Hypoxia-inducible factor 1 mediates intermittent hypoxia-induced migration of human breast cancer MDA-MB-231 cells

LITAO LIU[1,2], WENLAN LIU[1], LILI WANG[1,3], TING ZHU[1,4], JIANHUA ZHONG[1,5] and NI XIE[1,2]

[1]The Central Laboratory, Shenzhen Second People's Hospital, Shenzhen University First Affiliated Hospital;
[2]Institute of Translation Medicine, Shenzhen Second People's Hospital, Shenzhen University First Affiliated Hospital,
Shenzhen, Guangdong 518035; [3]Graduate School, Guangdong Medical College, Dongguan, Guangdong 523808;
[4]Graduate School, Guangzhou Medical University, Guangzhou, Guangdong 511436;
[5]Graduate School, Shantou University Medical College, Shantou, Guangdong 515041, P.R. China

Abstract. Metastasis is the major cause of triple-negative breast cancer (TNBC)-associated mortality. Hypoxia promotes cancer cell migration and remote metastasis, which occur with hypoxia inducible factor 1α (HIF-1α) stabilization and vimentin upregulation. However, the evolutionary dynamics that link the changes in HIF-1α and vimentin levels under hypoxic conditions are not well understood. In the present study, the effects of intermittent hypoxia (IH), continuous hypoxia (CH) and normoxia on the migration and proliferation of human TNBC MDA-MB-231 cells were investigated. The results demonstrated that IH significantly increased the migration of MDA-MB-231 cells, and this effect was dependent on the number of cycles of hypoxia-reoxygenation. Unexpectedly, IH significantly inhibited cell proliferation, while CH only caused such an effect if hypoxia extended for ≥3 days. IH and CH induced HIF-1α protein accumulation and vimentin upregulation, with a greater effect observed in IH. Knockdown of HIF-1α with siRNA abolished IH-induced cell migration and vimentin upregulation. In summary, multiple cycles of hypoxia and reoxygenation have a more pronounced effect on the promotion of TNBC invasiveness than CH; HIF-1α activation and downstream vimentin upregulation may account for this phenotypic change.

Correspondence to: Dr Ni Xie, Institute of Translation Medicine, Shenzhen Second People's Hospital, Shenzhen University First Affiliated Hospital, No. 3002, Sungang West Road, Shenzhen, Guangdong 518035, P.R. China
E-mail: xn100@szu.edu.cn

Abbreviations: HIF-1α, hypoxia-inducible factor 1α; IH, intermittent hypoxia; CH, continuous hypoxia; TNBC, triple-negative breast cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; PR, progesterone receptor; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; TBST, Tris-buffered saline with Tween-20; siRNA, small interfering RNA; si-HIF-1α, HIF-1α siRNA; si-NT, non-targeting siRNA

Introduction

Breast cancer is the most common type of cancer among women, accounting for 29% of all newly diagnosed cases of cancer and 15% of all cancer-associated mortalities in 2014 in the United States (1). Genetic analysis indicates that breast cancer is a disease of phenotypic heterogeneity, which includes various molecular subtypes associated with clinical prognoses. Triple-negative breast cancers (TNBCs), which are characterized by lacking expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (HER2), comprise 15-20% of breast cancer cases and are considered the most malignant subtype, with the highest risk of metastasis (2). TNBCs more frequently disseminate to the distant organs, including brain, lung and liver, than to regional lymph nodes (2,3). Metastasis is regarded as the key contributor to breast cancer-associated mortality. In general, the 5-year survival rates for patients with localized and regional breast cancer are 98.6 and 84.9%, respectively. However, if remote metastasis occurs, the 5-year relative survival rate is only 25.9% (4). Tumor progression and metastasis are complex processes that are influenced by a variety of extrinsic and intrinsic factors (5,6). Although the potential mechanisms underlying tumor metastasis remain incompletely defined, cell migration has attracted extensive attention as it is recognized as the first and fundamental step for the dissemination of a malignancy (7).

Hypoxia is an important component of the microenvironment of various types of solid tumor, including breast cancer (8). In hypoxia, whereas some tumor cells will undergo apoptosis, the majority of the tumor cells will adapt to the hypoxic conditions by favoring metabolic pathways that do not require oxygen, or by promoting angiogenesis and mutation to increase oxygen supply (9,10). It has been identified that hypoxia-inducible factor 1 (HIF-1) serves an important role in the response to hypoxia. HIF-1 is a transcription factor consisting of a constitutively expressed HIF-1β subunit and an oxygen-sensitive HIF-1α subunit. The transcriptional activity

of HIF-1 depends on the availability of HIF-1α protein, which is accumulated under hypoxic conditions, and quickly degraded under normoxic conditions. HIF-1 activates the transcription of numerous genes involved in cancer progression.

A pool of studies have demonstrated that hypoxia promotes cell migration; this process is associated with increased HIF-1α stability and activity, as well as the upregulation of vimentin, a marker for mesenchymal cells (11). Vimentin is a member of the intermediate filament family, the members of which constitute part of the cytoskeleton (12,13). In embryogenesis, vimentin serves a pivotal role in the differentiation of organs and tissues (13). In the development of tumors, vimentin may alter cellular polarity, regulate cell contact formation and transport signal proteins involved in cell mobility (6). However, the dynamics linking the changes in HIF-1α and vimentin levels in hypoxic conditions have not been fully investigated.

Hypoxia universally occurs in solid tumors; however, the duration of hypoxia varies greatly between and within tumors. Previous observations have revealed there are two major forms of hypoxia in tumors: Continuous hypoxia (CH) and intermittent hypoxia (IH). CH develops due to the imbalance between the rapid proliferation of cells and inadequate tumor angiogenesis/oxygen supply; this occurs because the blood supply is primarily located in tumor stroma, and the maximum oxygen diffusion distance in malignant tissues is 100-150 μm (14,15). Alternatively, in the tumor microenvironment, the structural abnormalities of tumor vasculature can produce unstable hemodynamics and cause IH (16,17). Histological analyses have shown that tumor vasculatures are characterized by an uneven thickness of the vascular basement membrane, a loose association or absence of vascular endothelial cells, a lack of vessel contractility, compression by tumor cells, vessel formation that is tortuous and dilated, and numerous dead ends. The duration of IH may range from min to days in various tumors, depending on the level of maturation and the structural complexity of the tumor vessel networks (18). Previous studies have focused on acute or chronic CH and the results are controversial (19,20). Although IH models have been tested in ovarian, lung and gastric cancer cells, the effects of IH on breast cancer cells remain unclear and require further investigation (16,21,22).

In the present study, human breast cancer MDA-MB-231 cells were cultured in an IH, CH or normoxic environment. The effect of the conditions on the migration and proliferation of MDA-MB-231 cells and the mechanisms involved were investigated. The data demonstrated that multiple cycles of hypoxia and reoxygenation induced a more invasive phenotype in breast cancer cells, mediated by HIF-1α activation and associated vimentin upregulation.

Materials and methods

Cell culture and hypoxia treatment. Human breast adenocarcinoma MDA-MB-231cells (a TNBC cell line) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; MRC Biological Technology Co., Ltd., Jiangsu China), 100 U/ml penicillin, and 100 μg/ml streptomycin. The

MDA-MB-231 cells were cultured under normoxic, CH or IH conditions. For normoxic cultures, the cells were grown in a humidified incubator (37°C, 21% O_2, 74% N_2, 5% CO_2). For CH cultures, the cells were grown in a sealed hypoxic incubator for 48 h (37°C, 1% O_2, 94% N_2, 5% CO_2). For IH cultures, the cells were subjected to a specified number of hypoxia (37°C, 1% O_2, 94% N_2, 5% CO_2) and reoxygenation (37°C, 21% O_2, 74% N_2, 5% CO_2) cycles; each hypoxia and reoxygenation cycle included 12 h of hypoxic incubation followed by 12 h of normoxic culture. When reoxygenated, the cell culture medium was replaced and the dishes were placed into a normoxic chamber.

Wound healing assay. To evaluate the mobility of MDA-MB-231 cells indifferent oxygenation conditions, cells were cultured in 6-well plates or 60-mm dishes with DMEM. For the IH group, the cells were subjected to 10 cycles of 12 h hypoxic incubation (37°C, 1% O_2, 94% N_2, 5% CO_2) and 12 h reoxygenation (37°C, 21% O_2, 74% N_2, 5% CO_2) cycles prior to testing and were continually exposed to IH conditions for 48 h during testing. For the CH group, the cells were cultured under hypoxia for 48 h (37°C, 1% O_2, 94% N_2, 5% CO_2) during the testing. For the normoxic group, the cells were exposed to normoxia for 48 h (37°C, 21% O_2, 74% N_2, 5% CO_2) during the testing, without the 10 cycles of hypoxia. When 90% confluence was reached, a 'wound' was created on the cell monolayer with a 200-μl pipette tip, the plates were washed three times with PBS and the medium was replaced with FBS-free DMEM. Cell migration at 0 and 48 h was assessed and photographed under an inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). The wound area was assessed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The relative mobility was calculated by the following migration index: Relative mobility=(wound width at 0 h-wound width at 48 h)/wound width at 0 h. Wound width was calculated wound area divided by image height.

Trans-well migration assay. A Boyden chamber system (Corning Life Sciences, Lowell, MA, USA) was applied to assess the migration ability of breast cancer cells. Cells were pre-treated with or without hypoxic conditions, then trypsinized and resuspended in serum-free DMEM at a density of $2\times10^4/250$ μl. The resuspended cells were placed on the upper layer of a cell-permeable membrane, and 500 μl complete medium was placed below the membrane. The plates were incubated for 48 h in IH, CH and normoxic conditions, respectively. The cells on the upper layer of the membrane were removed with a cotton swab; the migrated cells on the lower membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. The migrated cells were photographed and counted using an inverted microscope and random fields were scanned (5 fields/filter). Subsequently, cells were incubated in 10% glacial acetic acid for 20 min, and the medium was collected in 96-well plates. The optical density (OD) at 570 nm was measured by a microplate reader (Thermo Fisher Scientific, Inc.), which represented the relative levels of cell migration.

Cell proliferation assay. The proliferation of MDA-MB-231 cells in normoxia or hypoxia was assessed with a Cell Counting Kit-8

(CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). For the IH group, the cells were subjected to 10 cycles of hypoxia ($37°C$, 1% O_2, 94% N_2, 5% CO_2) and reoxygenation ($37°C$, 21% O_2, 74% N_2, 5% CO_2) cycles prior to the start of the CCK-8 assay and were continually cultured under IH conditions during the assay. For the CH group, the cells were continuously exposed to hypoxic conditions ($37°C$, 1% O_2, 94% N_2, 5% CO_2) during the CCK-8 assay. For the normoxic group, the cells were exposed to normoxic conditions ($37°C$, 21% O_2, 74% N_2, 5% CO_2) during the assay, without the 10 cycles of hypoxia. A total of 1.5×10^3 cells/well (or 3.0×10^3 cells/well when subsequent to transfection) were seeded in 96-well culture plates and cultured for 4-6 h at $37°C$ (21% O_2, 74% N_2, 5% CO_2). When the cells had adhered, CCK-8 reagents were added and incubated at $37°C$ (21% O_2, 74% N_2, 5% CO_2) for an additional 2 h. The OD was measured at 450 nm on a microplate reader to ensure an equal number of cells had been seeded, and this represented the number of cells on the 0th day. Then, the cells were cultured at $37°C$ under IH, CH and normoxic conditions for four days with DMEM and the OD was subsequently determined on the 1st, 2nd, 3rd and 4th days of culture.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), and the first-strand cDNA was synthesized using a Prime Script First Strand cDNA Synthesis Kit (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's protocol. PCR reactions were performed with SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) and Quant Studio Dx Real-Time PCR Instrument (Applied Biosystems, Thermo Fisher Scientific, Inc.). The reaction volume included 10 μl SYBR® Premix Ex Taq™ II, 2 μl cDNA, 7 μl H_2O, 0.5 μM forward and reverse primers and 1 μl template cDNA. The PCR conditions were as follows: $95°C$ for 30 sec, and 40 cycles of $95°C$ for 5 sec, $60°C$ for 1 min and $72°C$ for 30 sec. The forward and reverse primers (synthesized by Sangon Biotech Co., Ltd., Shanghai, China) are listed in Table I. Target mRNA expression was determined by normalizing to β-actin levels and was analyzed using the $2^{-\Delta\Delta Cq}$ method (21,23). The number of experimental repeats was three.

Western blotting. Total protein was extracted using radio immuno precipitation assay buffer (Solarbio; Beijing Soledad Co., Ltd., Beijing, China) supplemented with 1 m Mphenyl methanesul fonyl fluoride. Protein content was determined using BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal amounts of protein were separated with 8% SDS-PAGE and transferred to poly vinylidene fluoride membranes (pore size, $0.45\text{-}\mu$m; EMD Millipore, Billerica, MA, USA). The blots were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skimmed milk for 1.5 h at room temperature. Membranes were incubated with primary antibodies overnight at $4°C$. Primary antibodies included rabbit polyclonal anti-HIF-1α (dilution, 1:2,000; cat. no. NB100-479; Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti-vimentin (dilution, 1:1,000; cat. no. 3932s; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal anti-β-actin (dilution, 1:5,000; cat. no. ab6046; Abcam, Cambridge,

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence	Product size (bp)
HIF-1α		90
Forward	5'-CTGCCACCACTGATGAATTA-3'	
Reverse	5'-GTATGTGGGTAGGAGATGGA-3'	
Vimentin		106
Forward	5'-CCTTGAACGCAAAGTGGA ATC-3'	
Reverse	5'-GACATGCTGTTCCTGAATCTG AG-3'	
β-actin		101
Forward	5'-GATCATTGCTCCTCCTGAGC-3'	
Reverse	5'-ACTCCTGCTTGCTGATCCAC-3'	

HIF-1α, hypoxia-inducible factor 1α.

MA, USA) and mouse monoclonal anti-GAPDH (dilution, 1:5,000; cat. no. ab8245; Abcam). All membranes were washed three times with TBST and were further incubated with a peroxidase-conjugated anti-rabbit secondary antibody (dilution, 1:500; cat. no. DY3002; Shanghai Ex Cell Biology, Inc., Shanghai, China) at room temperature for 1 h. Blots were washed and detected using an enhanced chem ilumine scence reagent (WBKLS0100; EMD Millipore) and visualized using a Bio Spectrum Imaging System (version no., Alliance 4.7, UVItec, Cambridge, UK).

siRNA transfection. MDA-MB-231 cells that had been grown in IH conditions were seeded in a 6-well culture plate and incubated in antibiotic-free DMEM supplemented with 10% FBS. The cells were grown to subconfluence (60-70%) and transfected with HIF-1α siRNA (cat. no. sc-35561; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or control siRNA (cat. no. sc-13515; Santa Cruz Biotechnology, Inc.) using siRNA transfection reagent (cat. no. sc-29528; Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocol. Subsequent to transfection, the cells were harvested for western blotting or migration assays.

Statistical analysis. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data obtained from two groups were analyzed by two-tailed Student's t-tests, and data from three or more groups were analyzed using a one-way analysis of variance with a post hoc Fisher's least significant difference test. All values are expressed as the means ± standard deviation of ≥3 independent experiments. $P<0.05$ was considered to represent a statistically significant difference.

Results

Effect of IH on the migration of MDA-MB-231 cells. MDA-MB-231 cells were exposed to an IH environment for 5, 10, 15 or 20 cycles prior to a wound healing assay. As shown
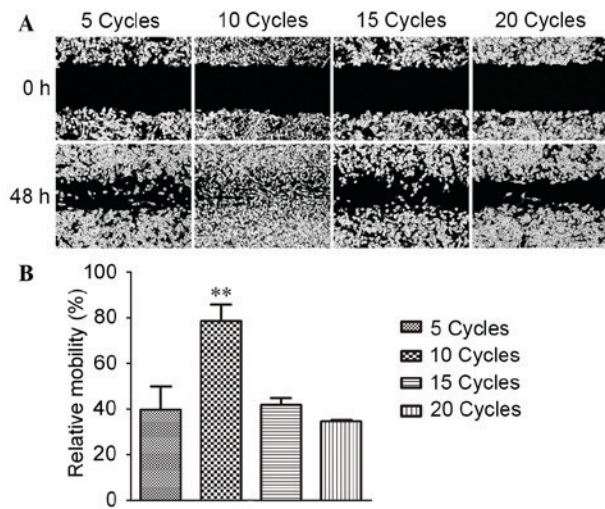
Figure 1. Effect of IH on the migration of MDA-MB-231 cells. MDA-MB-231 cells were exposed to 5, 10, 15 or 20 cycles of hypoxia and reoxygenation. (A) A wound-healing assay revealed that 10 cycles had the greatest effect on promoting cell migration. (B) The relative mobility was calculated from the wound healing assay data. Data are expressed as the means ± standard deviation of three repetitions. **P<0.01 vs. 10-cycle group. IH, intermittent hypoxia; CH, continuous hypoxia; N, normoxia.

in Fig. 1A, MDA-MB-231 cells on each side of the wound edge migrated into the wound area, and reached confluence at 48 h for the 10-cycle IH group, whereas cells in other groups did not reach confluence. The relative migration abilities of cells in the 5-, 10-, 15- and 20-cycle groups were 38.6, 76.3, 41.5 and 33.5%, respectively (Fig. 1B), indicating that 10 cycles of IH produced the greatest effect in promoting the migration of MDA-MB-231 cells (P<0.01, 10-cycle compared with the 5-, 15- and 20-cycle). Based on this result, 10 cycles was selected to represent IH in subsequent experiments.

Effects of CH and IH on MDA-MB-231 cell migration and proliferation. The effects of IH or CH on MDA-MB-231 cell migration and proliferation were compared. As shown in Fig. 2A, the IH group exhibited an accelerated rate of migration compared with CH and normoxic groups, whereas the CH and normoxic groups showed similar rates of cell migration, with relative mobility 76.1% for IH, 34.1% for CH and 31.7% for normoxic cells (P<0.01; Fig. 2B). A trans-well migration assay further verified the a for ementioned results. Direct cell number counting (Fig. 2C) and relative OD (Fig. 2D) measurements revealed that the number of cells migrating to the lower chamber in the IH group was significantly greater compared with that of the CH and normoxic groups, with ODs of 1.135100 for IH, 0.861450 for CH and 0.908983 for normoxic cells (P<0.05; Fig. 2C and D).

To determine whether IH-induced migration of MDA-MB-231 cells was due to accelerated cell proliferation, a CCK-8 assay was performed on MDA-MB-231 cells cultured in IH, CH or normoxic conditions. As demonstrated in Fig. 2E, the cells cultured under IH conditions revealed decreased proliferation compared with the normoxic cells (P<0.05), although such inhibition was not obvious until after the 3rd day of CH exposure. This indicated that IH promoted MDA-MB-231 cell migration by a mechanism other than increased cellular proliferation.

Effects of IH on HIF-1 and vimentin expression. As HIF-1α and vimentin are two important molecules implicated in cancer migration (6,12), it was next examined whether IH promoted MDA-MB231 cell migration through affecting these two molecules. As shown by the western blot analysis in Fig. 3A, HIF-1α protein was accumulated in MDA-MB231 cells in the IH group during hypoxia, and was degraded during reoxygenation, suggesting post-translational HIF-1α regulation; that is, in normoxic conditions, HIF-1α may be hydroxylated by prolyl hydroxylase and degraded by the proteasome, whereas hypoxia inhibits the activity of prolyl hydroxylase and, therefore, HIF-1α degradation.

For the cells exposed to CH, HIF-1α protein level remained low during the first 36 h of exposure, and was dramatically enhanced at 48 h. HIF-1α mRNA expression was assessed by RT-qPCR and the results revealed that HIF-1α mRNA expression was comparable between the IH (during hypoxia) and CH groups. However, HIF-1α mRNA expression in IH cells was significantly increased during reoxygenation when compared with during hypoxia (P<0.05; Fig. 3B).

Vimentin upregulation is associated with cancer cell migration (12,13). Western blotting data revealed that IH increased vimentin protein levels during the hypoxia and reoxygenation stages when compared with normoxic cells, indicating that the vimentin protein level remained high in the IH group. In CH cells, the increase in vimentin protein appeared to be transient; it increased at 12 h, peaked at 24 h, and returned back to the basal level (i.e. as in normoxic conditions) at 48 h (Fig. 3C). Vimentin mRNA expression was also examined. The data revealed that vimentin mRNA expression was significantly higher in IH cells than in CH cells (P<0.01); however, no difference in mRNA expression was observed in the groups between different time points (Fig. 3D).

Knockdown of HIF-1α expression abolishes hypoxia-induced vimentin expression and MDA-MB-231 cell migration. It was evaluated whether HIF-1α-mediated IH-induced MDA-MB-231 cell migration. The cells were cultured for 10 cycles of IH, and then transfected with HIF-1α (si-HIF-1α) or control (si-NT) siRNA. Western blot analysis verified the efficacy of si-HIF-1α; si-HIF-1α successfully knocked down HIF-1α protein expression, while si-NT had no effect (Fig. 4A). In addition to knockdown of HIF-1α, vimentin expression and cell migration (as assessed by wound healing or trans-well assay) were also inhibited by HIF-1α siRNA, when compared with si-NT (Fig. 4B-F). The relative migration into the wound are a was 75% for IH, 71.7% for si-NT and 49.2% for si-HIF-1α (P<0.01). The relative OD values, reflecting the number of cells that migrated to the lower chamber, were 1.214733 for IH, 1.109700 for si-NT, and 0.743567 for si-HIF-1α (P<0.05). Notably, knockdown of HIF-1α with siRNA had no effect on the proliferation of MDA-MB-231 cells (Fig. 4G). These results indicated that HIF-1α mediated IH-induced cell migration.

Discussion

IH, which is characterized by hypoxia and reoxygenation, is a result of fluctuations in oxygen perfusion caused by the inefficient structure of tumor microvasculature, and has a
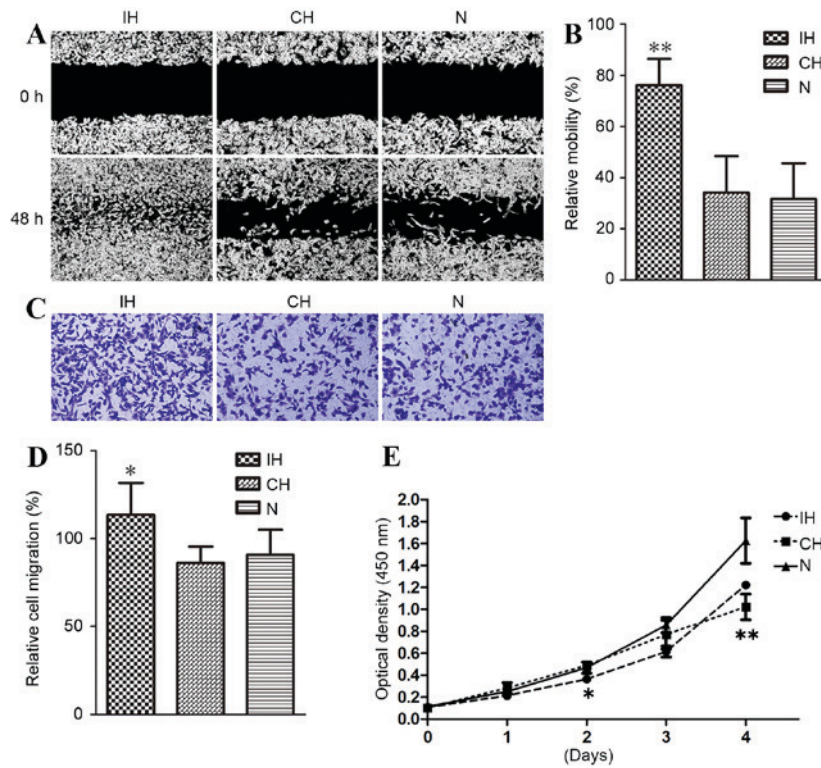
Figure 2. Effects of CH or IH on MDA-MB-231 cell migration and proliferation. MDA-MB-231 cells were exposed to IH, CH or N. (A) A wound-healing assay revealed that the IH group exhibited accelerated migration compared with the CH and N groups. (B) The relative mobility was calculated from the wound healing assay data. (C) A Transwell migration assay revealed that the proportion of cells migrating to the lower chamber in the IH group was significantly increased compared with the CH or N group. (D) The relative levels of cell migration were calculated. (E) A Cell Counting Kit-8 assay revealed that the IH group exhibited reduced proliferative activity compared with the N group. The CH group also exhibited reduced proliferative activity; however, this was not obvious until the 3rd day of culture. Data are expressed as the mean ± standard deviation of three repetitions. *P<0.05 vs. IH group; **P<0.01 vs. IH group. IH, intermittent hypoxia; CH, continuous hypoxia; N, normoxia.
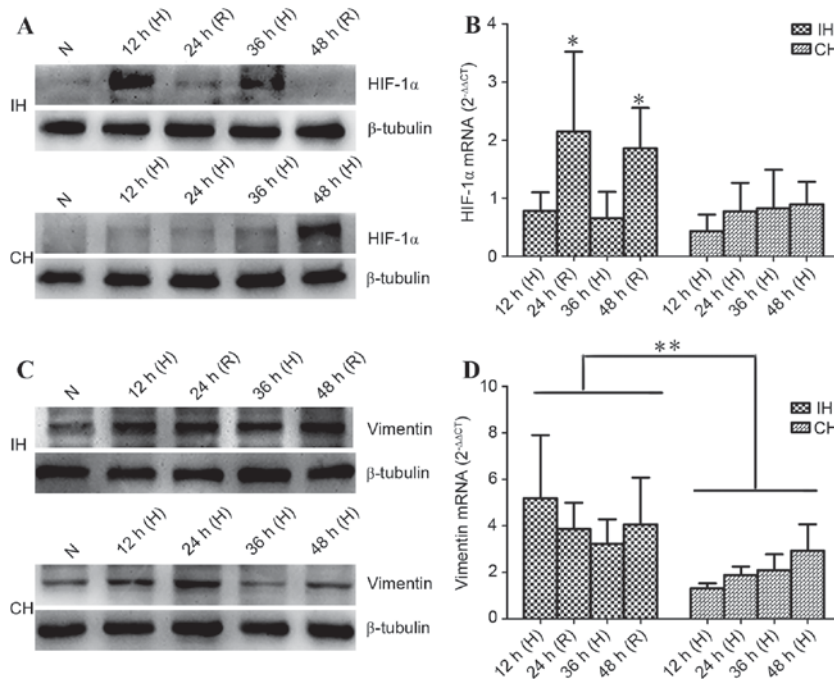

Figure 3. Effects of IH and CH on HIF-1 and vimentin expression. MDA-MB-231 cells were exposed to IH, CH and N for the times indicated, and then total proteins and mRNA were extracted. (A) In IH cells, HIF-1α protein was accumulated during H phases and was degraded during R. However, in CH cells, it remained low for the first 36 h of exposure and was enhanced at 48 h. (B) HIF-1α mRNA expression was comparable between the IH (H stage) and CH groups. However, it was significantly increased during R compared with H. (C) Vimentin protein level remained steady high in the IH group, while in CH cells, vimentin protein level appeared to be transient. (D) Vimentin mRNA expression was significantly higher in IH cells than in CH cells. Data are expressed as the mean ± standard deviation of three repetitions. *P<0.05 vs. H stage; **P<0.01 vs. IH group. IH, intermittent hypoxia; CH, continuous hypoxia; HIF-1, hypoxia-induced factor 1; N, normoxia; H, hypoxia; R, reoxygenation.
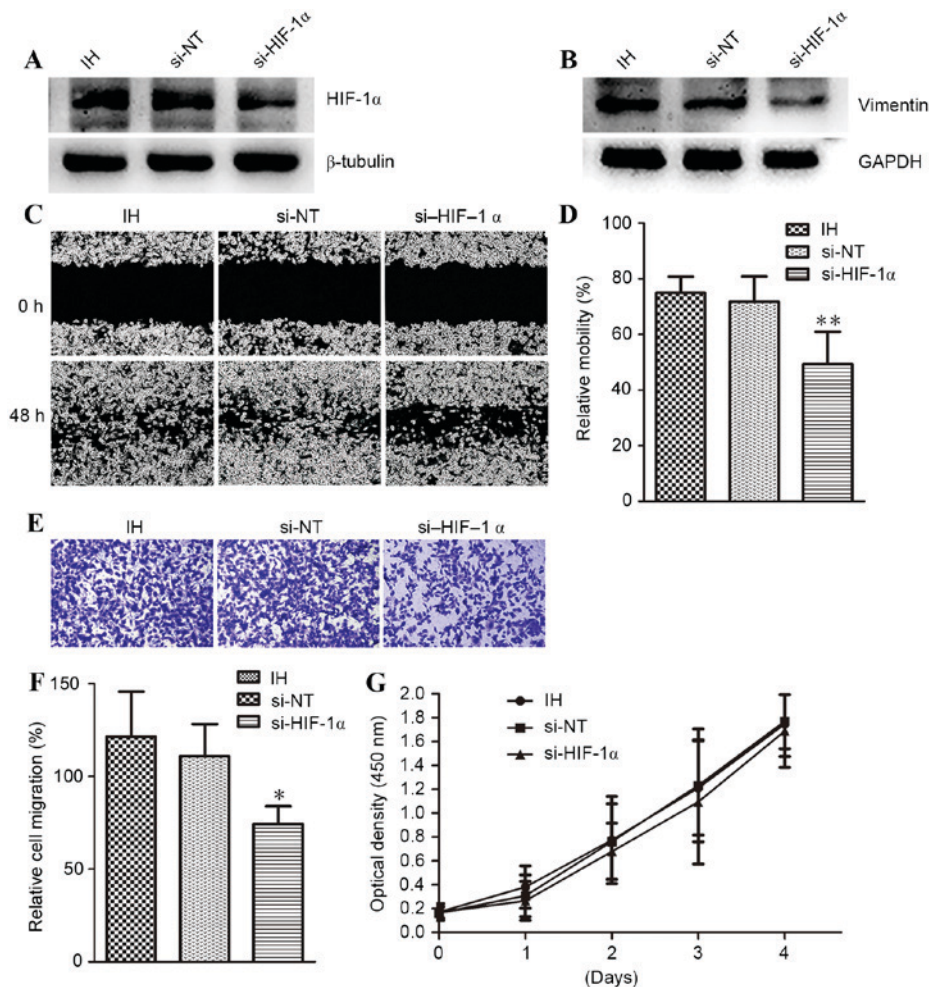
Figure 4. Knockdown of HIF-1α expression eliminates hypoxia-induced vimentin expression and MDA-MB-231 cell migration. MDA-MB-231 cells were cultured for 10 cycles of IH, then transfected with si-HIF-1α or si-NT. (A) si-HIF-1α knocked down HIF-1α protein expression whereas si-NT had no significant effect. (B) Vimentin expression was decreased along with the knockdown of HIF-1α. (C) A wound-healing assay revealed that cell migration was inhibited subsequent to transfection with si-HIF-1α. (D) The relative mobility was calculated from the wound-healing assay data. (E) A Transwell migration assay showed that cells migrating to the lower chamber were decreased subsequent tosi-HIF-1α transfection. (F) The optical density, which represented the relative level of cell migration, was measured (G) Knockdown of HIF-1α with siRNA had no effect on the proliferation of MDA-MB-231 cells. Data are expressed as the mean ± standard deviation of three repetitions. *P<0.05 vs. si-HIF-1α group; **P<0.01 vs. si-HIF-1α group. HIF-1α, hypoxia-induced factor 1α; IH, intermittent hypoxia; si-HIF-1α, small interfering RNA against HIF-1α; si-NT, non-targeting small interfering RNA.

significant effect on tumor biology (18). The present study demonstrated that IH significantly increased the migration of MDA-MB-231 cells, and that this effect was dependent on the number of cycles of hypoxia-reoxygenation. Unexpectedly, IH significantly inhibited cell proliferation, while CH did not if hypoxia persisted for >3 days. IH and CH induced HIF-1α protein accumulation and vimentin upregulation, with the greatest effect observed for IH. Knockdown of HIF-1α with siRNA eliminated IH-induced cell migration and vimentin upregulation. This was consistent with a number of previous studies, which reported that IH may positively modulate stem cell transformation, therapy resistance and cell autophagy in human cancer (17,24).

The effects of IH on cancer cells are diverse and contradictory (25). Accumulating evidence has demonstrated that multiple cycles of IH promote tumor metastasis by selecting cells with the invasiveness phenotype (17), which is consistent with the results of the present study. However, high-frequency exposure to IH or excessively long exposure to CH leads to the generation of reactive oxygen species

(ROS), which induce DNA strand breakage and cellular injury (26,27). In the present study, a decreased rate of migration was observed when IH cycles were increased to 15 and 20 cycles, indicating that there may be a ceiling effect for IH in promoting tumor migration. The data of the present study indicates that the CH and normoxic groups exhibit similar rates of cell migration, which is inconsistent with previous reports (9). This inconsistency may arise from the differences in cell type, hypoxia severity and hypoxia tolerance. A fluctuation in oxygen concentration in IH could inflict genotoxic stress upon cancer cells and inhibit proliferation, whereas CH induced cell death in an oxygen concentration-dependent and exposure time-dependent manner (28,29). In the CH group, the proliferation-inhibition effect became apparent on the 4th day, which may have arisen from an increased rate of apoptosis in MDA-MB-231 cells, whereas IH may improve hypoxia tolerance.

The data that cell migration decreased when HIF-1α was knocked down with siRNA implicates HIF-1α as being important in IH-induced metastasis. The regulation of HIF-1α

predominantly occurs at the post-transcriptional level (6). However, the data from the present study revealed that HIF-1α mRNA expression is significantly increased in IH cells during reoxygenation. A possible explanation for this finding is that HIF-1α protein degradation may trigger a feedback loop to promote expression of its mRNA. It is important to note that IH and CH are two distinct stimuli that activate different signal transduction pathways to affect HIF-1α protein level. For example, protein kinase A is reported to be involved in the regulation of HIF-1α phosphorylation in IH; however, it is not involved in CH (30). Conversely, inhibition of mitogen-activated protein kinase 1/3 or phosphatidylinositol-4,5-bisphosphate 3-kinase has no effect on HIF-1α stabilization and transcriptional activity in IH; however, if these kinases are inhibited in CH, gene regulation by HIF-1 is suppressed (31). HIF-1α protein additionally appears to be more stable in IH than CH, as HIF-1α protein is upregulated in two cycles of IH within 48 h, whereas equal protein expression only a rises after 48 h in CH cells, consistent with previous studies (18,21). Unexpectedly, HIF-1α protein level remained low within the first 36 h of exposure to CH. There are two possible explanations for this finding; either HIF-1α degradation by the proteasome continues to occur in CH conditions as previously reported (21,32), or CH may suppress HIF-1α synthesis at a transcriptional or translational level. Further study is required to explore these possibilities.

CH invoked a pronounced increase in HIF-1α protein after 48 h and a marked decrease in cell viability thereafter, which raises the possibility that a substantial increase in HIF-1α protein level may represent a turning point for stressed cells entering crisis. Typically, HIF-1α protein is degraded by the proteasome within 5 min of reoxygenation (33). However, in the present study, even if the HIF-1α protein was degraded completely, reoxygenation stimulated HIF-1α signaling and increased the expression of target genes such as vimentin. If HIF-1α is knocked down by siRNA, vimentin protein expression and cell migration are suppressed. A possible explanation for this observation is that a number of HIF-1α target gene transcripts may remain untranslated during hypoxic conditions to form HIF-1-mRNA complexes aggregated in stress granules; and, upon reoxygenation, these stress granules are depolymerized to allow the transcription of HIF-1α target genes (34,35).

The role of vimentin in cell migration is particularly complex and not yet fully understood. Vimentin has been reported to regulate cytoskeletal organization and the loss of cell polarization and adhesion (13). Fas and integrins act as receptors for the extracellular matrix, with vimentin-mediated signal transduction between cells and the extracellular matrix, to regulate cytoskeletal rearrangement and focal adhesion organization (36,37). Accumulating studies have demonstrated that increasing HIF-1α stability and activity lead to upregulation of vimentin expression, and this process is closely associated with epithelial-mesenchymal transition (7,11). In the present study, although IH and CH induced vimentin expression, the IH group exhibited significantly higher vimentin mRNA expression and more consistent protein levels than the CH group. In CH-exposed cells, vimentin protein elevation appears to be transient, as it increases for the first 24 h, and then decreases with continuing hypoxia. An important characteristic of vimentin is that it is prone to protease cleavage.

Calpains and caspases can be activated by hypoxia, and they have been reported to cleave and digest vimentin (12,38). It is thus speculated that long periods of hypoxia may induce calpains and caspases to target vimentin. Although sustained oxidative stress injury and toxic metabolites generated from anaerobic glycolysis may obstruct vimentin translation, future studies are required to elucidate how hypoxia dynamically regulates vimentin expression in CH.

In conclusion, the results presented in the present study clearly suggest that multiple cycles of hypoxia and reoxygenation have a more pronounced effect on promoting an invasive TNBC phenotype than CH, and that HIF-1α activation, together with vimentin upregulation, may account for this phenotypic change.

Acknowledgements

References

1. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.
2. Papa A, Caruso D, Tomao S, Rossi L, Zaccarelli E and Tomao F: Triple-negative breast cancer: Investigating potential molecular therapeutic target. Expert Opin Ther Targets 19: 55-75, 2015.
3. Surazynski A, Miltyk W, Prokop I and Palka J: The effect of estrogen on prolidase-dependent regulation of HIF-1α expression in breast cancer cells. Mol Cell Biochem 379: 29-36, 2013.
4. National Cancer Institute. SEER fact sheet for breast cancer. http://seer.cancer. gov/statfacts/html/breast.html. 2005-2011.
5. Quail DF and Joyce JA: Microenvironmental regulation of tumor progression and metastasis. Nat Med 19: 1423-1437, 2013.
6. Tsai YP and Wu KJ: Hypoxia-regulated target genes implicated in tumor metastasis. J Biomed Sci 19: 102, 2012.
7. Liu ZJ, Semenza GL and Zhang HF: Hypoxia-inducible factor 1 and breast cancer metastasis. J Zhejiang Univ Sci B 16: 32-43, 2015.
8. Agani F and Jiang BH: Oxygen-independent regulation of HIF-1: Novel involvement of PI3K/AKT/mTOR pathway in cancer. Curr Cancer Drug Targets 13: 245-251, 2013.
9. Du J, Sun B, Zhao X, Gu Q, Dong X, Mo J, Sun T, Wang J, Sun R and Liu Y: Hypoxia promotes vasculogenic mimicry formation by inducing epithelial-mesenchymal transition in ovarian carcinoma. Gynecol Oncol 133: 575-583, 2014.
10. He G, Jiang Y, Zhang B and Wu G: The effect of HIF-1α on glucose metabolism, growth and apoptosis of pancreatic cancerous cells. Asia Pac J Clin Nutr 23: 174-180, 2014.
11. Lei J, Fan L, Wei G, Chen X, Duan W, Xu Q, Sheng W, Wang K and Li X: Gli-1 is crucial for hypoxia-induced epithelial-mesenchymal transition and invasion of breast cancer. Tumour Biol 36: 3119-3126, 2015.
12. Dave JM and Bayless KJ: Vimentin as an integral regulator of cell adhesion and endothelial sprouting. Microcirculation 21: 333-344, 2014.

13. Chernoivanenko IS, Minin AA and Minin AA: Role of vimentin in cell migration. Ontogenez 44: 186-202, 2013 (In Russian).
14. Brown JM: Tumor hypoxia, drug resistance, and metastases. J Natl Cancer Inst 82: 338-339, 1990.
15. Helmlinger G, Yuan F, Dellian M and Jain RK: Interstitial pH and pO2 gradients in solid tumors in vivo: High-resolution measurements reveal a lack of correlation. Nat Med 3: 177-182, 1997.
16. Liu Y, Song X, Wang X, Wei L, Liu X, Yuan S and Lv L: Effect of chronic intermittent hypoxia on biological behavior and hypoxia-associated gene expression in lung cancer cells. J Cell Biochem 111: 554-563, 2010.
17. Verduzco D, Lloyd M, Xu L, Ibrahim-Hashim A, Balagurunathan Y, Gatenby RA and Gillies RJ: Intermittent hypoxia selects for genotypes and phenotypes that increase survival, invasion, and therapy resistance. PLoS One 10: e120958, 2015.
18. Bhaskara VK, Mohanam I, Rao JS and Mohanam S: Intermittent hypoxia regulates stem-like characteristics and differentiation of neuroblastoma cells. PLoS One 7: e30905, 2012.
19. Choi H, Gillespie DL, Berg S, Rice C, Couldwell S, Gu J, Colman H, Jensen RL and Huang LE: Intermittent induction of HIF-1α produces lasting effects on malignant progression independent of its continued expression. PLoS One 10: e125125, 2015.
20. Shen C, Beroukhim R, Schumacher SE, Zhou J, Chang M, Signoretti S and Kaelin WG Jr: Genetic and functional studies implicate HIF1α as a 14q kidney cancer suppressor gene. Cancer Discov 1: 222-235, 2011.
21. Miao ZF, Zhao TT, Wang ZN, Xu YY, Mao XY, Wu JH, Liu XY, Xu H, You Y and Xu HM: Influence of different hypoxia models on metastatic potential of SGC-7901 gastric cancer cells. Tumour Biol 35: 6801-6808, 2014.
22. Shi J, Wan Y and Di W: Effect of hypoxia and re-oxygenation on cell invasion and adhesion in human ovarian carcinoma cells. Oncol Rep 20: 803-807, 2008.
23. Zhou W, Wang G, Zhao X, Xiong F, Zhou S, Peng J, Cheng Y, Xu S and Xu X: A multiplex qPCR gene dosage assay for rapid genotyping and large-scale population screening for deletional α-thalassemia. J Mol Diagn 15: 642-651, 2013.
24. Zhu H, Wang D, Zhang L, Xie X, Wu Y, Liu Y, Shao G and Su Z: Upregulation of autophagy by hypoxia-inducible factor-1α promotes EMT and metastatic ability of CD133+ pancreatic cancer stem-like cells during intermittent hypoxia. Oncol Rep 32: 935-942, 2014.
25. Almendros I, Wang Y and Gozal D: The polymorphic and contradictory aspects of intermittent hypoxia. Am J Physiol Lung Cell Mol Physiol 307: L129-L140, 2014.
26. Pires IM, Bencokova Z, Milani M, Folkes LK, Li JL, Stratford MR, Harris AL and Hammond EM: Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. Cancer Res 70: 925-935, 2010.
27. Zepeda AB, Pessoa A Jr, Castillo RL, Figueroa CA, Pulgar VM and Farías JG: Cellular and molecular mechanisms in the hypoxic tissue: Role of HIF-1 and ROS. Cell Biochem Funct 31: 451-459, 2013.
28. Noh MY, Kim YS, Lee KY, Lee YJ, Kim SH, Yu HJ and Koh SH: The early activation of PI3K strongly enhances the resistance of cortical neurons to hypoxic injury via the activation of downstream targets of the PI3K pathway and the normalization of the levels of PARP activity, ATP, and NAD+. Mol Neurobiol 47: 757-769, 2013.
29. Chen PY, Ho YR, Wu MJ, Huang SP, Chen PK, Tai MH, Ho CT and Yen JH: Cytoprotective effects of fisetin against hypoxia-induced cell death in PC12 cells. Food Funct 6: 287-296, 2015.
30. Toffoli S, Feron O, Raes M and Michiels C: Intermittent hypoxia changes HIF-1alpha phosphorylation pattern in endothelial cells: Unravelling of a new PKA-dependent regulation of HIF-1alpha. Biochim Biophys Acta 1773: 1558-1571, 2007.
31. Mottet D, Dumont V, Deccache Y, Demazy C, Ninane N, Raes M and Michiels C: Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/akt/glycogen synthase kinase 3beta pathway in HepG2 cells. J Biol Chem 278: 31277-31285, 2003.
32. Lee JW, Bae SH, Jeong JW, Kim SH and Kim KW: Hypoxia-inducible factor (HIF-1)alpha: Its protein stability and biological functions. Exp Mol Med 36: 1-12, 2004.
33. Monti E and Gariboldi MB: HIF-1 as a target for cancer chemotherapy, chemosensitization and chemoprevention. Curr Mol Pharmacol 4: 62-77, 2011.
34. Dewhirst MW: Intermittent hypoxia furthers the rationale for hypoxia-inducible factor-1 targeting. Cancer Res 67: 854-855, 2007.
35. Moeller BJ, Cao Y, Li CY and Dewhirst MW: Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: Role of reoxygenation, free radicals, and stress granules. Cancer Cell 5: 429-441, 2004.
36. Chang IA, Oh MJ, Kim MH, Park SK, Kim BG and Namgung U: Vimentin phosphorylation by Cdc2 in schwann cell controls axon growth via β1-integrin activation. FASEB J 26: 2401-2413, 2012.
37. Chen WC, Hsu KY, Hung CM, Lin YC, Yang NS, Ho CT, Kuo SC and Way TD: The anti-tumor efficiency of pterostilbene is promoted with a combined treatment of Fas signaling or autophagy inhibitors in triple negative breast cancer cells. Food Funct 5: 1856-1865, 2014.
38. Nakajima E, Hammond KB, Rosales JL, Shearer TR and Azuma M: Calpain, not caspase, is the causative protease for hypoxic damage in cultured monkey retinal cells. Invest Ophthalmol Vis Sci 52: 7059-7067, 2011.