

# HIF-1 $\alpha$ Promotes A Hypoxia-Independent Cell Migration

Liyuan Li<sup>1</sup>, Chikezie O. Madu<sup>1</sup>, Andrew Lu<sup>2,3</sup> and Yi Lu<sup>\*1,2</sup>

<sup>1</sup>Department of Pathology and Laboratory Medicine, <sup>2</sup>Department of Medicine, University of Tennessee Health Science Center, Memphis, TN; <sup>3</sup>Princeton University, Princeton, NJ, USA

**Abstract:** Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is known as a transactivator for VEGF gene promoter. It can be induced by hypoxia. However, no study has been done so far to dissect HIF-1 $\alpha$ -mediated effects from hypoxia or VEGF-mediated effects. By using a HIF-1 $\alpha$  knockout (HIF-1 $\alpha$  KO) cell system in mouse embryonic fibroblast (MEF) cells, this study analyzes cell migration and HIF-1 $\alpha$ , hypoxia and VEGF activation. A hypoxia-mediated HIF-1 $\alpha$  induction and VEGF transactivation were observed: both HIF-1 $\alpha$  WT lines had significantly increased VEGF transactivation, as an indicator for HIF-1 $\alpha$  induction, in hypoxia compared to normoxia; in contrast, HIF-1 $\alpha$  KO line had no increased VEGF transactivation under hypoxia. HIF-1 $\alpha$  promotes cell migration: HIF-1 $\alpha$ -KO cells had a significantly reduced migration compared to that of the HIF-1 $\alpha$  WT cells under both normoxia and hypoxia. The significantly reduced cell migration in HIF-1 $\alpha$  KO cells can be partially rescued by the restoration of WT HIF-1 $\alpha$  expression mediated by adenoviral-mediated gene transfer. Interestingly, hypoxia has no effect on cell migration: the cells had a similar cell migration rate under hypoxic and normoxic conditions for both HIF-1 $\alpha$  WT and HIF-1 $\alpha$  KO lines, respectively. Collectively, these data suggest that HIF-1 $\alpha$  plays a role in MEF cell migration that is independent from hypoxia-mediated effects.

**Keywords:** VEGF, cell migration, hypoxia, HIF-1 $\alpha$ .

## INTRODUCTION

Hypoxia, a reduction of oxygen level in tissue due to insufficient delivery of oxygen to the demand of the tissue [1], can arise from either physiologic circumstances such as exercise and travel to high elevation, or from pathophysiologic conditions such as poorly formed tumor vasculature [2]. A key regulator of the cellular response to hypoxia is the hypoxia-inducible factor-1 (HIF-1), a master-transcriptional activator for a group of genes that are responsible for cellular adaptation to hypoxia [3, 4]. HIF-1 is composed of an inducible subunit, HIF-1 $\alpha$  and a constitutively expressed subunit, HIF-1 $\beta$  [5]. HIF-1 $\alpha$  is a protein of 120 Kd, member of the basic helix-loop-helix superfamily transcription factors, and its expression is highly sensitive to oxygen concentration [6, 7]. Under normal oxygen tension (normoxia), HIF-1 $\alpha$  is rapidly degraded by a posttranslational ubiquitination-triggered proteolysis, and this degradation process requires von Hippel Lindau (VHL) protein [8]. However, under low oxygen tensions (hypoxia), HIF-1 $\alpha$  becomes stabilized, translocates to the nucleus, and heterodimerizes with HIF-1 $\beta$  to form the complex HIF-1, which binds to hypoxic response elements (HRE, i.e., the DNA sequence where HIF-1 $\alpha$  binds) within the promoter regions of target genes and starts transcribing the target genes [3, 9, 10]. Thus, HIF-1 transcription activity is controlled by expression of HIF-1 $\alpha$ , the inducible subunit of the dimer complex. Hypoxia induces expression of HIF-1 $\alpha$ , which then transactivates a variety of its downstream genes including VEGF [5].

Cell migration plays a central role in a wide variety of biological phenomena. Migration/motility is essential for various cellular functions involved in both normal physiology such as embryogenesis, inflammatory response, wound healing and pathology such as tumor metastasis [11]. Vitexin, a HIF-1 $\alpha$  inhibitor, was shown to significantly inhibit the migration of rat pheochromocytoma PC12 cells along with hypoxia-induced activation of c-jun N-terminal kinase (JNK) [12], suggesting that hypoxia or HIF-1 $\alpha$  may play a role in cell migration. In another study whose purpose was to analyze the hypoxic effect on the development of tissue fibrosis, it was found that HIF-1 $\alpha$  enhanced epithelial-to-mesenchymal transition (EMT) *in vitro* and induced renal epithelial cell migration through upregulation of lysyl oxidase genes [13].

However, there is so far no dissect analysis that separates hypoxia's and HIF-1 $\alpha$ 's individual effect on cell migration, probably due to the fact that hypoxia always accompanies induction of HIF-1 $\alpha$  so it is difficult to dissect the differential roles between the two. In addition, there is no report directly on HIF-1 $\alpha$ 's effect on cell migration by rescue study (for example, functional regain of HIF-1 $\alpha$  on HIF-1 $\alpha$  knockout cells). Moreover, whether hypoxia directly affects migratory ability of the cells and whether the presence of HIF-1 $\alpha$  is required for the cell migration, have never been studied previously to our knowledge. In this report, in order to analyze whether hypoxia or the presence of HIF-1 $\alpha$  affect cell migration and dissect their individual roles in cell migration, respectively, we used MEF cell system that contains both cells expressing wild-type HIF-1 $\alpha$  (HIF-1 $\alpha$  WT) and cells lacking HIF-1 $\alpha$  expression (HIF-1 $\alpha$  knockout line or HIF-1 $\alpha$  KO), as well as the phenotypic rescued HIF-1 $\alpha$  KO cells that regain HIF-1 $\alpha$  expression by adenoviral-mediated gene transfer.

\*Address correspondence to this author at the Department of Pathology and Department of Medicine, University of Tennessee Health Science Center, Cancer Research Building, Room 218, 19 South Manassas Street, Memphis, TN 38163, USA; Tel: (901) 448-5436; Fax: (901) 448-5496; E-mail: ylu@utmem.edu

## MATERIALS AND METHODOLOGY

### Cell Lines, Culture Medium, and (Normoxic and Hypoxic) Incubation Conditions

Dulbecco's modified Eagle medium (DMEM) and RPMI-1640 were purchased from Gibco BRL (Gaithersburg, MD), and fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT). MEF RT  $\beta$ -gal line (HIF-1 $\alpha$  wild-type or HIF-1 $\alpha$  WT) and MEF RT CRE line (HIF-1 $\alpha$  knockout or HIF-1 $\alpha$  KO) (generous gifts from Drs. R. Johnson of UCSD and T. Seagroves of UTHSC, [14]) were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose and 25 mM HEPES (Gibco BRL). Human embryonic kidney 293 cells (American Type Culture Collection, Rockville, MD) were grown in D-MEM with 10% heat inactivated FBS. Human breast cancer cell lines MDA-MB-231 (ATCC) was grown in RPMI-1640 medium with 10% FBS. All cell lines were grown in medium containing 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in 5% CO<sub>2</sub>, 21% O<sub>2</sub> (normoxic conditions). Hypoxic conditions were induced by exposing the cells at 37°C to 5% CO<sub>2</sub> and 1% O<sub>2</sub>, balance with N<sub>2</sub>, in the incubator.

### Adenoviral Vector Construction

The construction of recombinant adenovirus expressing the HIF-1 $\alpha$ , Ad-HIF-1 $\alpha$ , was done as follows: The HaloTag pHT2 vector (Promega, Madison, WI) was cut with EcoR V and Not I to release a about 890-bp HT2 cDNA, which encodes a HaloTag reporter gene (Promega). The HT2 cDNA was subcloned into the pacAd5CMVNP $\alpha$  (University of Iowa, Iowa City, IA) to generate the adenoviral shuttle vector pacAd5-HT2. A 2.5-kb PCR product of human HIF-1 $\alpha$  cDNA was generated from HIF-1 $\alpha$  expression vector (pHIF-1 $\alpha$ , a generous gift from Dr. B. Jiang of West Virginia University, Ref. 5) using a set of primers specific to the human HIF-1 $\alpha$  cDNA gene, the primers contained an introduced Bam HI site at both ends of the PCR products. After digestion with Bam HI on both the 2.5-kb PCR product of HIF-1 $\alpha$  and pacAd5-HT2, the HIF-1 $\alpha$  cDNA was inserted into at the 5'-terminal of the HT2 gene to create an adenoviral shuttle vector pacAd5-HIF-1 $\alpha$ /HT2. The pacAd5-HIF-1 $\alpha$ /HT2 was cotransfected with adenoviral genome vector into the 293 cells to generate the recombinant adenovirus Ad-HIF-1 $\alpha$ . The construction of the control virus, AdRSVlacZ, in which a bacterial  $\beta$ -galactosidase gene was under the control of the RSV promoter, was described previously [15].

### Adenoviral Vector Preparation, Titration and Transduction

Individual clones of Ad-HIF-1 $\alpha$  and AdRSVlacZ were obtained by a serial of plaque purification [16]. Single clone of Ad-HIF-1 $\alpha$  and AdRSVlacZ, respectively, was propagated in HEK 293 cells. The culture medium of the HEK 293 cells showing the complete cytopathic effect was collected, and adenoviral vectors were purified by BD Adeno-X Virus Purification Kits (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. The viral titration and transduction were performed as standard procedures as previously described [17].

### Western Blot

Cells were lysed in lysis buffer containing 10mM Tris-HCl (pH 8.0), 0.25 M Sucrose, 0.05 mM CaCl<sub>2</sub>, 0.02% Azide, 0.5% NP-40, 1x protease inhibitor cocktail (Sigma, St. Louis, MO), and 1x phosphatase inhibitor cocktail I and II (Sigma), and processed for gel electrophoresis as previous described [18]. Protein concentration was determined by using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Protein extracts were loaded on a precast 4-12% gradient polyacrylamide gel (NuPAGE gel, Invitrogen, Carlsbad, CA) (100  $\mu$ g/per lane) and subject to SDS-gel electrophoresis, followed by transferring to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Transfer Membrane, Millipore). The membrane was incubated with blocking solution (5% nonfat milk and 0.02% sodium azide in phosphate-buffered saline) overnight at 4°C. The membrane was incubated for 16 h at 4°C with the mouse anti-human HIF-1 $\alpha$  antibody (1:1,000 dilution, cat # 61958, BD Biosciences). The membrane was then incubated for 1h at room temperature with goat anti-mouse secondary coupled to peroxidase, followed by detection with chemiluminescent reagents (ECL kit, Amersham).

### Immunohistochemistry

The culture cells were grown on SlideFlasks with detachable bottom slides (Nalge Nunc, Naperville, IL) that could be directly used for immunohistochemistry staining later. After fixing with 10% formalin and treating with hydrogen peroxide to inactivate endogenous peroxidase, the samples (slides) were incubated with mouse anti-human HIF-1 $\alpha$  antibody (1:60 dilution, 36.5  $\mu$ g/ml, cat# NB 100-105, Novus Biologicals, Littleton, CO) for 16 h at 4°C, then by a corresponding second antibody (1:200 dilution) of the Universal Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's protocol. The reaction was visualized with DAB solution (75 mg 3,3'-Diaminobenzidine and 30 ml 50% H<sub>2</sub>O<sub>2</sub> in 150 ml PBS) for 3-10 min.

### Transactivation Assay of VEGF Promoter

HIF-1 $\alpha$  WT and KO cells were cultured in 24-well plates. A chimeric construct containing a 2.4-kb VEGF promoter and a luciferase reporter gene (pVEGF/Luc) [5] (a generous gift from Dr. B. Jiang of West Virginia University) were co-transfected with pRLuc-TK (Promega) (20:1 ratio, w/w), a plasmid expressing Renilla luciferase as an internal control for normalization of transfection efficiency. Eighteen h after co-transfection, the transfected cells were incubated at either normoxia (37°C in 5% CO<sub>2</sub>, 21% O<sub>2</sub>) or hypoxia (37°C in 5% CO<sub>2</sub>, 1% O<sub>2</sub>) conditions for another 30 h at 37°C. The cell extracts were then harvested and analyzed for luciferase activity using Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions. The luciferase activity was detected by using a luminometer (Turner Designs Instrument, Model TD2020, Sunnyvale, CA). The normalized luciferase activity was represented as ratio of Firefly luciferase activity (pVEGF/ Luc) over Renilla luciferase activity (pRLuc-TK). At least two independent experiments were done with each performed in triplicates.

## Cell Migration Assay

The cell migration was measured by a modified Boyden's chamber (transwell) method using a 24-well plate that contains inserts with polyethylene terephthalate (PET) track-etched membrane with a pore size of 8.0  $\mu\text{m}$  (BD Biosciences). The inserts were precoated on the undersurface (between upper and lower chambers) with 10  $\mu\text{g/ml}$  fibronectin (Sigma) at 37°C for 3 h. The HIF-1 $\alpha$  WT and KO cells, or KO cells transduced by Ad-HIF-1 $\alpha$  (HIF-1 $\alpha$  KO/Ad-HIF-1 $\alpha$ ) were cultured for 24 h under normoxic (37°C in 5% CO<sub>2</sub>, 21% O<sub>2</sub>) and hypoxic (37°C in 5% CO<sub>2</sub>, 1% O<sub>2</sub>) conditions, respectively. The cells were then harvested for the following transwell assay. After briefly incubating the cells at 37°C for 30 min (normoxia or hypoxia) in serum-free DMEM medium with 1% BSA (Sigma), the cell suspension was seeded into the upper chamber of an insert at a density of 10,000 cells per well, and the insert was placed onto the well of a 24-well plate that contains serum-free medium with 1% BSA. The plate was then incubated at normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>), correspondingly, for 3 h at 37°C for the transwell motility assay. The inserts were then removed and nonmigratory cells were removed from the upper chamber by gentle scraping. The cells that had migrated to the lower surface of the insert were stained using Giemsa staining solution (Sigma). After extensive washing, the migrated cells were counted in five different fields under a microscope at x200 magnification. Migratory activity was expressed as the mean number of cells that migrated to the lower side of the filter, and results were represented as sum of total cell numbers in five randomly selected fields of view. At least three independent experiments were done with each performed in duplicates.

## Statistical Analysis

All statistical analysis was performed using Student's t-test.

## RESULTS

### Ad-HIF-1 $\alpha$ Successfully Transduces and Expresses HIF-1 $\alpha$ Protein in Mammalian Cells

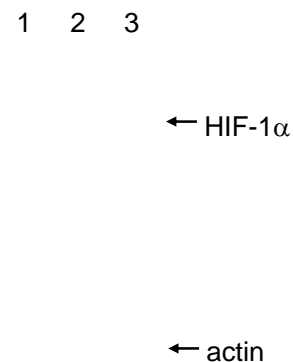
To examine the HIF-1 $\alpha$  effect on cell migration, both HIF-1 $\alpha$  WT and KO lines were used in this study. In addition, to confirm that a loss of cellular function in KO line is indeed due to the lack of HIF-1 $\alpha$ , we have generated a system that enables the restoring of HIF-1 $\alpha$  expression in KO line, namely, a recombinant adenoviral vector expressing HIF-1 $\alpha$  gene (Ad-HIF-1 $\alpha$ ) was constructed. To demonstrate that this Ad-HIF-1 $\alpha$  is functional and able to transduce and express HIF-1 $\alpha$  protein in mammalian cells generally, breast cancer MDA-MB-231 cells were transduced with Ad-HIF-1 $\alpha$  for 72 h and the cell extracts were subjected to a Western blot analysis using anti-HIF-1 $\alpha$  as the primary antibody. As shown in Fig. (1), cells transduced with Ad-HIF-1 $\alpha$  indeed expressed HIF-1 $\alpha$  protein at an expected size around 120 Kd (lane 2 and lane 3, Fig. 1). The cell extracts from MDA-MB-231 cells incubated under the hypoxia conditions were used as positive control to show the induced endogenous expression of HIF-1 $\alpha$  protein (lane 1, Fig. 1).



**Fig. (1). Ad-HIF-1 $\alpha$  successfully transduces and expresses HIF-1 $\alpha$  protein in mammalian cells**

MDA-MB-231 cells were transduced with either Ad-HIF-1 $\alpha$  at moi of 200 (lane 2) or moi of 50 (lane 3), control virus AdRSVlacZ (lane 4), or no virus (lane 5) for 72 h. Cell extracts (100  $\mu\text{g}$  per lane) were loaded for Western blot analysis and proceeded using anti-HIF-1 $\alpha$  antibody as the primary antibody. As a positive control, cell extracts of MDA-MB-231 cells that were incubated under hypoxic conditions (1% O<sub>2</sub>, 5% CO<sub>2</sub>) for 18 h were used for demonstrating endogenous HIF-1 $\alpha$  protein induction (lane 1). In addition, a housekeeper protein, actin, was used for normalization of equal protein loading in each lane.

The Western blot of cell extracts derived from MEF HIF-1 $\alpha$  WT and HIF-1 $\alpha$  KO lines showed an endogenous HIF-1 $\alpha$  expression in HIF-1 $\alpha$  WT cells (lane 1, Fig. 2), whereas no detectable expression of HIF-1 $\alpha$  protein was found in HIF-1 $\alpha$  KO cells (lane 2, Fig. 2). This result has verified and confirmed that KO line does not express detectable endogenous HIF-1 $\alpha$  protein, even under hypoxic conditions. However, when HIF-1 $\alpha$  KO cells were transduced by Ad-HIF-1 $\alpha$  at multiplicity of infection (moi) of 200 for 72 h, the



**Fig. (2). Endogenous and exogenous expression of HIF-1 $\alpha$  protein in MEF HIF-1 $\alpha$  WT and HIF-1 $\alpha$  KO lines**

Western blot of MEF cells that were incubated under hypoxic conditions were immunoblotted for expression of HIF-1 $\alpha$  protein in HIF-1 $\alpha$  WT (lane 1), HIF-1 $\alpha$  KO (lane 2), and HIF-1 $\alpha$  KO cells transduced by Ad-HIF-1 $\alpha$  (lane 3).



**Fig. (3).** *In vitro* immunohistochemical staining of MEF cells with anti-HIF-1 $\alpha$  as the primary antibody

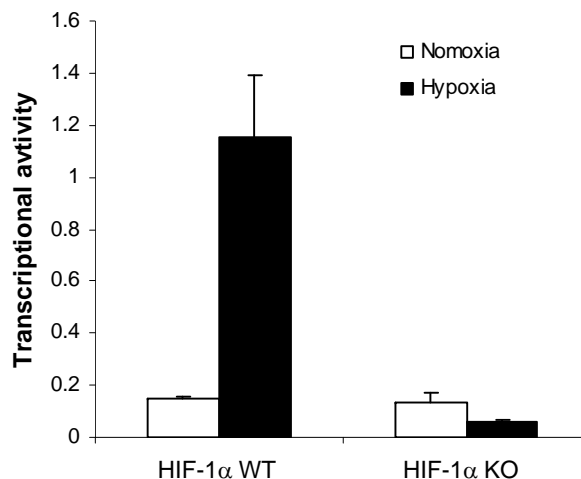
Immunohistochemical staining of HIF-1 $\alpha$  expression in MEF cells of HIF-1 $\alpha$  wild-type line (HIF-1 $\alpha$  WT) (a), HIF-1 $\alpha$  knock out line (HIF-1 $\alpha$  KO) (b), and HIF-1 $\alpha$  KO line transduced by Ad-HIF-1 $\alpha$  (c). The original magnification is x40.

viral transduced cells regained an overexpression of exogenous HIF-1 $\alpha$  protein (lane 3, Fig. 2). The similar results were also observed by *in vitro* immunohistochemical (IHC) staining for HIF-1 $\alpha$  protein on MEF HIF-1 $\alpha$  WT and KO cells. As shown in Fig. (3), we found that there is high percentage of HIF-1 $\alpha$  expression in HIF-1 $\alpha$  WT line (brown color, see arrows for the representative staining, Fig. 3a). In contrast, there is no detectable HIF-1 $\alpha$  expression in HIF-1 $\alpha$  KO cells, indicating a clear knockout background (Fig. 3b). There is a strong expression of HIF-1 $\alpha$  in KO line after they have been transduced by Ad-HIF-1 $\alpha$  at moi of 200 for 72 h (see representative arrows, Fig. 3c). Together, these results demonstrate that Ad-HIF-1 $\alpha$  can successfully transduce and express HIF-1 $\alpha$  protein in mammalian cells as evidenced by both Western blot analysis (Figs. 1, 2) and IHC staining (Fig. 3); and it enables MEF HIF-1 $\alpha$  KO cells to regain HIF-1 $\alpha$  expression after viral transduction (Figs. 2, 3).

#### Hypoxia Stimulates VEGF Promoter Transactivation in HIF-1 $\alpha$ WT but not in HIF-1 $\alpha$ KO Cells

HIF-1 $\alpha$  is known as a transcriptional activator for VEGF gene promoter [5, 36-38]. Therefore, a chimeric construct, pVEGF-Luc, that contains a 2.4 kb VEGF promoter (with a HRE element) fused to a luciferase reporter gene can be used as the readout for measuring the function and activity of HIF-1 $\alpha$  (Forsythe *et al.*, 1996). We transiently cotransfected pVEGF/Luc with phRLuc-TK into both HIF-1 $\alpha$  WT and KO cells for 18 h at 37°C at 21% O<sub>2</sub>, then the cells were either kept at 21% O<sub>2</sub> (normoxia) or 1% O<sub>2</sub> (hypoxia) for another 30 h before harvesting for luciferase activity. We observed a huge induction of VEGF promoter transactivity by hypoxia in HIF-1 $\alpha$  WT cells, with an almost 6 fold increase in VEGF promoter transactivity in hypoxia compared to that in normoxia, indicating a dramatic induction of the transactivator, HIF-1 $\alpha$ , by hypoxia (compare lane 1 with lane 2, Fig. 4). This result demonstrates that hypoxia stimulates a HIF-1 $\alpha$ -mediated transactivation of VEGF promoter. In contrast, in HIF-1 $\alpha$  KO cells, hypoxia did not increase, if not actually somehow decreased, the VEGF promoter transactivation. These results demonstrate that (A) the MEF HIF-1 $\alpha$  KO line we used is indeed a HIF-1 $\alpha$  knockout line with no HIF-1 $\alpha$ -mediated function; (B) HIF-1 $\alpha$  is required for

hypoxia-mediated transactivation of VEGF promoter. Hypoxia stimulates HIF-1 $\alpha$  and its regulated downstream genes (i.e., VEGF).

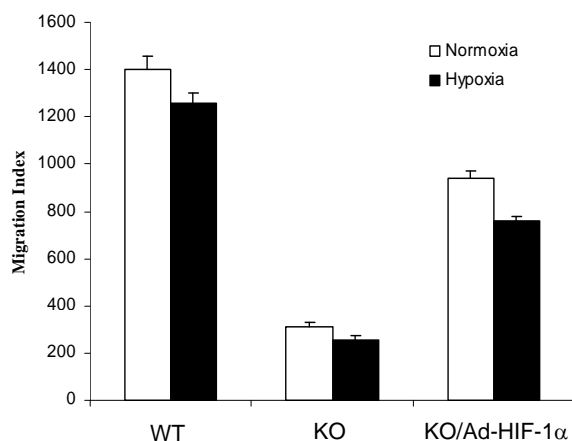


**Fig. (4).** HIF-1 $\alpha$  stimulates VEGF gene expression at transcription level

VEGF promoter-luciferase reporter chimeric construct pVEGF-luc was co-transfected into HIF-1 $\alpha$  WT and HIF-1 $\alpha$  KO cells with phRLuc-TK, a Renilla luciferase-expressing plasmid by Fugene 6 for 48 h under either normoxic or hypoxic conditions. After lysis of the cells, aliquots of cell extracts were used for dual-luciferase assay. The relative transcriptional activity was represented as ratio of Firefly luciferase activity (pVEGF/Luc) over Renilla luciferase activity (phRLuc-TK) for normalization of transfection efficiency. The results represent data from three independent experiments with duplicate tests. The difference between normoxia and hypoxia in HIF-1 $\alpha$  WT group is statistically significant (i.e., column 1 vs column 2,  $p < 0.05$ ), whereas the difference between normoxia and hypoxia in HIF-1 $\alpha$  KO group is statistically insignificant (i.e., column 3 vs. column 4,  $p > 0.05$ ). Some error bars are too small to show in this scale.

## HIF-1 $\alpha$ Promotes a Hypoxia-Independent Cell Migration

Cell migration is an important aspect of the normal cell functions and also involved in the tumor metastatic process. To analyze the effect of HIF-1 $\alpha$  on MEF cell migration, both HIF-1 $\alpha$  WT and KO cells, as well as KO cells that had been transduced by Ad-HIF-1 $\alpha$  at moi of 200 for 72 h, were harvested and plated in the upper compartment of a chamber which has been precoated with fibronectin on the undersurface of the chamber/filter. By comparison with the HIF-1 $\alpha$  WT cells, the HIF-1 $\alpha$  KO cells had significantly reduced migration ability (at astonishing 77.8% and 79.8% reduction rate at normoxia and hypoxia, respectively) (Fig. 5, compare column 1 to column 3 and column 2 to column 4, respectively), indicating HIF-1 $\alpha$  is critical for maintaining a high basal level of cell motility. Interestingly, KO cells that regained HIF-1 $\alpha$  expression by exogenous Ad-HIF-1 $\alpha$ -mediated transduction (Figs. 2, 3) could partially rescue their ability for cell motility, namely, recovered at 67.0% and 60.6% motility ability compared to those of their HIF-1 $\alpha$  WT counterparts in normoxia and hypoxia, respectively (Fig. 5, compare column 1 to column 5, and column 2 to column 6, respectively). This indicates that HIF-1 $\alpha$  contributes a direct, but not complete, migratory ability to the HIF-1 $\alpha$  KO cells (Fig. 5). These data indicate that HIF-1 $\alpha$  plays an important role in cell migration. Cells that lack HIF-1 $\alpha$  have a significantly reduced cell migration. Restoration of HIF-1 $\alpha$  in KO line enables cells to regain high-level cell migration, although it is not a 100% recovery.



**Fig. (5). HIF-1 $\alpha$  expression promotes cell migration**

The cell motility of HIF-1 $\alpha$  WT (WT) and HIF-1 $\alpha$  KO (KO) cells, or HIF-1 $\alpha$  KO cells transduced by Ad-HIF-1 $\alpha$  (KO/Ad-HIF-1 $\alpha$ ) were analyzed by modified Boyden's chamber methods as described in Materials and methods section. The sum of total cell numbers in five randomly selected fields was presented. The results represent data from three independent experiments with duplicate tests. HIF-1 $\alpha$  KO transduced by control virus AdRSVlacZ showed a similar result as that of untreated HIF-1 $\alpha$  KO (not shown). The differences between HIF-1 $\alpha$  WT and HIF-1 $\alpha$  KO groups (i.e., column 1 vs. column 3; or column 2 vs. column 4, respectively), and between HIF-1 $\alpha$  KO and KO/Ad-HIF-1 $\alpha$  groups (i.e., column 3 vs. column 5; or column 4 vs. column 6, respectively) at corresponding conditions (normoxia or hypoxia, respectively) are statistically significant ( $p < 0.05$ ). Some error bars are too small to show in this scale.

The other interesting phenomenon of this experiment is that hypoxia *per se* does not increase the cell migration of MEF cells, regardless of HIF-1 $\alpha$ 's presence. In contrast, there is actually an 11% and 18.6% reduction of cell migration in hypoxia compared to that of normoxia in HIF-1 $\alpha$  WT cells and KO cells, respectively (Fig. 5, compare column 1 to column 2, and column 3 to column 4, respectively). Although these reductions are statistically not significant (both  $p$  value  $> 0.5$ ), at least they showed that there is no increase of cell migration by hypoxia. Even hypoxia stimulated HIF-1 $\alpha$ -mediated transactivation of its downstream target gene VEGF (Fig. 4), hypoxia *per se* did not increase the cell migration of HIF-1 $\alpha$  WT cells (compare column 1 and column 2 in Fig. 5). These results suggest that HIF-1 $\alpha$ -promoted cell motility is independent of hypoxia- or VEGF-mediated pathway. This is the first such report that dissects separately HIF-1 $\alpha$ 's cellular function and hypoxia effect on MEF cell migration. Taken together, these results suggest that HIF-1 $\alpha$  promotes MEF cell migration under a setting of a chemo-attractant migration assay, whereas hypoxia *per se* does not affect cell migration. In summary, the HIF-1 $\alpha$  promotes a cell migration that is hypoxia independent.

## DISCUSSION

In summary, we have shown in this report that the presence of HIF-1 $\alpha$  protein is required for a high-level cell motility: HIF-1 $\alpha$  KO cells showed a significantly reduced cell migration (up to 80%) as compared to that of the HIF-1 $\alpha$  WT cells, and this reduced migratory ability can be rescued partially by regaining HIF-1 $\alpha$  protein mediated by adenoviral-mediated ectopic expression (Fig. 5). Interestingly, while hypoxia induces its expected downstream VEGF expression as shown by transactivation of the VEGF promoter in our study (Fig. 4), hypoxia *per se* does not stimulate cell migration regardless of endogenous HIF-1 $\alpha$  status (Fig. 5). Taken together, we have demonstrated that HIF-1 $\alpha$  promotes fibroblast cell migration *in vitro*, and this HIF-1 $\alpha$ -mediated promotion in cell migration is independent of hypoxia. Our results imply that the genes involved in the control of cell motility are induced through a HIF-1 $\alpha$  dependent mechanism that is independent of hypoxia. To our knowledge, this is the first report to analyze and dissect hypoxia and HIF-1 $\alpha$ , respectively and separately, for their effects on cell migration.

However, ectopic expression of HIF-1 $\alpha$  in HIF-1 $\alpha$  KO/Ad-HIF-1 $\alpha$  did not recover 100% of the cell migration in HIF-1 $\alpha$  KO cells, indicating that other components other than HIF-1 $\alpha$  may be required to fully regain the migratory ability. Alternatively, HIF-1 $\alpha$ -regulated downstream genes or secondary-effect genes, which contribute to or regulate the cell migration, may respond to the exogenous HIF-1 $\alpha$  expression in HIF-1 $\alpha$  KO/Ad-HIF-1 $\alpha$  rescued cells in a more delayed manner than they would to the endogenous HIF-1 $\alpha$  expression in HIF-1 $\alpha$  WT cells. Whether this speculation is true or not remains unknown at this stage.

Hypoxia can arise from either physiologic circumstances such as exercise and travel to high elevation, or from pathophysiologic conditions such as poorly formed tumor vasculature [2]. One general characteristic of fast-growing solid tumors is the development of intratumoral hypoxia.

Adaptation to the hypoxic environment is critical for tumor cells' survival and growth. For example, to prevent cellular damage and avoid cell death, the hypoxic cells in tumors must undergo modification of their gene expression profiles in order to form new blood vessels to supply oxygen and nutrition. Accumulated evidence indicates that hypoxia plays an important role in tumor progression and metastasis [19-21]. Studies both *in vitro* and *in vivo* demonstrated that transient hypoxic exposure of tumor cells enhanced the cells' metastatic ability [22-26]. Although the mechanism by which hypoxia amplifies the metastatic potential is not fully understood, it is likely that HIF-1, the main mediator of hypoxia response and a master-regulator at the transcription level [3, 4], drives expression of a set of downstream genes including VEGF that control processes involved in the metastatic cascade such as proliferation, angiogenesis, survival, migration, and invasion. Hypoxia in the tumor microenvironment is sufficient for activating HIF-1-dependent gene expression [27]. Up-regulation of HIF-1 $\alpha$  (the regulatory subunit of HIF-1) signaling pathways has been associated with the molecular expression signature of micrometastasis in human breast cancer [28, 29]. High levels of HIF-1 $\alpha$  have been consistently correlated with tumor progression and poor prognosis in several types of cancers [30].

Cell migration plays a central role in a wide variety of biological phenomena in both normal physiology and pathophysiology. In particular, cell migration is an important aspect of the tumor metastatic process and it is considered as one of key steps that transform the tumor cells from local, noninvasive confined cells to the migrating, metastatic cancer cells. The transition process from the local, confined tumor cells to metastatic cancer cells is initiated when the cells obtain the ability to dissociate from intracellular adhesions and become motile [19, 20], which is usually driven by complex regulatory signaling cascades that transiently and/or permanently alter the expression of a multitude of genes that act to reorganize the cytoskeletal network [20, 21]. Several motility/migration related proteins have been implicated to play important roles in either promoting or inhibiting the tumor metastatic process [31-35].

While the effects of hypoxia (or HIF-1 $\alpha$ ) on, and roles of cell migration in, tumor progression and metastasis, respectively, have been extensively studied, the effect of hypoxia (or HIF-1 $\alpha$ ) on cell migration has not been explored in depth. As cell migration plays an important role in tumor cell's progression and metastasis, and hypoxia enhances the metastatic ability of tumor cells, we are interested to know whether hypoxia-mediated metastatic phenotype is partially due to the altered cell migration controlled by genes regulated by HIF-1 $\alpha$ . To address the issue of whether it is hypoxia or HIF-1 $\alpha$  *per se* that contributes to the altered migration of tumor cells, the appropriate model would be a tumor cell line lacking HIF-1 $\alpha$  expression. Through collaboration with others, we are currently performing the similar studies on a HIF-1 $\alpha$  KO breast cancer cell line that derives from epithelial cells of a mouse primary breast tumor. Our study on MEF HIF-1 $\alpha$  KO line provides a new research strategy to dissect the roles of hypoxia and HIF-1 $\alpha$ , individually and separately, on cell migration. This may lead to the new findings of HIF-1 $\alpha$ 's intrinsic roles in cellular function (i.e., migration) and a novel HIF-1 $\alpha$  mediated

signaling pathway that is independent of well-known hypoxia/HIF-1 $\alpha$ /VEGF signaling pathway.

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