxic conditions (Seagroves et al., 2001). To determine the extent to which HIF-1α was required during hypoxic growth of EC, we evaluated growth rates following loss of HIF-1α in immortalized EC lines.

We isolated EC lines from animals with a conditional mutation in HIF-1α. These animals were also homozygous for a deletion of the p53 gene, allowing rapid outgrowth and EC line establishment. These EC lines were used in this study because primary EC do not proliferate well at passage 2. Following establishment, cells were treated with adenovirus expressing Cre recombinase, in order to delete the HIF-1α gene.

As seen in Figure 5A, the EC cell line lacking HIF-1α (labeled null) was able to grow at approximately the same rate as a wild-type control EC line (wt) under normoxic conditions. As seen in Figure 5B, wild-type EC have the same growth rate under hypoxia and normoxia. However, HIF-1α null cells grew more slowly

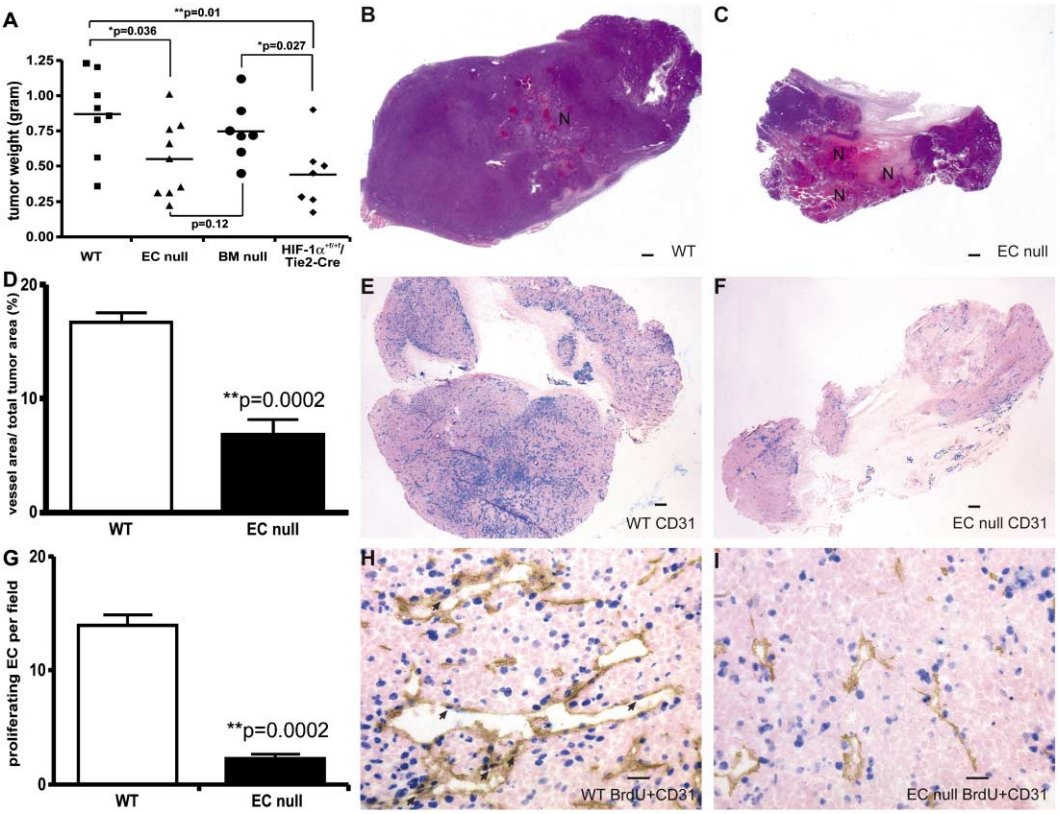

Figure 4. Reduced tumor size in HIF-1α EC null mice

A: Wild-type (wt) mice (closed square), EC null mice (closed triangle), BM null mice (closed circle), and non-bone marrow-transplanted HIF-1$\alpha^{+f/+f}$/Tie2-cre mice (closed diamond) were inoculated subcutaneously with 8×10^6 Lewis lung carcinoma cells, and tumors were dissected after 15 days and weighed (lines indicate mean tumor weight). Statistical analysis was performed using the unpaired Student's test, $^*p < 0.05$, $^{**}p < 0.01$.

B and C: Hematoxylin & eosin (H&E) staining of tumors from wt mice (**B**) and HIF-1α EC null mice (**C**) (scale bar equals 500 μm). Note necrotic areas (N) in the tumors from HIF-1 α EC null mice.

D: Mean vessel density in tumors from wt (white bar) and HIF-1α EC null (black bar) mice was determined with Image J software. Error bars indicate standard deviation (SD).

E and F: The CD31 staining (blue color) of frozen sections of tumors from wt (E) and HIF-1α EC null mice (**F**) (scale bar equals 500 μm). Counterstain is nuclear fast red.

G: Proliferating endothelial cells in tumors from wt mice (white bar) and HIF-1α EC null mice (black bar) were determined by labeling and counting cells with both BrdU- and CD31-positive staining per 200× field. Error bars indicate standard deviation (SD).

H and I: The BrdU (blue nuclei) and CD31 (brown) double staining of sections of tumors from wt (**H**) and HIF-1α EC null (**I**) mice. Scale bar equals 50 μm. Black arrows indicate cells stained with both colors. Counterstain is nuclear fast red.

Statistical analysis was performed using the unpaired Student's test.

in the exponential phase of culture under hypoxia. As seen in Figure 5C, the growth of both wild-type and null EC was arrested after 48 hr culture under anoxic conditions, although the wild-type cells still had a significant survival advantage under these conditions.

HIF-1α null EC and VEGF null EC have defects in capillary structure formation on matrigel substratum under hypoxia

A major attribute of new blood vessel formation is capillary sprouting and tube formation by migrating EC, which may be stimulated by hypoxia (Kroon et al., 2001; Phillips et al., 1995), a process thought to be regulated in part by paracrine or autocrine VEGF. A requirement for HIF-1α in motility might be due to a defect in autocrine or paracrine production of VEGF, in a HIF-1α-dependent manner. To determine the extent to which loss of VEGF production affects motility toward VEGF, we isolated and assayed EC from VEGF conditionally deleted animals (Gerber et al., 1999), in a manner similar to that described for isolation and characterization of HIF-1α null EC.

To determine whether HIF-1α is involved in this process, and the extent to which it might be dependent on VEGF expression induced by hypoxia, we tested the capacity of primary wild-type, HIF-1α null, or VEGF null primary EC to organize into tubes in vitro. Under normoxia, both wild-type and, to a lesser extent, HIF-1α and VEGF null EC (Figure 6A) elongated on matrigel substrata within 6 hr of plating. At hypoxia, tube formation was induced, and tubular networks formed in the wild-type EC (Figures 6A and 6B) at an increased rate relative to normoxia, demonstrating the role of hypoxic induction in capillary formation in primary EC. In contrast, tubular structures were greatly reduced in the HIF-1α null EC and were inhibited to a slightly greater extent in VEGF null EC.

Due to the similarity of the tube formation phenotype under

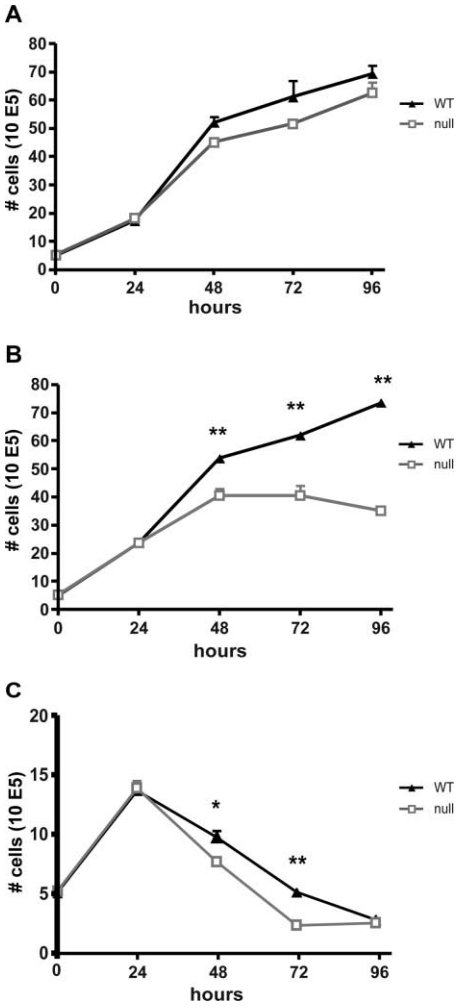

Figure 5. Growth of HIF-1α null EC is reduced during hypoxia

Following overnight seeding at a low density, HIF-1α null EC were incubated under 20% (**A**), 0.5% (**B**), or 0% (**C**) oxygen conditions in DMEM high-glucose with 10% FCS medium and harvested every 24 hr until the cultures reached confluence. The average cell number of triplicate plates for each condition (mean ± SEM) was determined by counting by hemacytometer following trypsinization. The maximal difference in growth rates between wt (closed triangle) and null (open square) EC was noted at 72 hr posthypoxia. Statistical analysis was performed using the unpaired Student's test, *$p < 0.05$, **$p < 0.01$.

hypoxia in HIF-1α null EC and VEGF null EC, we determined the rate at which hypoxia induces VEGF secretion in HIF-1α null EC. Analysis of VEGF protein levels in the EC-conditioned medium of cells cultured under normoxia, hypoxia, and anoxia for 24 hr (Figure 6C) shows that the levels of VEGF protein are approximately similar in wild-type and HIF-1α null EC under normoxic conditions but are greatly reduced under hypoxia or anoxia.

Autocrine VEGF in EC regulates motility, but hypoxia-mediated VEGF production regulates directed chemotaxis

Migration of endothelial cells toward tissues or solid tumors that are secreting VEGF or other angiogenic factors is a critical aspect of angiogenesis (Ferrara, 2002). To determine the functional role of HIF-1α in EC migration, we used recombinant mouse VEGF 164 (rmVEGF164) as a chemotactic factor to analyze the directional migration of wild-type, HIF-1α null, and VEGF null primary EC through a matrigel-coated Boyden chamber under hypoxic conditions. We assayed random motility through assays where serum was at high levels in both the upper and lower chambers (Figure 7B) and directed motility/chemotaxis, where VEGF was placed only in the lower chamber under low serum conditions (Figure 7A).

The motility of both VEGF null and HIF-1α null EC is greatly reduced under hypoxia in the directed assay (Figure 7A). However, in an assay of random motility (Figure 7B), the HIF-1α null EC are essentially as motile as wild-type cells, whereas the VEGF null EC have a greatly reduced level of migration, presumably due to defects in autocrine VEGF-induced cell movement.

Hypoxia-induced expression of VEGF receptors in EC requires HIF-1α

Hypoxia has been proposed to play an important role in the upregulation of VEGF receptor gene expression. It has been shown that VEGFR-1 is transcriptionally upregulated by HIF-1α under hypoxic conditions (Gerber et al., 1997), while VEGFR-2 receptor expression is mediated by posttranscriptional mechanisms such as enhanced protein stability (Waltenberger et al., 1996). To investigate the expression of VEGFR-1 and VEGFR-2 in primary wild-type and mutant EC, they were cultured under normoxia, hypoxia, and anoxia for 24 hr, and total RNA and protein were isolated. The mRNA expression of *VEGFR-1* and *VEGFR-2* were determined by real-time PCR following normalization to 18S rRNA. We found a 2.5-fold increase of *VEGFR-1* mRNA in wild-type EC under hypoxia, and this hypoxic induction of *VEGFR-1* mRNA was partially blocked by the loss of HIF-1α; levels in VEGF null EC were similar to those seen in wild-type cells (Figure 7C). This was correlated with the protein expression levels of VEGFR-1, which were upregulated under hypoxia and anoxia in wild-type and VEGF null EC, but not in HIF-1α null EC (data not shown). *VEGFR-2* mRNA levels were decreased slightly in HIF-1α and VEGF null EC under normoxic conditions.

In wild-type EC, the protein expression levels of VEGFR-2 were increased about 2.5-fold by hypoxia, while in both HIF-1α null and VEGF null EC, VEGFR-2 levels were not changed by culture in hypoxia (Figure 7D). These results demonstrate that loss of HIF-1α induction/regulation of EC-produced VEGF disrupts an autocrine loop, interrupting increased expression of VEGFR-2 caused by increased VEGF expression.

Discussion

There is a great deal of experimental evidence that the growth of transformed cells in vivo requires a hypoxic response and that this occurs primarily through the action of HIF-1α (Semenza, 2003). Recent data has also demonstrated that there is a site-specific aspect for the requirement for HIF-1α in malignant cells, where the vascular network available at the site of tumor growth is a critical determinant of the overall rate and invasiveness of tumorigenic progression (Blouw et al., 2003). The data presented here argue that targeting of HIF-1α response in the endothelium also has to be taken into account.

Endothelial cells are able to withstand large fluxes in oxygen and maintain physiological integrity; this presumably allows them to migrate into hypoxic and even anoxic sites during wound healing to reestablish oxygenation (Ten and Pinsky,

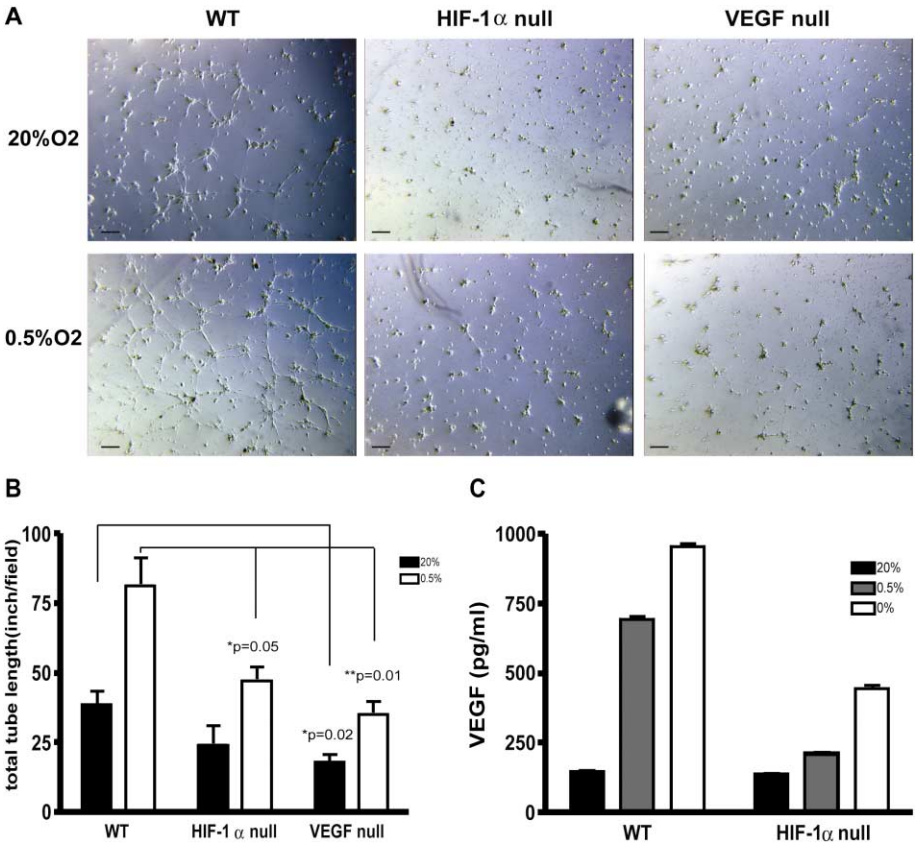

Figure 6. HIF-1α regulation of endothelial cell autocrine VEGF is essential for capillary-like tube formation in vitro

A: EC tube formation assay: 5×10^4 primary EC in 0.5 ml medium containing 0.5% FCS were plated on growth factor-reduced matrigel substratum. Then the cells were left at 20% oxygen (top) or were transferred to 0.5% oxygen conditions (bottom) for 8 hr. The network formation was viewed by phase-contrast microscopy at 40× magnification. At 20% oxygen conditions, after 8 hr plating, both wild-type (left top) and HIF-1α null (middle top) EC began to form capillary-like structures. Fewer capillary-like tubes were observed in VEGF null EC (right top). At 0.5% oxygen conditions, tube formation was induced in wild-type EC (left bottom). Few tubes were induced in the HIF-1α null EC (middle bottom) and VEGF null EC (right bottom) incubated under 0.5% oxygenation. Scale bar equals 200 μm.
B: The length of tube-like structures was measured by analyzing digitized images (4 random images per well) (40×) by Image J software. *$p < 0.05$, **$p < 0.01$.
C: Expression of VEGF protein in wild-type (wt) and HIF-1α null EC. Confluent monolayer cells of both cell types were cultured under 20% (black bars), 0.5% (gray bars), or 0% (white bars) oxygen conditions for 24 hr, conditioned supernatant was harvested, and VEGF protein levels analyzed by ELISA.

2002; Tretyakov and Farber, 1995). As part of the response to hypoxia, all cells require a robust capacity for anaerobic generation of energy, and endothelial cells are particularly dependent on the glycolytic pathway (Culic et al., 1997). We describe here that loss of HIF-1α has a deleterious effect on hypoxic ATP levels in primary endothelium. This likely gives rise to alterations in the overall capacity of the endothelium to proliferate and invade into tissues where oxygen levels are limited, although our data indicate that random motility of the cells is not affected during hypoxia.

A possible mechanism to compensate for the absence of HIF-1α is the redundancy of the HIF-1 pathway. A close homolog of HIF-1α, HIF-2α, has been connected in the literature extensively to endothelial cells, and in fact was labeled endothelial PAS protein-1 (EPAS1) by one of the groups that first cloned this transcription factor (Ema et al., 1997; Flamme et al., 1997; Jain et al., 1998; Tian et al., 1997). It has been shown that HIF-1α shares some common targets with HIF-2α, but HIF-1α also has unique targets different from HIF-2α (Hu et al., 2003). HIF-2α is highly expressed in endothelial cells in vivo but can be found in a wide range of other cell types as well. Its function, however, is generally unclear, although one recent paper has shown that HIF-2α has a role in regulating the response to oxidative stress (Scortegagna et al., 2003). We have shown previously that in cells lacking HIF-1α, HIF-2α is unable to compensate during hypoxic stress; this appears to be due to a cytoplasmic segregation of the HIF-2α protein, which is refractory to hypoxia (Park et al., 2003). We see no compensation in hypoxic response in HIF-1α null endothelial cells in this study, relative to other HIF-1α null cell types, arguing against a specific role for HIF-2α in endothelial cell hypoxic response. This is in agreement with a previous study (Sowter et al., 2003). It remains to be determined how HIF-2α regulates endothelial cell response to hypoxia, or what its exact role is, given the evidence for its EC-specific expression.

Cutaneous wound healing requires a coordinated migration of endothelial cells into the wound margin, and a subsequent reorganization of the EC, with a concomitant formation of new capillaries to reestablish circulation under the reformed epithelial layer (Ferrara, 2002; Li et al., 2003b; Tonnesen et al., 2000). We have shown that HIF-1α-mediated endothelial cell function is important for early events in this process, but that the absence of HIF-1α can be compensated for in later stages of wound healing. However, some wounds in HIF-1αEC null animals remain unhealed for long periods; and the resolution of these wounds is also typically less successful than is the case in wild-type animals (N.T. and R.S.J., unpublished observation). This argues that endothelial cell migration stimulated by hypoxia is required for successful resolution of wound healing, and not just for the initial process of reepithelialization.

Loss of HIF-1α in all tissues results in an early embryonic lethality, with significant deficits in embryonic and yolk sac vascularization (Iyer et al., 1998; Ryan et al., 1998). Capillaries in the global HIF-1α knockout embryos are largely absent and are disorganized in the embryonic yolk sac of the HIF-1α null mutants (Iyer et al., 1998; Ryan et al., 1998). The Tie2 transgene is expressed in hemagioblasts at early E7.5 stage of development and is able to induce recombination of the conditional

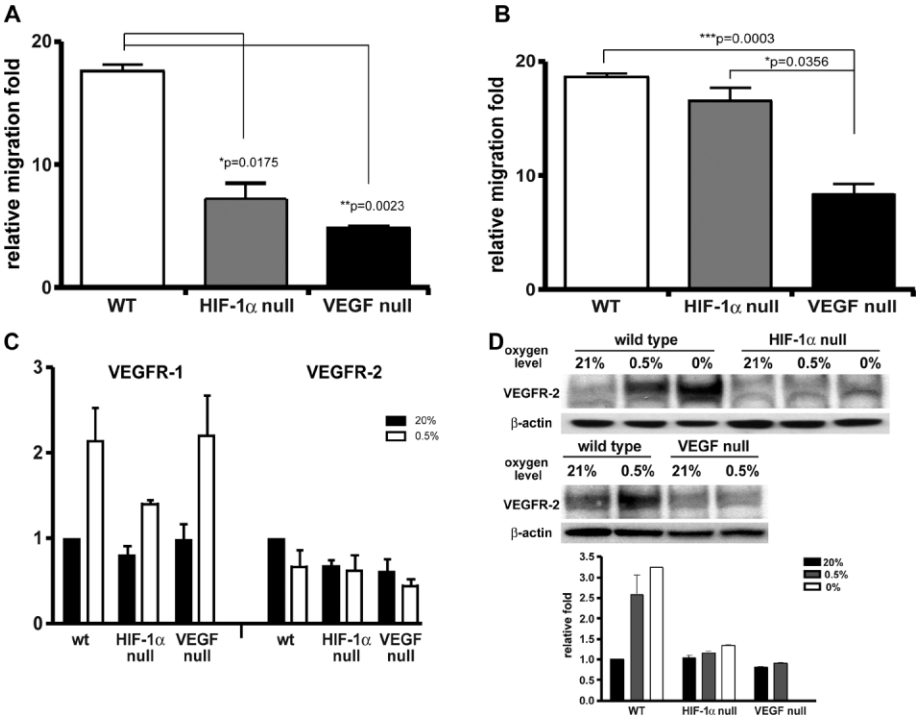

Figure 7. Loss of HIF-1α impairs EC movement toward rmVEGF164

A: Migration of EC to VEGF was analyzed in Boyden chambers. Wild-type (white bars), HIF-1α null (gray bars), and VEGF null (black bars) EC in transwells with medium containing 0.5% FCS were allowed to migrate toward 20 ng/ml rmVEGF164 in lower wells with medium containing 0.5% FCS for 24 hr under 0.5% oxygen conditions. The migrating cells were stained as described in Experimental Procedures.

B: Random motility of EC was analyzed in Boyden chambers with medium containing higher levels of serum (5% FCS) in both upper and lower wells. Wild-type (white bars), HIF-1α null (gray bars), and VEGF null (black bars) EC in transwells were allowed to migrate randomly for 24 hr under 0.5% oxygen culture conditions.

C: Primary wild-type (wt) EC, HIF-1α null EC, and VEGF null EC were cultured under 20% (black bars) or 0.5% (white bars) oxygen conditions for 24 hr. The mRNA expression levels of VEGFR-1 and VEGFR-2 were examined by real-time PCR, with gene expression normalized to 18S rRNA.

D: Expression of VEGFR-2 protein in wild-type, HIF-1α null, and VEGF null EC was influenced by oxygen level. Primary wild-type and HIF-1α null EC were cultured under 20%, 0.5%, or 0% oxygen levels for 24 hr (top). Primary wild-type and VEGF null EC were cultured at 20% or 0.5% oxygen conditions for 24 hr (bottom). Western blot analysis was detected using anti-mouse VEGFR-2 antibody and a chemiluminescence-based detection system for visualization. Equal protein loading controls were shown by β-actin blots. Western results were analyzed by a Typhoon scanner and ImageQuant software and shown in the bar graph. Similar results were obtained in at least three independent experiments.

gene in vivo at least at E8.5 (Cattelino et al., 2003; Kisanuki et al., 2001). The normal development of the HIF-1α EC null mice indicates that the defects in capillarization seen in early mutant embryos may be due to changes in the hypoxic response of the underlying tissues, and not in the EC. It is an interesting aspect of this mutation that there is a profound effect on wound healing and tumorigenesis, but no discernible effect on normal vascularization during development. Further work will be needed to determine if there are more subtle effects on developmental vascularization caused by deletion of EC HIF-1α. We also cannot totally exclude the possibility of a functional redundancy of HIF-1α and HIF-2α during embryonic development. The study of the development of HIF-2α EC null mice will be helpful to elucidate this question.

Perhaps most strikingly, we have found that the loss of the hypoxia-responsive transcription factor HIF-1α severely alters the morphology, vascularization, necrosis, and growth of tumors. These data demonstrate that loss or inhibition of hypoxic response as a component of cancer therapy may need to target endothelium in the first instance, where it may indeed be more efficacious then the targeting of that response in malignant cells themselves. In addition, the overall normal viability and lack of overt phenotype in uninjured mice with HIF-1α null endothelium demonstrates that inhibition of the transcription factor should not adversely affect the overall health of treated patients, if the treatment itself is targeted to EC.

Further experiments on tumor growth in different sites in this model are critical; they will help determine how tumorigenesis is affected by endothelial response when the endothelial network itself is handicapped in its hypoxic response. In addition, the use of this model in conjunction with tumor cells that lack HIF-1α, or lack one of its targets, such as VEGF, will help to model targeted treatments much better than currently experimentally possible. In such experiments, it will be possible to assess effects on both transformed cells and critical stromal compartments. In this way, genetic models can be created that more truly mimic the pharmacology of inhibited hypoxic response.

In summary, we have shown that loss of HIF-1α or VEGF in EC interrupts an autocrine loop necessary for the posttranscriptional hypoxic induction of VEGFR-2 expression. This demonstrates that loss of HIF-1α affects function of EC during their directed migration toward regions of hypoxia and, thus, higher levels of VEGF. This feedback mechanism of hypoxic response in EC is necessary for the proper neovascularization of wounds and the growth of one type of experimental solid tumor and indicates that HIF-1α-directed hypoxic response can be critical in indirect as well as direct control of cellular responses in vivo.

Experimental procedures

Animals

All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Endothelial cell-specific inactivation of HIF-1α was achieved by cross-breeding Tie2-Cre transgenic mice (Kisanuki et al., 2001) with HIF-1$\alpha^{+f/+f}$ mice homozygous for the HIF-1α allele with exon2 flanked by loxp sites (Ryan et al., 1998). In all experiments, littermates from the same breeding pair were used as controls.

In vitro endothelial cell culture

Mouse lung endothelial cells were isolated from HIF-1$\alpha^{+f/+f}$ mice, VEGF$^{+f/+f}$ mice, or p53$^{-/-}$ HIF-1$\alpha^{+f/+f}$ mice and cultured as previously described (Dong et al., 1997).

Quantitative real-time PCR analysis

Gene expression of VEGF, PGK, Glut-1, VEGFR-1, and VEGFR-2 was quantified by real-time PCR. Results were normalized to the expression level of ribosome RNA, as described previously (Seagroves et al., 2003).

Antibodies and Western blot analysis

The source and dilution of primary antibodies used are the following: HIF-1α, a gift from Abraham lab at 1:500; HIF-2α, from Novas at 1:2000; and VEGFR-1 and VEGFR-2, both from Sigma-Aldrich at 1:500.

For detecting VEGFR-1 and VEGFR-2, endothelial cells were cultured in 1% FCS medium under normoxic (20% O_2), hypoxic (0.5% O_2), or anoxic (0%) conditions for 22 hr. Cytoplasmic extracts (CE) and nuclear extracts (NE) were then prepared as described previously (Ryan et al., 1998).

Growth curves

Triplicate plates of $p53^{-/-}$HIF-1α wt and $p53^{-/-}$HIF-1α null EC were seeded in DMEM-high-glucose medium, supplemented with 10% fetal bovine serum (FBS) and 25 mM HEPES (pH 7.4). The following day, cells were left under normoxia or transferred to hypoxic or anoxic chambers (0 hr). During the experiments, the cell culture medium was not changed. Cells were harvested every 24 hr by trypsinization. A minimum of 100 cells from each plate were counted using a hemocytometer and the average cell number per treatment was determined. All growth assays described were repeated three times and the values described in the figures are representative of one triplicate culture experiment ± the standard error of the mean (SEM).

Endothelial cell migration assay

The migration of primary EC was tested by using COSTAR transwells with a polycarbonate-filter (PVP free, 6.5 mm diameter, pore size 8 μM; Corning Costar Corporation, Corning, NY). The transwells were coated with 5 $\mu g/cm^2$ collagen I (BD Bioscience, Bedford, MA). To test the migration toward VEGF, 2×10^5 primary EC resuspended in 200 μl of M199 medium supplemented with 0.5% FCS were seeded in the upper chamber, with the lower chamber containing M199 medium supplemented with 0.5% FCS plus 20 ng/ml rmVEGF164. To test random migration, 2×10^5 primary EC resuspended in 200 μl M199 medium supplemented with 5% FCS were seeded in the upper chamber, with the lower chamber also containing M199 medium supplemented with 5% FCS. Cells were allowed to migrate across 8.0 μm pore size polycarbonate membrane under 20% (normoxia) or 0.5% (hypoxia) oxygen conditions for 24 hr. Data were expressed as one triplicate culture experiment ± the standard error of the mean (SEM).

In vitro matrigel tube formation

Growth factor reduced matrigel 10 mg/ml (BD Biosciences) was applied at 300 μl/well in 24-well plates and incubated at 37°C for at least 1 hr to allow hardening. 5×10^4 primary lung capillary EC were resuspended in 0.5% FCS growth medium at 1×10^5 concentration. 500 μl cells were added to the matrigel-coated plates. Plates were incubated at 20% or 0.5% oxygen conditions at 37°C for 8 hr. The assays were repeated at least three times. The pictures described in the figures are representative of one experiment. Bar data were expressed as one duplicate culture experiment ± the standard error of the mean (SEM).

In vivo matrigel plug implantation

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into matrigel plugs containing the test sample as in Passaniti et al. (1992).

Wound healing

Wound healing experiments were performed on mice 8 weeks after bone marrow transplantation. 14-week-old sex- and age-matched mice were used in the experiment with 5 males and 5 females per group. The detailed procedure was followed as previously described (Li et al., 2003a).

Tumorigenesis

A total of 8×10^6 Lewis lung carcinomas cells on a BL6 background (ATCC) were injected subcutaneously into EC null mice, BM null mice, and wild-type control littermates 8 weeks after bone marrow transplantation. A control group of nonirradiated, non-bone marrow-transplanted HIF-$1\alpha^{+f/+f}$/Tie2-cre mice were also injected with the same number of LLC cells subcutaneously in parallel. The experiment was stopped at the day 15 postinjection because the animals showed signs of lack of well being, and tumors were removed from 8 wt, 9 EC null, 7 BM null, and 7 HIF-$1\alpha^{+f/+f}$/Tie2-cre mice and weighed.

Acknowledgments

N.T. is sponsored by NIEHS-Superfund predoctoral fellowship. We would like to thank B. Blouw, D. Liao, W. McNulty, G. Li, T. Seagroves, and members of Johnson laboratory for helpful suggestion and assistance and Dr. Robert Abraham for generously providing us anti-mouse HIF-1α antibody. We thank Pfizer Corporation and the UC BioStar program, as well as Michael North, Steven Bender, and Michael Niesman, for support.

Received: March 2, 2004
Revised: July 22, 2004
Accepted: September 15, 2004
Published: November 15, 2004

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