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Regulation of Colon Carcinoma Cell Invasion by Hypoxia-Inducible Factor 1[1]

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ABSTRACT

Hypoxia-inducible factor 1 (HIF-1) transactivates genes the products of which mediate tumor angiogenesis and glycolytic metabolism. Overexpression of the HIF-1α subunit, resulting from intratumoral hypoxia and genetic alterations, has been demonstrated in common human cancers and is correlated with tumor angiogenesis and patient mortality. Here we demonstrate that hypoxia or HIF-1α overexpression stimulates Matrigel invasion by HCT116 human colon carcinoma cells, whereas this process is inhibited by a small interfering RNA directed against HIF-1α. We show that HIF-1 regulates the expression of genes encoding cathepsin D; matrix metalloproteinase 2; urokinase plasminogen activator receptor (uPAR); fibronectin 1; keratins 14, 18, and 19; vimentin; transforming growth factor α; and autocrine motility factor, which are proteins that play established roles in the pathophysiology of invasion. Neutralizing antibodies against uPAR block tumor cell invasion induced by hypoxia or HIF-1α overexpression. These results provide a molecular basis for promotion of the invasive cancer phenotype by hypoxia and/or HIF-1α overexpression.

INTRODUCTION

Genetic alterations promote tumor cell proliferation and survival by inducing physiological alterations within tumor cells, *e.g.,* dysregulation of apoptosis, cell cycle, and growth factor signaling pathways, as well as in stromal cells, *e.g.,* stimulation of angiogenesis (1). The resulting pathological increase in cell number defines a tumor. In contrast, cancer is defined by the ability to penetrate the ECM[3] of basement membrane and underlying stroma and to invade into surrounding tissue (2). Important properties of invasive cancer cells include decreased cell-cell adhesion, cytoskeletal remodeling, increased motility, increased production of ECM proteases, and synthesis of new ECM components (ECM remodeling).

A consequence of increased cell number within a tumor is a corresponding increase in O_2 consumption. Tumor progression and patient mortality are correlated with both microvascular density (3–6) and intratumoral hypoxia (7). The basis for this apparent paradox is that although angiogenesis is stimulated within tumors, the resulting vessels are structurally and functionally abnormal, resulting in a failure to deliver adequate O_2. Tumor cell survival is thus dependent on the stimulation of angiogenesis and the metabolic adaptation of tumor cells to hypoxia.

HIF-1 is a transcriptional activator, composed of O_2-regulated HIF-1α and constitutively expressed HIF-1β subunits (8), that functions as a master regulator of O_2 homeostasis (9). Four lines of evidence indicate that HIF-1 plays important roles in tumor progression. First, immunohistochemical analyses indicate that HIF-1α is overexpressed in primary and metastatic human cancers and that the level of expression is correlated with tumor angiogenesis and patient mortality (10–17). Second, in addition to intratumoral hypoxia, genetic alterations in tumor suppressor genes (*p53*, *VHL*, *PTEN*) and oncogenes (*SRC*, *HER2*^{neu}, H-*RAS*) induce HIF-1 activity (18–25). Third, in mouse xenograft assays, genetic manipulations that increase or decrease HIF-1 activity are associated with increased or decreased tumor growth and angiogenesis, respectively (19, 23, 26–28). Fourth, HIF-1 controls the expression of gene products that stimulate angiogenesis, such as VEGF, and that promote metabolic adaptation to hypoxia, such as glucose transporters and glycolytic enzymes, providing a molecular basis for its effects on tumor growth and angiogenesis (9, 29–31).

Intratumoral hypoxia is correlated with an increased risk of invasion in human cancer (7) and rodent xenografts (32), indicating that the hypoxic tumor microenvironment may select for mutations that promote survival (33) and invasion. An alternate, but not mutually exclusive, hypothesis is that hypoxia acts as a physiological stimulus to induce expression of genes the products of which promote invasion. This model is supported by studies demonstrating that tumor cells that are transiently subjected to hypoxia manifest increased rates of invasion through basement membrane *ex vivo* (34). HIF-1α overexpression was observed in human brain and colon cancer biopsies at the invading tumor margin (16, 17). We hypothesized that HIF-1α overexpression, induced either by intratumoral hypoxia or by genetic alterations, activates programs of gene expression controlling invasion by cancer cells.

MATERIALS AND METHODS

Cell Culture and Transfection. HCT-116 cells were cultured in McCoy's 5A medium with 10% FBS and 1% penicillin-streptomycin (Life Technologies, Inc.). *Hif1a*$^{+/+}$ and *Hif1a*$^{-/-}$ ES cells were maintained in high-glucose DMEM with 15% FBS, 1% penicillin-streptomycin, nonessential amino acids, sodium pyruvate, and 200 μg/ml of G418 (9). 786-0 RCCs and the WT-8 subclone expressing VHL (provided by William Kaelin, Harvard Medical School, Boston, MA) were cultured in DMEM with 10% FBS, 1% penicillin-streptomycin and 1 mg/ml of G418 (35). Five $\times 10^5$ HCT116 cells were plated per 6-cm dish and transfected with 2 μg of pCEP4 or pCEP4/HIF-1α (23) using LipofectAMINE-Plus (Life Technologies, Inc.). To generate siRNA$_{HIF\text{-}1\alpha}$, two oligonucleotides consisting of ribonucleosides except for the presence of 2′-deoxyribonucleosides (dTdT) at the 3′ end, 5′-AGAGGUGGAUAUGUGUGGGdTdT-3′ and 5′-CCCACACAUAUCCACCUCUdTdT-3′, were synthesized and annealed (Dharmacon Research, Inc.). HCT116 cells were exposed to 100 nM siRNA$_{HIF\text{-}1\alpha}$ in the presence of Oligofectamine (Invitrogen) for 4 h (36). Control experiments were performed using siRNA directed against UFP3B mRNA (provided by Josh Mendell and Hal Dietz, Johns Hopkins University, Baltimore, MD).

Invasion Assays. For invasion assays, 12-mm-diameter Transwell polycarbonate filters (12-μm pore size, Costar) in a modified Boyden chamber were coated with 100 μl of Matrigel (Sigma) at 1:20 dilution in serum-free medium and air-dried for 24 h. Five $\times 10^4$ HCT116 cells in 200 μl of complete medium were seeded into the inner chamber. Six hundred μl of medium were added to the lower chamber, and the plate was incubated at 37°C in a 5% CO_2/95% air

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[3] The abbreviations used are: ECM, extracellular matrix; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; RCC, renal carcinoma cell; VHL, von Hippel-Lindau protein; uPA, urokinase plasminogen activator; uPAR, uPA receptor; MMP2, matrix metalloprotease 2; ES, embryonic stem; TGF, transforming growth factor; K19, keratin 19; AMF, autocrine motility factor; siRNA, small interfering RNA.

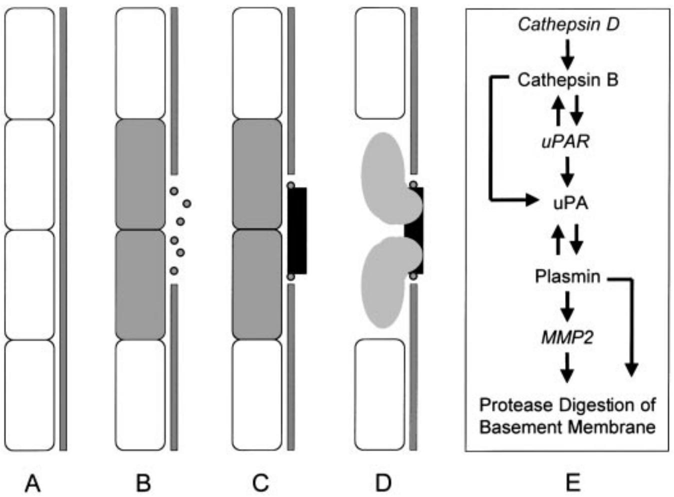

Fig. 1. Molecular and cellular aspects of tumor cell invasion. *A,* the spatial localization of epithelial cells (*ovals*) are normally constrained by cell-cell contacts and by basement membrane (*thin shaded rectangle*). *B,* tumor cells (*shaded ovals*) elaborate proteases that digest ECM/basement membrane. *C,* tumor cells also produce specific ECM proteins such as fibronectin (*thick solid rectangle*) for which they possess specific integrin receptors. *D,* tumors cells switch their production of intermediate filaments from keratin subtypes expressed by fixed epithelial cells to vimentin and keratin subtypes that are expressed by motile mesenchymal cells. The combination of ECM remodeling, altered expression of integrins and intermediate filaments, loss of cell-cell contacts, and increased motility results in invasion through the basement membrane that is the defining characteristic of cancer. *E,* ECM digestion involves a cascade (with multiple positive feedback loops) of proteases (active forms shown).

incubator (20% O_2). For hypoxic treatment, plates containing the Boyden chambers were placed in a modular incubator chamber (Billups-Rothenberg) that was flushed for 3 min with a gas mixture consisting of 1% O_2, 5% CO_2, and 94% N_2; sealed; and incubated at 37°C for 24 h. Cells on the lower surface of the filter were scraped with a rubber scraper into the medium from the lower chamber, pelleted, resuspended in 50 μl of medium, and counted using a hemocytometer. Each condition was performed in quadruplicate, and the experiment was performed twice. Transfected cells were plated 24 h after the removal of the transfection reagent. Neutralizing and non-neutralizing antibodies against uPAR were used in Boyden chamber assays as described previously (34).

RNA and Protein Assays. Total RNA was isolated from cells maintained at 20% or 1% O_2 for 24 h (starting 24 h after the removal of Oligofectamine for transfected cells) using Trizol reagent (Life Technologies, Inc.). RNA (10 μg) was fractionated by 2.2 M formaldehyde- 1.4% agarose gel electrophoresis and transferred to Hybond N^+ membrane (Amersham-Pharmacia) in 20× SSC. IMAGE Consortium cDNAs were isolated from plasmids (Research Genetics, Inc.) and ^{32}P-labeled probes were synthesized by random primer-labeling (Roche). Prehybridization and hybridization were performed at 67°C for 1 and 2.5 h, respectively, in QuikHyb (Stratagene). The filters were washed in 0.1× SSC/1% SDS at 56°C for 1 h. Immunoblot assays were performed using monoclonal antibody H1α67 (17).

RESULTS

Analysis of Basement Membrane Invasion by Human Colon Carcinoma Cells. In order for cancer cells of epithelial origin to invade surrounding tissue, the cells must degrade the underlying basement membrane (Fig. 1). We previously demonstrated increased growth and angiogenesis of tumor xenografts after s.c. injection of HCT116 human colon carcinoma cells that were transfected with an expression vector encoding HIF-1α (23). To assay the effects of hypoxia and HIF-1α overexpression on invasion, HCT116 cells were transiently transfected either with empty vector or HIF-1α expression vector. Transfected cells were seeded onto a filter that was coated with Matrigel, an experimental basement membrane, and exposed to 20% or 1% O_2 for 24 h. The number of cells that digested Matrigel and migrated through the 12-μm pores in the filter were counted 24 h later. The invasiveness of tumor cells transfected with empty vector was significantly increased under hypoxic conditions ($P < 0.005$; Fig. 2*A*). Compared with empty vector, transfection of cells with HIF-1α expression vector resulted in a highly significant increase in invasiveness under both nonhypoxic and hypoxic conditions ($P < 0.00005$; Fig. 2*A*). The combination of hypoxia and HIF-1α overexpression resulted in the greatest number of cells invading through Matrigel. These effects were observed under transfection conditions that resulted in only a modest increase in HIF-1α protein levels (Fig. 2*D*).

As a complementary approach, we synthesized a siRNA$_{HIF\text{-}1\alpha}$ that, when transfected into cells, targets HIF-1α mRNA for degradation, thus reducing the expression of HIF-1α mRNA and protein. The invasion of HCT116 cells transfected with siRNA$_{HIF\text{-}1\alpha}$ was significantly reduced as compared with mock-transfected cells under both

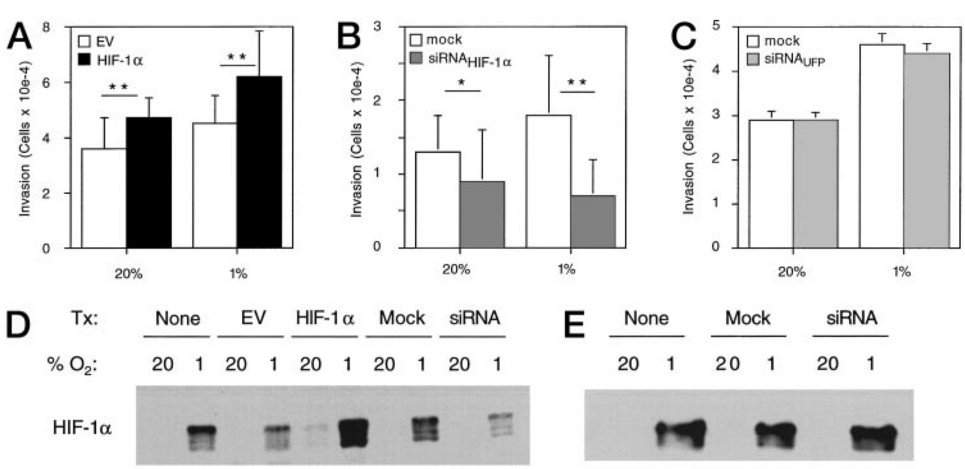

Fig. 2. Effect of hypoxia and HIF-1α expression on invasion of human colon cancer cells. *A–C,* HCT116 cells were transiently transfected, seeded onto Matrigel-coated filters in a Boyden chamber and incubated for 24 h in 20% or 1% O_2, and the number of cells on the underside of the filter was determined. For each condition, data are presented as mean and SD ($n = 8$). Statistically significant differences in tumor cell invasion are indicated: *, $P < 0.05$; **, $P < 0.00005$ (paired Student's *t* test). *A,* cells were transfected with expression vector that was either empty (*EV*) or encoded HIF-1α. *B,* cells were exposed to Oligofectamine reagent in the absence (*mock*) or presence of siRNA directed against HIF-1α (*siRNA$_{HIF\text{-}1\alpha}$*). *C,* cells were exposed to Oligofectamine in the absence (*mock*) or presence of siRNA directed against an unrelated mRNA (*siRNA$_{UFP}$*). *D,* HCT116 cells were exposed to the following treatment (*Tx*): *None*; transfection with EV or HIF-1α expression vector; transfection with Oligofectamine in the absence (*Mock*) or presence of siRNA$_{HIF\text{-}1\alpha}$. Cell lysates were subject to immunoblot assay using an anti-HIF-1α monoclonal antibody. *E,* HCT116 cells were untreated (*None*), mock-transfected (*Mock*), or transfected with siRNA$_{UFP}$; and HIF-1α expression was analyzed.

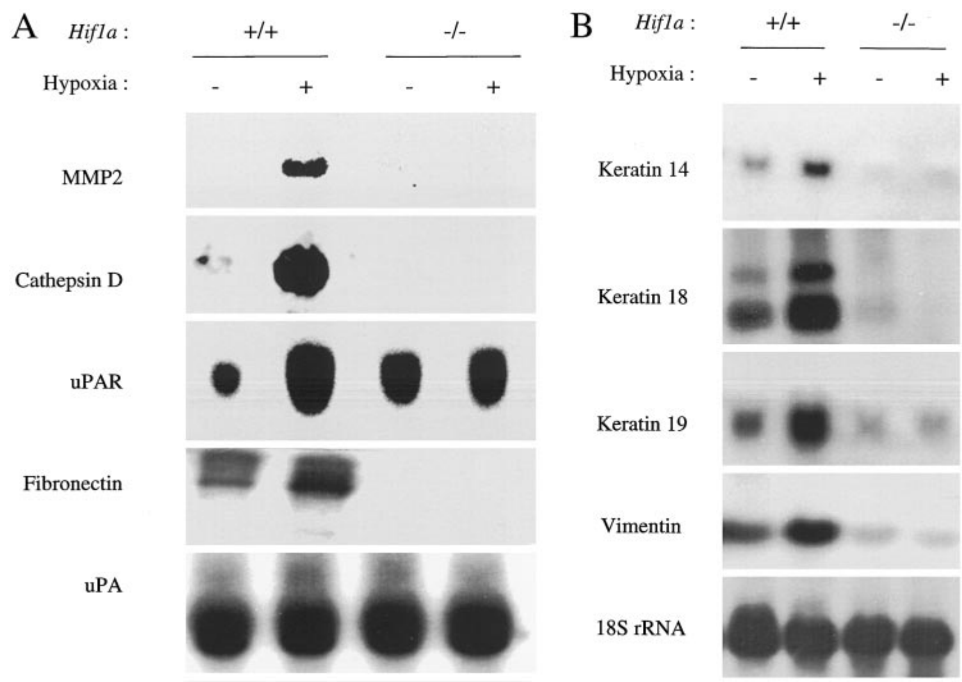

Fig. 3. Effect of HIF-1α deficiency on gene expression in mouse ES cells. ES cells that were either wild-type (+/+) or homozygous for a loss-of-function allele (−/−) at the *Hif1a* locus were exposed to non-hypoxic (−) or hypoxic (+) culture conditions (20% and 1% O_2, respectively) for 24 h; and aliquots of total RNA were analyzed by blot hybridization. *A*, expression of mRNAs encoding proteins involved in ECM remodeling. *B*, expression of mRNAs encoding intermediate filaments.

nonhypoxic and hypoxic conditions (Fig. 2*B*). These effects were observed under transfection conditions that resulted in only a modest decrease in HIF-1α protein levels (Fig. 2*E*). Transfection of siRNA targeted to an irrelevant mRNA ($siRNA_{UFP}$) had no effect on HIF-1α protein expression (Fig. 2*E*) or on invasion (Fig. 2*C*), indicating that impaired invasion of cells transfected with $siRNA_{HIF\text{-}1\alpha}$ was a specific result of HIF-1α loss-of-function. Thus, both gain- and loss-of-function experiments demonstrate that modest changes in HIF-1α levels have significant effects on basement membrane invasion by HCT116 colon cancer cells.

Analysis of Gene Expression in Mouse ES Cells. To provide a molecular basis for the observed effects of hypoxia and HIF-1α overexpression on tumor cell invasion, we sought to identify HIF-1 target genes that encode proteins with established roles in this process. Degradation of basement membrane (Fig. 1*B*) requires the production of ECM proteases (2). The protein uPAR binds to uPA, and the complex catalyzes the conversion of plasminogen to plasmin, which degrades ECM (Fig. 1*E*), both by acting directly and by activating latent metalloproteases, including MMP2. MMP2 degrades type IV collagen, the principal basement membrane protein. MMP2 expression is detectable in most invasive colon adenocarcinomas but not in normal colonic epithelium or benign polyps (2). Cathepsin D is a protease that activates cathepsin B (Fig. 1*E*), an activator of uPAR (37). Mouse ES cells that were wild-type (*Hif1a*$^{+/+}$) or homozygous for a knockout allele resulting in loss of HIF-1α expression (*Hif1a*$^{-/-}$; Ref. 9) were exposed to 20% or 1% O_2 for 24 h, and total RNA was isolated for blot hybridization assays. Expression of mRNAs encoding uPAR, MMP2, and cathepsin D was induced by hypoxia in wild-type ES cells, whereas no induction was observed in *Hif1a*$^{-/-}$ cells (Fig. 3*A*). In contrast, uPA mRNA was constitutively expressed in wild-type and *Hif1a*$^{-/-}$ cells.

Invasion also involves altered expression of integrins on the surface of cancer cells (1, 2) and the production of ECM proteins recognized by these receptors (Fig. 1, *C–D*). One of the major ECM proteins associated with tumor invasion is fibronectin 1 (38, 39). As observed for mRNAs encoding ECM proteases, fibronectin 1 mRNA expression was induced by hypoxia in wild-type ES cells but not in HIF-1α-null cells (Fig. 3*A*).

Cells in the center of a colorectal cancer maintain an epithelial phenotype, whereas cells at the invasive front exhibit a mesenchymal phenotype (Fig. 1*D*) characterized by a loss of cell-cell contacts and increased expression of fibronectin and vimentin (40). Reprogramming of intermediate filament expression leading to the production of vimentin, either alone or in combination with specific keratins, promotes tumor cell invasion (41). Expression of mRNAs encoding vimentin and keratins 14, 18, and 19 was induced by hypoxia in *Hif1a*$^{+/+}$ cells, whereas their expression was markedly reduced in *Hif1a*$^{-/-}$ cells (Fig. 3*B*).

Analysis of Gene Expression in Human Cancer Cells. To complement the data obtained by analysis of mouse ES cells with HIF-1α loss-of-function, we analyzed gene expression in human 786-0 RCCs that lack functional VHL protein (35). VHL is required for the O_2-dependent ubiquitination and proteasomal degradation of HIF-1α and of HIF-2α, which also dimerizes with HIF-1β and activates HIF-1 target genes (22, 42–44). In the absence of VHL, HIF-1 target genes are constitutively expressed under nonhypoxic conditions (35). Transfection of a wild-type VHL expression vector into 786-0 cells corrects the defect and restores O_2-regulated gene expression. As reported previously (35), expression of the HIF-1 target gene *VEGF* was induced by hypoxia in VHL-expressing RCCs, whereas VHL-null RCCs expressed VEGF mRNA at high levels under nonhypoxic conditions (Fig. 4). Similarly, expression of MMP and uPAR mRNAs was also regulated by hypoxia and VHL. Expression of mRNA encoding TGF-α, which stimulates cell proliferation, migration, and uPAR synthesis, was also regulated by hypoxia in a VHL-dependent manner (Fig. 4). In contrast, expression of Ku86 mRNA was not induced by hypoxia or VHL loss-of-function in 786-0 cells.

To further establish a connection between hypoxia- and HIF-1-regulated gene expression and tumor cell invasion, we analyzed HCT116 cells in which HIF-1α protein expression was partially inhibited by RNA interference, as described above (Fig. 2*D*). Expression of mRNAs encoding VEGF, TGF-α, uPAR, and K19 was induced by hypoxia in untreated cells or mock-transfected cells that were exposed to transfection reagent alone (Fig. 5). Compared with mock-transfected cells, transfection of cells with $siRNA_{HIF\text{-}1\alpha}$ resulted in reduced HIF-1α mRNA levels and a comparable reduction in

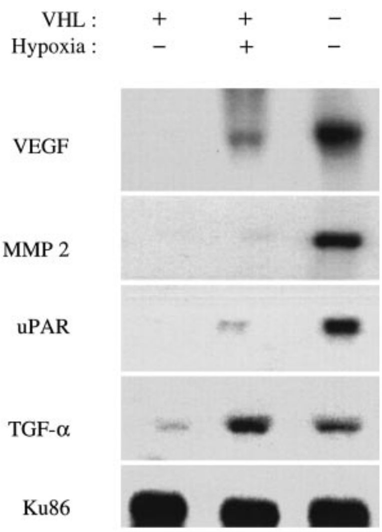

Fig. 4. Effect of VHL deficiency on gene expression in human RCCs. 786-0 cells that either lacked VHL expression (*Lane 3*) or were transfected with a VHL expression vector (*Lanes 1* and *2*) were incubated under nonhypoxic (−) or hypoxic (+) culture conditions for 24 h; and aliquots of total RNA were analyzed by blot hybridization.

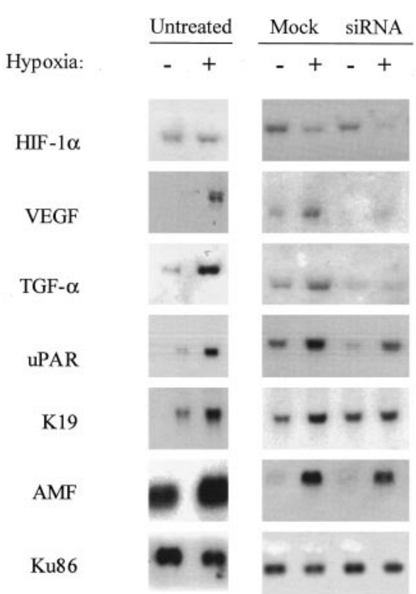

Fig. 5. Effect of RNA interference on the expression of HIF-1α and HIF-1 target genes. HCT116 cells were untreated, mock-transfected, or transfected with $siRNA_{HIF\text{-}1\alpha}$ and, 24 h later. were incubated under nonhypoxic (−) or hypoxic (+) culture conditions for 24 h before RNA isolation for analysis by blot hybridization.

the expression of VEGF, uPAR, TGF-α, and K19 mRNAs under hypoxic conditions. mRNA encoding AMF, which has been shown to promote tumor cell invasion (45), was also expressed in a HIF-1-dependent manner in HCT116 cells. These results are in agreement with recent reports that hypoxia-induced AMF mRNA expression and motility in pancreatic cancer cells is mediated by HIF-1 (46). In contrast, expression of Ku86 mRNA was not induced by hypoxia in HCT116 cells and was unaffected by transfection of $siRNA_{HIF\text{-}1\alpha}$, thus confirming the specific inhibitory effect of $siRNA_{HIF\text{-}1\alpha}$ on HIF-1-dependent gene expression.

Involvement of uPAR in Tumor Cell Invasion. To explore the role of a specific HIF-1 target gene product in the invasive process, we chose to analyze uPAR. In human colorectal cancers, uPAR expression is localized at the invasive front (47) and is correlated with patient mortality (48). A neutralizing antibody against uPAR that blocks its association with uPA has been shown to inhibit hypoxia-induced invasion of MDA-MB-231 breast cancer cells (34). HCT116 cells were transfected with empty vector or HIF-1α expression vector, plated on Matrigel, and incubated for 24 h at 20% or 1% O_2 in the presence or absence of a neutralizing antibody against uPAR. The anti-uPAR antibody significantly inhibited the stimulatory effect of hypoxia and/or HIF-1α overexpression on HCT116 cell invasion (Fig. 6). In contrast, a nonneutralizing anti-uPAR antibody that does not disrupt the interaction of uPAR with uPA had no inhibitory effect on invasion (data not shown). These results indicate that uPAR

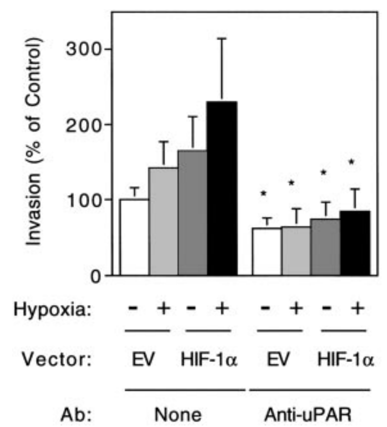

Fig. 6. Effect of inhibiting uPAR activity on invasion of human colon cancer cells. HCT116 cells were transfected with empty vector (*EV*) or HIF-1α expression vector, were plated onto Matrigel in medium containing 0 or 5 μg of an antibody (*Ab*) that binds to uPAR and blocks its interaction with uPA, and were incubated under nonhypoxic (20% O_2) or hypoxic (1% O_2) culture conditions for 24 h. The number of cells invading through Matrigel to the bottom side of the filter was counted and normalized to the number of invading cells transfected with EV and incubated at 20% O_2 in the absence of Ab. Mean and SD ($n = 6$) are shown. *, significant inhibition of invasion relative to cells subjected to the same transfection and culture conditions in the absence of Ab ($P < 0.001$, Student's *t* test).

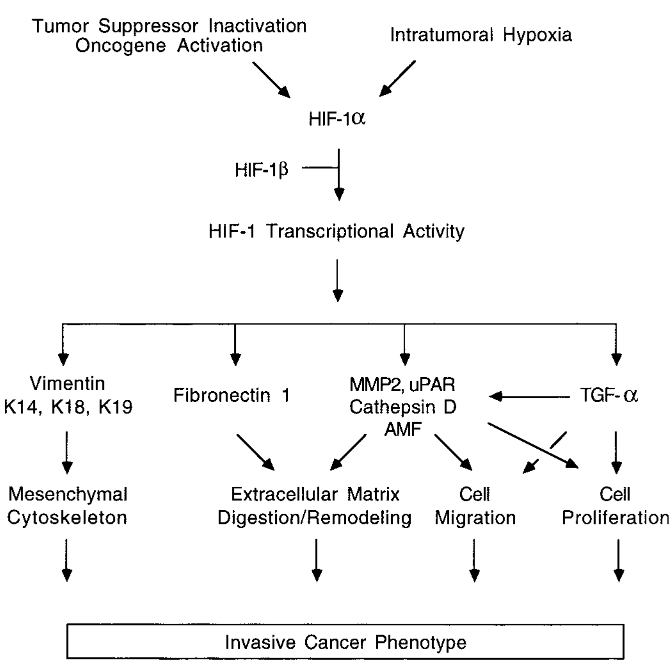

Fig. 7. Pathophysiological roles of HIF-1 target genes in tumor cell invasion. HIF-1 activity is induced as a result of intratumoral hypoxia and genetic alterations. The 10 novel HIF-1 target genes identified in this study (vimentin; keratins K14; K18, K19; fibronectin 1; MMP2; uPAR; cathepsin D; AMF; and TGF-α) have well-established roles in invasion.

activity is required for the stimulatory effect of hypoxia or HIF-1α overexpression on the invasion of HCT116 colon cancer cells through basement membrane.

DISCUSSION

The results presented above provide evidence that HIF-1α overexpression, either as a result of intratumoral hypoxia or genetic alterations, leads to the increased transcription of genes the protein products of which contribute to basement membrane invasion, the defining feature of cancer cells (Fig. 7). The use of transient transfection to alter HIF-1α expression provides a stringent test by eliminating the possible selection of stable transfectants that, in addition to the transgene, contain other genetic alterations that affect their biological behavior. Furthermore, the level of HIF-1α overexpression that was achieved by this method was modest, both in comparison with the hypoxic response and in comparison with the level of HIF-1α overexpression observed in many human cancers. As a result, it is unlikely that our results are caused by high levels of HIF-1α saturating the available amounts of VHL and thus preventing the latter from performing other unrelated functions (an hypothesis that, in any case, could not explain the data from HIF-1α loss-of-function experiments using siRNA).

The data presented in this article provide a molecular basis for clinical and experimental evidence associating tumor invasion and patient mortality with hypoxia and/or HIF-1α overexpression. These results should be interpreted with four caveats in mind. First, transactivation of target genes by HIF-1 is cell-type specific, and it is not expected that the battery of genes reported here will be transactivated by HIF-1 in every cancer. Second, the data presented here do not distinguish between direct and indirect regulation of the identified target genes by HIF-1. Additional studies are required to identify, within each target gene, a functional HIF-1 binding site to conclude that HIF-1 is directly regulating gene expression. Third, for each of these target genes, HIF-1 is not the only transcription factor contributing to its regulation, *i.e.*, HIF-1 exerts a modulating rather than an absolute effect on gene expression. Fourth, these data should not be considered a complete compendium of HIF-1 target genes that contribute to the invasive properties of human cancer cells.

With these caveats in mind, our results, nevertheless, indicate that HIF-1α overexpression affects multiple steps in the complex process of invasion by promoting the ability of cells to undergo mesenchymal transformation and to degrade, remodel, and migrate through the ECM. In particular, our demonstration that exposure of cells to either siRNA against HIF-1α or neutralizing antibodies against uPAR blocked the stimulatory effect of hypoxia or HIF-1α overexpression on invasion provides a direct link between HIF-1 target gene activation and basement membrane invasion by colon cancer cells. The functional relationship between HIF-1α and uPAR established here is consistent with the immunohistochemical detection of these proteins at the invasive front of human colorectal cancers (17, 47).

Combined with its well-established roles in regulating angiogenesis and metabolic adaptation, these results add yet another dimension to the multifaceted involvement of HIF-1 in tumor progression. The coordinated activation by HIF-1 of a large battery of target genes, the protein products of which perform diverse but related functions contributing to tumor invasion, suggests that inhibitors of HIF-1 activity (49) may have therapeutic utility as anticancer agents.

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