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Human enhancer of filamentation 1 is a mediator of hypoxia-inducible factor-1 α -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-1 α (HIF-1 α) 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 specimens. Moreover, our data demonstrates that HIF-1 α mediates the effects of hypoxia on induction of HEF1 expression via binding to a hypoxia-responsive element (HRE) of the HEF1 promoter in CRC cells. Importantly, the induction of HEF1 expression significantly enhances hypoxia-stimulated HIF-1 α transcriptional activity by modulating the interaction between HIF-1 α 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 in encountering hypoxia during cancer progression.

Keywords

Human enhancer of filamentation 1 (HEF1); hypoxia; HIF-1 α ; colorectal cancer; carcinoma cell migration

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 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

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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-1 α 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-1-activating genes can facilitate the adaption 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 referred to as NEDD9 and Cas-L. Recent studies have suggested that HEF1 is an essential regulator for metastasis of melanoma and glioblastoma (4,5). Moreover, over-expression of HEF1 promotes cancer cell growth, migration, and invasion by activating various signal transduction pathways such as ERK, 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 TGF- 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 up-regulated 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 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 show that hypoxia induces HEF1 expression through HIF-1 α and the induction of HEF1 by hypoxia leads to increased cell migration. Importantly, inhibition or over-expression of HEF1 significantly affected hypoxia-stimulated HIF-1 α transcriptional activity by modulating the interaction between HIF-1 α and its transcriptional coactivator p300. Our findings suggest that a crosstalk between HEF1 and HIF-1 α 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 the ATCC (Manassas, VA, USA) 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 μ g/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 (St. Louis, MO).

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. Culture medium containing virus particles was collected 24 h later and passed through a 0.45- μ m filter to remove cell debris. Cells were plated in a 60-mm dish 24 h before they were infected. Medium containing virus was added to the

cells and the cells were re-infected with freshly collected virus particles 24 h later. Cells were sorted by green fluorescent protein (GFP) positivity to eliminate uninfected cells.

Western blotting

Whole cell lysates were prepared for Western blot analyses using 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 PMSF. Phosphatase inhibitors consisting of 50 mmol/L NaF and 1 mmol/L Na_3VO_4 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 pH6.8, 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% non-fat dry milk in phosphate-buffered saline containing 0.1% Triton X-100 and incubated with primary antibodies that recognize HIF-1 α (Becton Dickinson, Franklin Lakes, NJ), HIF1 (SantaCruz Biotechnology, Santa Cruz, CA, USA), and actin (Sigma-Aldrich, St. Louis, MO). Secondary antibody conjugated to horseradish peroxidase (Vector Laboratories Inc, Burlingame, CA, USA) was used at 1:2,000 to detect primary antibodies and enzymatic signals were visualized by chemiluminescence. Quantification of Western blots was analyzed densitomerically by using Quantity One[®] software (Bio-Rad Laboratories).

Real-time quantitative PCR (RT-qPCR)

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, Foster City, CA). 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 PAI-1, MMP9, LRP-1, HIF-1 α , and HIF1. Specific primers for Fast SYBR[®] Green (Applied Biosystems) are described in Supplementary table. 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, Carlsbad, CA). Paraffin-embedded specimens were treated with xylene and ethanol to remove the paraffin. The slides were immersed in Borg decloaker solution (Biocare Medical, Inc.) and boiled in a pressure cooker at 125°C for 5 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubating in 3% H_2O_2 containing PBS solution for 10 min. The slides were blocked with TSA blocking reagent and incubated with anti-HEF1 (1:100, Santa Cruz Biotech), and anti-HIF-1 α (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-HRP (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 DAPI.

Human colorectal tissue samples

Human colorectal carcinoma specimens were obtained from Tissue Procurement and Banking Facility (TPBF) at The University of Texas MD Anderson Cancer Center.

RNA interference

siGENOME SMARTpool siRNAs targeting HEF1 (M-019466-02-0005) and HIF-1 α (M-004018-05-0005) were purchased from Dharmacon, Inc. (Chicago, IL). *SMARTpool* strategies utilize a multi-component 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-1 α siRNA, or non-targeting siRNA using LipofectamineTM RNAiMAX (Invitrogen) according to the manufacturer's specifications. The efficacy of knock-down was confirmed by Western blot analysis.

DNA constructs

The HEF1 promoter (-850/+4) reporter construct was graciously provided by Dr. Raingeaud (14). The HRE1 mutant and HRE2 mutant in the HEF1 promoter were all prepared with the Stratagene Quikchange Site-Directed Mutagenesis Kit (Stratagene; San Diego, CA). The following primers were designed to generate HRE1 and HRE2 mutants: HRE1 mutant, forward 5'-GTGATGATGTATAGGGCATACACTGGCCAACCCCATC-3' and reverse 5'-GATGGGGTTGGCCAGTGTATGCCCTATACATCATCAC-3'; HRE2 mutant, forward, 5'-CAGGAAAACGCTGGTGCATATGCTAGAGGGTTCCTTTTC-3', and reverse, 5'-GAAAAGGAACCCTCTAGCATATGCACCAGCGTTTTCCTG-3'. The HRE reporter construct was prepared by inserting the consensus HRE element of the erythropoietin gene promoter (gcctacgtgctgtctca, (15)) as a three tandem repeat into pGL3 promoter vector (Dr. Pann-Ghill Suh, POSTEC, Pohang, South Korea).

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 LipofectamineTM (Invitrogen). After treatment, cells were lysed with cell lysis buffer provided by the dual-luciferase reporter assay kit (Promega, Madison, WI). Luciferase activity was then measured according to the manufacture's instruction.

Nuclear extraction

Cells were washed with ice-cold PBS and resuspended in 500 μ l 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 and 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'-ATGATGTATAGGGCGTGCCTGGCCAACCC-3'); HRE2 (5'-GAAAACGCTGGTGCCTGTGCTAGAGGGTTCC-3'); HRE2 mutant (5'-GAAAACGCTGGTGCATATGCTAGAGGGTTCC-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, Rockford, IL) 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 denatured in SDS sample buffer as described above and HIF-1 α was detected by Western blotting.

Immunoprecipitation

500 µg of cell lysate were incubated with 2 µg of p300 antibody (SantaCruz Biotechnology) for 6 hrs. Protein A/G-agarose (Pierce) was added to each IP reaction. After the IP, 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) were prepared by coating with type I collagen (Chemicon/Millipore, Burlington, MA). Cells were seeded in the upper well of each insert at 50,000 cells 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 hrs. Non-migrating cells were removed from the upper surface by scrubbing with a cotton swab, after which the membrane was fixed with methanol for 5 min and stained with crystal violet. The number of migrating cells was analyzed by computerized image analysis using a Nikon SMZ1000 dissecting scope and NIS elements software (Nikon, Melville, NY). 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 CRC

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 RT-qPCR and Western blotting. 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 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 as 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, demonstrating 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, demonstrating that the HEF1 induction following hypoxia relies on new synthesis of mRNA (Fig. 2A). Since HIF-1α is a critical transcription factor in regulating the cellular transcriptional

response to hypoxia (2), we examined whether HIF-1 α mediates the hypoxia-induced expression of HEF1. As shown in Fig. 2B, LS174T cells treated with a HIF-1 α inhibitor, YC-1, abolished hypoxia-induced HEF1 expression. YC-1 has been shown by others to inhibit HIF-1 α expression (17). Moreover, inhibition of HIF-1 α 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-1 α (18), increased HEF1 protein levels. Inhibition of HIF-1 α by its siRNA completely inhibited the CoCl₂ induction of HEF1 (Fig. 2D), demonstrating that HIF-1 α mediates the effects of CoCl₂ on induction of HEF1 expression.

As HIF-1 α binds to the hypoxia response element (HRE) in order to activate transcription, we found that two HRE consensus sites, HRE1 (-779/-771) and HRE2 (-647/-639), are present within 1kb of the HEF1 promoter. Therefore, we examined whether hypoxia induces the promoter activity of the HEF1. Exposure of LS174T to hypoxia for 6 hrs resulted in a ~3 fold increase in HEF1 promoter reporter activity compared with normoxia, whereas inhibition of HIF-1 α 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-1 α strongly bound to the HRE2 oligonucleotide and weakly bound to the HRE1, whereas hypoxia-induced HIF-1 α failed to bind mutated HRE2 oligonucleotides in LS174T cells (Fig. 3C). Taken together, these results demonstrated that HIF-1 α is a transcription factor that activates HEF1 transcription when colon carcinoma cells were exposed to hypoxia.

HEF1 enhances HIF-1 α transcriptional activity under hypoxic conditions

We next determined whether HEF1 affects hypoxia-induced HIF-1 α expression and/or its transcriptional activity. We found that knockdown of HEF1 expression by its siRNA significantly reduced hypoxia-stimulated HIF-1 α transcriptional activity (Fig. 4A), but did not affect HIF-1 α expression at both the protein (Fig. 4B, top) and mRNA (Fig. 4B, middle) levels and hypoxia-induced HIF-1 α DNA binding activity in LS-174T cells (Fig. 4B, bottom). Since transcriptional activity of HIF-1 α requires recruiting co-transcriptional factors such as CBP/p300 (3), we examined whether HEF1 plays a role in recruiting CBP/p300 to form the transcriptional complex with HIF-1 α . Our results showed that hypoxia induced the formation of a complex that includes p300, HIF-1 α and HEF1 (Fig. 4C, left). Furthermore, HEF1 silencing significantly decreased the level (about 36% inhibition) of interaction of HIF-1 α and p300 induced by hypoxia (Fig. 4C, right). In contrast, over-expression of HEF1 results in induction of HIF-1 α transcriptional activity (Fig. 4D, left panel) and led to an increase in the interaction of HIF-1 α with p300 (Fig. 4D, right panel) in LS174T cells under normoxic conditions. These results demonstrate that induction of HEF1 following hypoxia in colon carcinoma cells regulates HIF-1 α transcriptional activity by enhancing the interaction of HIF-1 α with its co-activator. To evaluate if HEF1 mediates the effects of HIF-1 α , we assessed the downstream target genes for both HEF1 and HIF-1 α under hypoxia. We selected 33 genes that were induced by hypoxia in LS174T cells. HIF-1 α silencing blocked all of hypoxia-induced genes we selected while HEF1 silencing attenuated 26 of 33 hypoxia-induced genes including VEGF, suggesting that HEF1 can regulate a large cluster of hypoxia-inducible genes via HIF-1 α (Table 1). These results suggest that HEF1 is an important enhancer for HIF-1 α -induced gene expression under hypoxic conditions.

HEF1 mediates hypoxia-induced colon carcinoma cell migration

Since HEF1 is involved in regulating cell motility (5) and our findings show that HEF1 is involved in regulating genes that are involved in cell motility such as PAI-1, LRP1, MMP9,

and integrin $\alpha 2$ (Table 1), we examined whether HEF1 affects the migration of colon carcinoma cells under hypoxic conditions. Hypoxia increased cell migration, which was dramatically blocked by knockdown of HEF1 (Fig. 5). These findings indicate that hypoxia-induced HEF1 promotes cancer cell migration via induction of the genes involved in the regulation of cell motility.

Discussion

Among its many functions, HEF1 is associated with cancer invasion and metastasis. However, the role of HEF1 in the hypoxic tumor microenvironment remains unknown. Our study is the first to suggest that HEF1 is a new member of the HIF-1 transcriptome. Furthermore, hypoxia-induced HEF1 expression via HIF-1 α positively enhances HIF-1 α transcriptional activity. This positive feedback loop between HEF1 and HIF-1 α promotes colon tumor cell migration.

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 demonstrate 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-1 α directly binds one HRE element (-647 to -639) of HEF1 promoter to activate HEF1 transcription in colon cancer cells (Fig. 2 and 3), which provides the first evidence to show that HEF1 is a direct HIF-1 α target gene.

It is well established that p300 is a critical transcriptional co-activator of the HIF-1 α (3,20, 21). Our novel findings demonstrate that hypoxia induces formation of a complex that includes p300, HIF-1 α and HEF1. The hypoxia-induced HEF1 enhances HIF-1 α transcriptional activity by promoting the interactions between HIF-1 α and p300 (Fig. 4), suggesting that HEF1 modulates the assembly of transcriptional machinery of HIF1 and is involved in the expression of a large population of hypoxia-inducible genes (Table 1). These results demonstrate that HEF1 acts as a positive feedback regulator for HIF-1 α under hypoxic conditions. This feedback mechanism between HIF-1 α 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.

Extensive data supports 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 PAIs, MMPs, and integrins (24-26). Our data that hypoxia induction of HEF1 promotes CRC cell migration through regulating the expression of genes (Table 1 and Fig. 5) might help explain how the over-expression 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-1 α 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 crosstalk between HIF-1 α 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 the 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 demonstrates that hypoxia induces HEF1 expression via HIF-1 α in CRC. The induction of HEF1 regulates HIF-1 α 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 colorectal cancer 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 2 of 5 human colorectal cancer specimens. Nuclei were stained with DAPI (blue) (X10). Circled regions show the area stained by both anti-HEF1 and HIF-1α in the adjacent slides.

Figure 2.

HIF-1 α is responsible for hypoxia-induced HEF1 expression. *A*, LS174T cells were treated with 1 μ g/ml actinomycin D under normoxia/hypoxia for 6 hrs and then HEF1 mRNA levels were determined using RT-qPCR analyses. *B*, LS174T cells were exposed to hypoxia in the presence of increasing concentration of YC-1 for 12 hrs, followed by Western blotting for HEF1 and HIF-1 α . *C*, Cells were transfected with non-targeting and HIF-1 α siRNA, respectively. After twenty-four hours, transfecting cells were exposed to hypoxia for 12 hrs and subjected to analysis of Western blotting for targeting protein levels (*top*) and RT-qPCR for mRNA levels (*bottom*). *D*, Cells transfected with non-targeting or HIF-1 α siRNA were

treated with 100 $\mu\text{mol/L}$ CoCl_2 for 3 hrs and then the protein levels of HIF-1, HIF-1 α and actin were determined by using Western blotting.

Figure 3.

Direct involvement of HIF-1 α for HEF1 expression. *A*, Cells were transfected with non-targeting or HIF-1 α siRNA, HEF1 promoter luciferase reporter, and renilla luciferase (pRL-CMV) plasmids and then were incubated under hypoxic conditions for 24 hrs. Cell lysates were analyzed by luciferase assay. Luciferase activity represents data that have been normalized into cotransfected renilla luciferase activity. *B, top*, A diagram of the promoter construct. *Bottom*, Transient transfection of cells was performed with HEF1 promoter wild type (WT), HRE1 mutant (HRE1 M), or HRE2 mutant (HRE2 M) luciferase reporter plasmids and then cells were incubated for 24 hrs under hypoxic conditions. The luciferase activity was determined. *C*, LS174T cells were exposed to normoxia (nor)/hypoxia (hy) for 12 hrs followed by the isolation of nuclear protein extract. The oligonucleotide pull-down assay was performed as described in Materials and Methods.

Figure 4. 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 non-targeting or HEF1 siRNA were exposed to hypoxia for 12 hrs and then analyzed by Western blotting (*top*) and by RT-qPCR (*middle*) for HEF1 and HIF-1α. *B*, *bottom*, Nuclear protein extract was prepared from cells transfected with non-targeting or HEF1 siRNA and incubated with HRE oligonucleotides for pull-down assays. *C*, *left*, LS174 T cells were exposed to hypoxia for 8 hrs and 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 non-targeting or HEF1 siRNA were exposed to hypoxia for 8 hrs. The interaction between HIF-1α and p300 was determined by using IP 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 hrs and then the luciferase activity was determined. *Right*, Cells were exposed to hypoxia for 8 hrs and then cell extracts were subjected to immunoprecipitation (IP) with anti-p300 antibody and the precipitates were analyzed by Western blot (IB) using indicated antibodies.

Figure 5.

Silencing of HEF1 inhibits hypoxia-induced cell migration. Cells were transfected with non-targeting and HEF1 siRNA, respectively. *A*, After twenty-four hours, transfecting cells were exposed to hypoxia for 48 hrs and subjected to Western blotting for HEF1. *B*, Representative images show migrated tumor cells after being exposed to normoxia or hypoxia for 48 hrs (crystal violet, X200). *C*, Histogram represents the number of migrated tumor cells. *D*, A model for a HIF-1 α /HEF1 positive feedback loop. Our results indicate that HIF-1 α can directly up-regulate HEF1 under hypoxic conditions. HIF-1 α induction of HEF1 in turn enhances HIF-1 α transcriptional activity through increasing the interaction of p300 and HIF-1 α . The activation of this loops results in up-regulation of genes such as PAI-1, LRP2 and MMP9, which leads to promotion of migration of CRC cells.

Table 1

Effect of HIF-1 α or HIF1 silencing on genes up-regulated by hypoxia. LS174T cells transfected with non-targeting HIF-1 α , or HIF1 siRNA were exposed to normoxia or hypoxia for 12 hrs and then analyzed by RT-qPCR for changes in hypoxia-inducible gene expression. Results shown are expressed as fold increase of hypoxia versus normoxia. The values represent the mean of three different experiments done in triplicate $\pm$ SD. §, not significant.

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Target gene	Non-targeting siRNA	HIF-1 α siRNA	HEF1 siRNA
Integrin α -2	12.02 $\pm$ 2.62	3.64 $\pm$ 0.52	2.54 $\pm$ 0.43
PLAC8	4.77 $\pm$ 0.25	3.080 $\pm$ 0.08	1.85 $\pm$ 0.22
ELF3	14.80 $\pm$ 1.08	1.52 $\pm$ 0.03	6.06 $\pm$ 0.15
STC1	14.94 $\pm$ 0.53	2.86 $\pm$ 0.17	6.81 $\pm$ 0.22
ADM	34.42 $\pm$ 4.88	9.99 $\pm$ 2.37	20.17 $\pm$ 1.84
MKNK2	9.60 $\pm$ 0.50	1.94 $\pm$ 0.08	5.01 $\pm$ 0.49
PFKP	4.20 $\pm$ 0.55	1.30 $\pm$ 0.15	2.27 $\pm$ 0.29
PAI	13.03 $\pm$ 0.47	3.12 $\pm$ 0.57	7.18 $\pm$ 0.50
ADFP	12.59 $\pm$ 1.94	2.69 $\pm$ 0.52	7.12 $\pm$ 1.60
ALOXE3	4.260 $\pm$ 0.32	1.28 $\pm$ 0.16	2.49 $\pm$ 0.10
VEGF	15.260 $\pm$ 1.57	6.39 $\pm$ 1.98	8.57 $\pm$ 0.75
BMP2	8.60 $\pm$ 0.50	2.73 $\pm$ 0.33	5.15 $\pm$ 0.32
KIT	3.93 $\pm$ 0.09	1.25 $\pm$ 0.08	2.42 $\pm$ 0.18
CA9	18.85 $\pm$ 2.72	3.54 $\pm$ 0.26	11.72 $\pm$ 1.88
MMP9	2.42 $\pm$ 0.24	1.56 $\pm$ 0.24	1.51 $\pm$ 0.23
FABP1	8.86 $\pm$ 0.32	3.07 $\pm$ 0.30	6.11 $\pm$ 0.49
PCK1	63.21 $\pm$ 5.06	29.98 $\pm$ 2.25	40.61 $\pm$ 2.73
NRN1	8.01 $\pm$ 0.08	2.76 $\pm$ 0.49	5.17 $\pm$ 0.54
FOS	27.840 $\pm$ 1.26	12.81 $\pm$ 0.58	16.89 $\pm$ 0.68
ALOX5	2.73 $\pm$ 0.07	1.19 $\pm$ 0.11	1.760 $\pm$ 0.08
DDIT4	12.60 $\pm$ 1.06	5.35 $\pm$ 0.36	8.79 $\pm$ 0.05
BHLHE40	24.20 $\pm$ 1.09	10.03 $\pm$ 0.56	17.360 $\pm$ 0.07
AOAH	5.190 $\pm$ 0.19	2.48 $\pm$ 0.15	3.79 $\pm$ 0.13
LRP1	9.28 $\pm$ 0.40	1.03 $\pm$ 0.09	6.82 $\pm$ 0.34
GDF15	9.00 $\pm$ 0.46	5.55 $\pm$ 0.33	7.70 $\pm$ 0.26
BNIP2	8.60 $\pm$ 4.51	2.14 $\pm$ 0.42	4.70 $\pm$ 0.58
TXNIP	6.59 $\pm$ 1.06	1.81 $\pm$ 0.29	5.06 $\pm$ 0.54§
ERRFI1	22.24 $\pm$ 1.29	9.01 $\pm$ 0.22	20.80 $\pm$ 0.34§
ANKRD37	57.83 $\pm$ 6.00	19.44 $\pm$ 2.61	54.20 $\pm$ 4.49§
PLOD2	12.02 $\pm$ 3.09	3.21 $\pm$ 0.37	11.80 $\pm$ 0.89§
PGK1	5.78 $\pm$ 1.10	1.63 $\pm$ 0.15	4.92 $\pm$ 0.29§
PPP1R3B	4.580 $\pm$ 0.53	2.7 $\pm$ 0.33	4.52 $\pm$ 0.03§
SLC2A	21.632 $\pm$ 2.45	5.80 $\pm$ 0.63	18.57 $\pm$ 0.17§