Human Enhancer of Filamentation 1 Is a Mediator of Hypoxia-Inducible Factor-1α–Mediated Migration in Colorectal Carcinoma Cells

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Abstract

Human enhancer of filamentation 1 (HEF1; also known as NEDD9 or Cas-L) is a scaffolding protein that is implicated in regulating diverse cellular processes, such as cellular attachment, motility, cell cycle progression, apoptosis, and inflammation. Here, we identify HEF1 as a novel hypoxia-inducible factor-1α (HIF-1α)–regulated gene and reveal that HEF1 mediates hypoxia-induced migration of colorectal carcinoma cells. HEF1 is highly expressed in cultured colorectal carcinoma cells exposed to hypoxia and in the hypoxic areas of human colorectal cancer (CRC) specimens. Moreover, our data show that HIF-1α mediates the effects of hypoxia on induction of HEF1 expression via binding to a hypoxia-responsive element of the HEF1 promoter. Importantly, the induction of HEF1 expression significantly enhances hypoxia-stimulated HIF-1α transcriptional activity by modulating the interaction between HIF-1α and its transcriptional cofactor p300. Inhibition of HEF1 expression also reduced the levels of hypoxia-inducible genes, including those that regulate cell motility. Cell migration was reduced dramatically following knockdown of HEF1 expression under hypoxic conditions. Thus, this positive feedback loop may contribute to adaptive responses of carcinoma cells encountering hypoxia during cancer progression. Cancer Res; 70(10); 4054–63. ©2010 AACR.

Introduction

Hypoxia is a condition resulting from decreased oxygen levels, which frequently occurs in the microenvironment of solid tumors. Due to the rapid growth of solid malignancies, a large part of the tumor is far away from the existing blood vessels that provide oxygen and nutrients. Hypoxia in the tumor microenvironment is associated with cancer progression and causes carcinoma cells to become resistant to therapy. Cells in the hypoxic microenvironment of a solid tumor undergo genetic and adaptive changes that enable them to survive and become more malignant or invasive over time (1). Hypoxia can induce the activation of a transcription factor, hypoxic-inducible factor-1 (HIF-1), consisting of a heterodimeric protein complex that contains an oxygen-dependent α subunit and constitutively expressed β subunit. HIF-1α is the key regulatory component that rapidly degrades during normoxia but becomes stabilized and activated when subjected to hypoxia (2).

HIF-1 must recruit the transcriptional cofactor/histone acetyltransferase protein CBP/p300 to form active transcriptional complexes (3). HIF-1α–activating genes can facilitate the adaptation of the tumor cells and the tumor microenvironment to hypoxia by regulating glycolysis, angiogenesis, pH, and metastasis (1).

Human enhancer of filamentation 1 (HEF1) is a scaffolding protein also called NEDD9 and Cas-L. Recent studies have suggested that HEF1 is an essential regulator for metastasis of melanoma and glioblastoma (4, 5). Moreover, overexpression of HEF1 promotes cancer cell growth, migration, and invasion by activating various signal transduction pathways, such as extracellular signal-regulated kinase, p38, RhoA, and Aurora A (6–9). In addition, several studies reveal that HEF1 is transcriptionally regulated in response to intracellular or extracellular signals (6). Treatment of human skin fibroblasts with transforming growth factor-β led to increased HEF1 expression at both mRNA and protein levels (10). In SH-SY5Y neuroblastoma cells, all-trans retinoic acid stimulated neurite outgrowth and mediated an increase of HEF1 mRNA expression (11). Furthermore, hypoxia is emerging as another regulator of HEF1 expression. For example, HEF1 was upregulated after transient global ischemia in rats (12) and in cultured mesenchymal stem cells in response to hypoxia (13). However, the molecular basis for regulation and function of HEF1 in cancer tissues under hypoxic conditions remains unknown.

This study is the first to analyze the regulatory mechanisms that control HEF1 expression and its functional role in colorectal carcinoma cells exposed to hypoxia. Our results

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show that hypoxia induces HEF1 expression through HIF-1α, and the induction of HEF1 by hypoxia leads to increased cell migration. Importantly, inhibition or overexpression of HEF1 significantly affected hypoxia-stimulated HIF-1α transcriptional activity by modulating the interaction between HIF-1α and its transcriptional coactivator p300. Our findings suggest that a cross-talk between HEF1 and HIF-1α through a positive feedback loop regulates hypoxia-induced migration.

Materials and Methods

Cell culture and treatment. LS174T, DLD-1, SW480, HCT-15, and SW620 cells were purchased from American Type Culture Collection, and HCA-7 cells were provided by Susan Kirkland. All cells were routinely maintained in McCoy’s 5A medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO2 humidified incubator at 37°C. Cells were exposed to hypoxia by placing them in a mixed gas incubator that was infused with an atmosphere consisting of 94% N2, 5% CO2, and 1% O2. Actinomycin D, CoCl2, and YC-1 were purchased from Sigma-Aldrich.

Establishment of stable cell line. LZRS-Ires-GFP-HEF1 and control LZRS-Ires-GFP retroviral vectors (9) were transfected into Phoenix cells in 60-mm dishes using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. After overnight incubation, the transfection medium was removed and replaced by 3 mL of growth medium. Cultured medium containing virus particles was collected 24 hours later and passed through a 0.45-μm filter to remove cell debris. Cells were plated in a 60-mm dish 24 hours before they were infected. Medium containing virus was added to the cells, and the cells were reinfected with freshly collected virus particles 24 hours later. Cells were sorted by green fluorescent protein positivity to eliminate uninfected cells.

Western blotting. Whole-cell lysates were prepared for Western blot analyses using a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% TX-100, 1 mmol/L EDTA (pH 8.0), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Phosphatase inhibitors consisting of 50 mmol/L NaF and 1 mmol/L Na3VO4 were included in the lysis buffer to prevent dephosphorylation of proteins and to preserve the integrity of the binding complexes. Samples were denatured in a SDS sample buffer [30 mmol/L Tris-HCl (pH 6.8), 5% glycerol, 2.5% SDS, 2.5% 2-mercaptoethanol, 0.01% Bromophenol blue]. Total proteins were separated by loading 20 μg of total cell lysate on a denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Triton X-100 and incubated with primary antibodies that recognize HIF-1α (Becton Dickinson), HEF1 (SantaCruz Biotechnology), and actin (Sigma-Aldrich). Secondary antibody conjugated to horseradish peroxidase (Vector Laboratories, Inc.) was used at 1:2000 to detect primary antibodies, and enzymatic signals were visualized by chemiluminescence. Quantification of Western blots was analyzed densitometrically by using Quantity One software (Bio-Rad Laboratories).

Real-time quantitative PCR. Total RNA was isolated by using TRIzol (Invitrogen). cDNA was synthesized from 2 μg of total RNA by using high-capacity cDNA reverse transcription kits (Applied Biosystems). cDNA was mixed with TaqMan Gene Expression Assay Mix, sterile water, and TaqMan Fast Universal PCR Master Mix (Applied Biosystems) to detect mRNA levels of plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase 9 (MMP9), LRP-1, HIF-1α, and HEF1. Specific primers for Fast SYBR Green (Applied Biosystems) are described in Supplementary Table S1. Real-time PCR was carried out using 7900 HT Fast (Applied Biosystems) system, and expression of target genes mRNA relative to 18s rRNA was calculated.

Immunofluorescent staining. Immunofluorescent staining was performed on paraffin-embedded sections using the Tyramide Signal Amplification System (Invitrogen). Paraffin-embedded specimens were treated with xylene and ethanol to remove the paraffin. The slides were immersed in Borg de-cloaker solution (Biocare Medical, Inc.) and boiled in a pressure cooker at 125°C for 5 minutes for antigen retrieval. Endogenous peroxidase activity was blocked by incubating in PBS solution containing 3% H2O2 for 10 minutes. The slides were blocked with TSA blocking reagent and incubated with anti-HEF1 (1:100, Santa Cruz Biotech) and anti-HIF-1α (1:100, Becton Dickinson) at 4°C overnight. After washing with PBS, the slides were incubated with 1:200 biotinylated goat anti-mouse IgG (Vector Laboratories). Streptavidin–horseradish peroxidase (1:200) was then applied to the slides. Thereafter, Alexa Flour 488–labeled tyramide (1:100 in TSA amplification diluents) was used to detect the specific signals. The nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI).

Human colorectal tissue samples. Human colorectal carcinoma specimens were obtained from Tissue Procurement and Banking Facility at University of Texas M.D. Anderson Cancer Center.

RNA interference. siGENOME SMARTpool siRNAs targeting HEF1 (M-019466-02-0005) and HIF-1α (M-004018-05-0005) were purchased from Dharmacon, Inc. SMARTpool strategies use a multicomponent algorithm to identify siRNAs with a significant probability of exerting potent and specific silencing. The SMARTpool approach combines four siRNAs into a single pool, with a high probability of eliminating target mRNA. LS174T cells were transfected with 20 nmol/L of HEF1 siRNA, HIF-1α siRNA, or nontargeting siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s specifications. The efficacy of knockdown was confirmed by Western blot analysis.

DNA constructs. The HEF1 promoter (~850/+4) reporter construct was graciously provided by Dr. Raingeaud (14). The hypoxia-responsive element 1 (HRE1) mutant and HRE2 mutant in the HEF1 promoter were all prepared with the Stratagene Quikchange Site-Directed Mutagenesis kit (Stratagene). The following primers were designed to generate HRE1 and HRE2 mutants: HRE1 mutant, forward 5'-GATGGATGATGTATAGGGCACAATCAGCTGCGACAACCCCATC-3' and reverse 5'-GATGGATGATGTATAGGGCACAATCAGCTGCGACAACCCCATC-3'; HRE2 mutant, forward 5'-CAGGAAAAAGCTGGTGCAATGCTAGAGGGTTCTTTTCT-3' and reverse
5′-GAAAAGGAAACCCTGATGACCGCTTTCCTG-3′. The HRE reporter construct was prepared by inserting the consensus HRE element of the erythropoietin gene promoter (gccctacgtgctgtctca; ref. 15) as a three tandem repeat into pGL3 promoter vector (Dr. Pann-Ghill Suh, POSTEC).

**Luciferase assay.** For dual luciferase reporter assays, LS174T cells were transfected with the firefly luciferase reporter constructs and the control Renilla luciferase reporter pRL-CMV using Lipofectamine (Invitrogen). After treatment, cells were lysed with cell lysis buffer provided by the dual-luciferase reporter assay kit (Promega). Luciferase activity was then measured according to the manufacture’s instruction.

**Nuclear extraction.** Cells were washed with ice-cold PBS and resuspended in 500 μL low-salt buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, 1 mmol/L PMSF]. The nuclei were collected by centrifugation, resuspended in half the packed nuclei equivalent volume of high-salt buffer [20 mmol/L HEPES (pH 7.9), 450 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 25% glycerol, 0.2 mmol/L DTT, 1 mmol/L PMSF], and incubated on ice for 30 minutes with gentle agitation. The nuclear extract was collected by centrifugation.

**Oligonucleotide pull-down assay.** Nuclear extracts were incubated at room temperature for 20 minutes with 5′-biotinylated double-stranded oligonucleotides (16): HRE1 (5′-ATGATGTATAGGGCGTGCACTGGCCAACCC-3′), HRE2 (5′-GAAAACGCTGCGTGTGCTAGAGGGTTCC-3′), and HRE2 mutant (5′-GAAAACGCTGCGTACATGCTAAGGGGTCC-3′) in binding buffer [12 mmol/L HEPES (pH 7.9), 4 mmol/L Tris-HCl (pH 7.9), 150 mmol/L NaCl, 12% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT]. Prewashed streptavidin agarose beads (Pierce) were then added to samples and incubated with agitation for 3 hours at 4°C. Following incubation, the biotinylated oligonucleotide-coupled streptavidin beads were washed four times. Samples were de-natured in SDS sample buffer as described above, and HIF-1α was detected by Western blotting.

**Immunoprecipitation.** Cell lysates (500 μg) were incubated with 2 μg of p300 antibody (SantaCruz Biotechnology) for 6 hours. Protein A/G-agarose (Pierce) was added to each immunoprecipitation reaction. After immunoprecipitation, a 2× SDS sample buffer was added, and the samples were boiled and subjected to Western blotting as described above.

**Collagen-based migration assay.** Transwell (8-μm pores; BD Biosciences) was prepared by coating with type I collagen (Chemicon/Millipore). Cells were seeded in the upper well of each insert at 50,000 per 0.5 mL in serum-free medium. Medium containing 5% serum as chemoattractant was added to the well. The cells were placed in hypoxia or normoxia for 48 hours. Nonmigrating cells were removed from the upper surface by scrubbing with a cotton swab, after which, the membrane was fixed with methanol for 5 minutes and stained with crystal violet. The number of migrating cells was analyzed by computerized image analysis using a Nikon

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**Figure 1.** Hypoxia induces HEF1 expression. A and B, LS174T cells were exposed to normoxia or hypoxia for the indicated times, and then HEF1 mRNA (A) and protein (B) levels were determined using RT-qPCR analysis and Western blots, respectively. C, total RNA was isolated from 16 individual human CRC tissues and corresponding normal mucosa. Equal amounts of mRNA were analyzed by RT-qPCR for HEF1 expression. D, two representative sections show immunofluorescent staining of HEF1 (green) and HIF-1α (green) in two of five human CRC specimens. Nuclei were stained with DAPI (blue; ×10). Circled regions show the area stained by both anti-HEF1 and HIF-1α in the adjacent slides.
SMZ1000 dissecting scope and NIS elements software (Nikon). Digital images of each transwell were acquired, and segmentation analyses were performed to identify the crystal violet stained cells.

**Statistical analysis.** Statistical tests were determined by Student’s *t* test. *P* value of <0.05 was considered significant (*, *P* < 0.05; **, *P* < 0.01).

**Results**

**Hypoxia induces HEF1 expression in colorectal cancer.** We initially analyzed the gene expression profiles in LS174T colon carcinoma cells exposed to normoxia or hypoxia by using microarray assays and found that HEF1 is one of the hypoxia-induced genes (data not shown). To confirm whether hypoxia induces HEF1 expression in LS174T cells, we performed real-time quantitative PCR (RT-qPCR) and Western blotting assays. Our results showed that hypoxia induced HEF1 expression at both the mRNA and protein levels in a time-dependent manner (Fig. 1A and B). Induction of HEF1 protein expression under hypoxic conditions was also observed in several colorectal carcinoma cell lines based on densitometric analyses of Western blots (not shown) as follows: DLD1 (1.92-fold increase), SW620 (2.45-fold increase), HCT-15 (4.34-fold increase), SW480 (1.53-fold increase), and HCA-7 (1.35-fold increase). The greatest increase in HEF1 protein expression occurred in the LS174T cells (7.47-fold), which led us to use the LS174T cells as a model for our studies. To examine whether the expression of HEF1 is relevant to colorectal cancer (CRC) progression, RT-qPCR was performed to measure HEF1 levels in human colorectal carcinomas with the matched normal tissues. HEF1 mRNA levels were elevated in 11 of 16 (69%) cancer specimens compared with those in adjacent normal mucosa (Fig. 1C). To assess the induction of HEF1 expression in hypoxic regions of human CRC specimens, we performed immunofluorescent staining to examine the localization of HEF1 and HIF-1α. HIF-1α is a good indicator of hypoxic regions. As shown in Fig. 1D, the pattern of HEF1 expression correlates well with the expression pattern of HIF-1α in the consecutive sections, showing that HEF1 is also induced in hypoxic regions of the tumors.
**HIF-1α mediates hypoxia-induced HEF1 expression.** To further explore the underlying mechanisms responsible for induction of HEF1, LS174T cells were treated with actinomycin D under either normoxic or hypoxic conditions. Treatment with actinomycin D completely inhibited hypoxia-induced HEF1 mRNA synthesis, showing that HEF1 induction following hypoxia relies on new synthesis of mRNA (Fig. 2A). Because HIF-1α is a critical transcription factor in regulating the cellular transcriptional response to hypoxia (2), we examined whether HIF-1α mediates the hypoxia-induced expression of HEF1. As shown in Fig. 2B, LS174T cells treated with a HIF-1α inhibitor, YC-1, abolished hypoxia-induced HEF1 expression. YC-1 has been shown by others to inhibit HIF-1α expression (17). Moreover, inhibition of HIF-1α by its siRNA blocked hypoxia-induced HEF1 expression at both the protein (Fig. 2C, top) and mRNA levels (Fig. 2C, bottom). Treatment of CoCl2, a chemical inducer of HIF-1α (18), increased HEF1 protein levels. Inhibition of HIF-1α by its siRNA completely inhibited the CoCl2 induction of HEF1 (Fig. 2D), showing that HIF-1α mediates the effects of CoCl2 on induction of HEF1 expression.

As HIF-1α binds to a HRE to activate transcription, we found that two HRE consensus sites, HRE1 (−779/−771) and HRE2 (−647/−639), are present within 1 kb of the HEF1 promoter. Therefore, we examined whether hypoxia induces the promoter activity of the HEF1. Exposure of LS174T to hypoxia for 6 hours resulted in a ~4-fold increase in HEF1 promoter reporter activity compared with normoxia, whereas inhibition of HIF-1α by its siRNA abolished activation of HEF1 promoter following hypoxia (Fig. 3A). Moreover, a mutation in the HRE2 element of HEF1 promoter led to a loss of responsiveness to hypoxia, whereas a mutant HRE1 element in the HEF1 promoter had little effect on HEF1 promoter activity following hypoxia (Fig. 3B). Consistent with above results, our oligonucleotide pull-down assays showed that hypoxia-induced HIF-1α strongly bound to the HRE2 oligonucleotide and weakly bound to the HRE1, whereas hypoxia-induced HIF-1α failed to bind mutated HRE2 oligonucleotides in LS174T cells (Fig. 3C). Taken together, these results verified that HIF-1α is a transcription factor that activates HEF1 transcription when colon carcinoma cells were exposed to hypoxia.
HEF1 regulates HIF-1α transcriptional activity. A, LS174T cells were transiently cotransfected with HEF1-specific siRNA, HRE luciferase, and pRL-CMV plasmids. The luciferase activity was determined. B, LS-174T cells transfected with nontargeting or HEF1 siRNA were exposed to hypoxia for 12 h and then analyzed by Western blotting (top) and RT-qPCR (middle) for HEF1 and HIF-1α. B, bottom, nuclear protein extract was prepared from cells transfected with nontargeting or HEF1 siRNA and incubated with HRE oligonucleotides for pull-down assays. C, left, LS174 T cells were exposed to hypoxia for 8 h, then cell extracts were subjected to immunoprecipitation (IP) with anti-p300 antibody, and the precipitates were analyzed by Western blot (IB) using indicated antibodies. C, right, LS174T cells transfected with nontargeting or HEF1 siRNA were exposed to hypoxia for 8 h. The interaction between HIF-1α and p300 was determined by using immunoprecipitation and Western blotting. D, LS174T cells were stably transfected with vector or HEF1. Left, these cell lines were transfected with HRE luciferase and pRL-CMV plasmids and exposed to hypoxia for 6 h, and then the luciferase activity was determined. Right, cells were exposed to hypoxia for 8 h, then cell extracts were subjected to immunoprecipitation (IP) with anti-p300 antibody, and the precipitates were analyzed by Western blot (IB) using indicated antibodies.
HEF1 enhances HIF-1α transcriptional activity under hypoxic conditions. We next determined whether HEF1 affects hypoxia-induced HIF-1α expression and/or its transcriptional activity. We found that knockdown of HEF1 expression by its siRNA significantly reduced hypoxia-stimulated HIF-1α transcriptional activity (Fig. 4A) but did not affect HIF-1α expression at both the protein (Fig. 4B, top) and mRNA (Fig. 4B, middle) levels or hypoxia-induced HIF-1α DNA binding activity in LS-174T cells (Fig. 4B, bottom). Because transcriptional activity of HIF-1α requires recruiting cotranscriptional factors, such as CBP/p300, we examined whether HEF1 plays a role in recruiting CBP/p300 to form the transcriptional complex with HIF-1α. We found that knockdown of HEF1 expression by its siRNA significantly reduced hypoxia-induced HIF-1α transcriptional activity (Fig. 4A) but did not affect HIF-1α expression at both the protein (Fig. 4B, top) and mRNA (Fig. 4B, middle) levels or hypoxia-induced HIF-1α DNA binding activity in LS-174T cells (Fig. 4B, bottom). Because transcriptional activity of HIF-1α requires recruiting cotranscriptional factors, such as CBP/p300, we examined whether HEF1 plays a role in recruiting CBP/p300 to form the transcriptional complex with HIF-1α. We found that knockdown of HEF1 expression by its siRNA significantly reduced hypoxia-induced HIF-1α transcriptional activity (Fig. 4A) but did not affect HIF-1α expression at both the protein (Fig. 4B, top) and mRNA (Fig. 4B, middle) levels or hypoxia-induced HIF-1α DNA binding activity in LS-174T cells (Fig. 4B, bottom). Hypoxia in the tumor microenvironment contributes to cancer progression by activating adaptive programs that promote cell survival, motility, and tumor angiogenesis. Many cellular responses to hypoxia are mediated through changes in gene expression. In this study, we are the first to show hypoxia induction of HEF1 in cancer cells (Fig. 1), although hypoxia induces HEF1 expression in normal mesenchymal and
neuronal tissues (13). Our current results (Fig. 1C) and the recent report published by our group (9) show that HEF1 expression is elevated in human CRC. Importantly, elevation of HEF1 expression is present in the hypoxic region of human CRC specimens (Fig. 1D). The molecular mechanisms of HEF1 expression in normoxic conditions have been studied, and several transcription factors that regulate HEF1 expression have been identified, including aryl hydrocarbon receptor, SOX2, and RXR/PAR (6, 11, 19). However, the molecular basis of HEF1 regulation under hypoxic conditions is not well understood. Our observation that treatment of CRC cells with actinomycin D under hypoxic conditions reduced HEF1 mRNA expression indicates that hypoxia induction of HEF1 requires new HEF1 mRNA synthesis (Fig. 2A). We found that HIF-1α directly binds to one HRE element (−647 to −639) of the HEF1 promoter to activate HEF1 transcription in colon cancer cells (Figs. 2 and 3), which provides the first evidence to show that HEF1 is a direct HIF-1α target gene.

It is well established that p300 is a critical transcriptional coactivator of HIF-1α (3, 20, 21). Our novel findings show that hypoxia induces formation of a complex that includes p300, HIF-1α, and HEF1. Hypoxia-induced HEF1 enhances HIF-1α transcriptional activity by promoting the interactions between HIF-1α and p300 (Fig. 4), suggesting that HEF1 modulates the assembly of transcriptional machinery of HIF1 and is involved in regulating the expression of a number of hypoxia-inducible genes (Table 1). These results show that HEF1 acts as a positive feedback regulator for HIF-1α under hypoxic conditions. This feedback mechanism between HIF-1α and its target gene is also observed in several other studies (22, 23). This positive feedback signaling can facilitate tumor cells and tumor microenvironment to adapt to the hypoxic conditions.

**Figure 5.** Silencing of HEF1 inhibits hypoxia-induced cell migration. Cells were transfected with nontargeting and HEF1 siRNA, respectively. A, after 24 h, transfecting cells were exposed to hypoxia for 48 h and subjected to Western blotting for HEF1. B, representative images show migrated tumor cells after being exposed to normoxia or hypoxia for 48 h (crystal violet, ×200). C, histogram represents the number of migrated tumor cells. D, a model for a HIF-1α/HEF1–positive feedback loop. Our results indicate that HIF-1α can directly upregulate HEF1 under hypoxic conditions. HIF-1α induction of HEF1 in turn enhances HIF-1α transcriptional activity through increasing the interaction of p300 and HIF-1α. The activation of this loop results in upregulation of genes such as PAI-1, LRP1, and MMP9, which results in promotion of migration of CRC cells.
Extensive data support the notion that hypoxia promotes cancer cell migration and invasion. HIF-1 regulates matrix remodeling that disrupts cell-cell and cell-matrix interactions, which promotes cell migration and invasion through regulating the expression of genes, such as *PAI-1, MMP9*, and *integrin α2* (24–26). Our data that hypoxia induction of HEF1 promotes CRC cell migration through regulating the expression of genes (Table 1; Fig. 5) might help explain how the overexpression of HEF1 leads to metastatic progression in glioblastoma, melanoma, and other cancers. It has been reported that activation of focal adhesion kinase (FAK), a critical mediator of cell adhesion and migration, is regulated by HIF-1α as a regulatory control over adhesion and migration of smooth muscle cells during hypoxia (27). In addition, the observation that HEF1 interacts directly with FAK (4, 28, 29) prompts us to hypothesize that the possible cross-talk between HIF-1α and HEF1 potentially modulates FAK activation during hypoxia-induced migration and invasion. Although our current study provides an explanation for the role of HEF1 in cell migration, other cellular functions of HEF1 during hypoxia need to be evaluated further because HEF1-regulated gene expression is associated with survival (BMP2, FOS), angiogenesis (VEGF), metabolism (PFKP, FABP1, PCK1), and growth (CA9, GDF15, PLAC8, KIT) as shown in the Table 1.

In summary, this study shows that hypoxia induces HEF1 expression via HIF-1α in CRC. The induction of HEF1 regulates HIF-1α transcriptional activity and promotes colon carcinoma cell migration. Identification of this positive feedback loop may help to understand how intratumoral hypoxia promotes metastatic spread of carcinoma cells during progression of CRC.

**Disclosure of Potential Conflicts of Interest**

R.N. DuBois: consultant/advisory board, Tragara. The other authors disclosed no potential conflicts of interest.

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