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Claudin-7 Mediated Metastasis of Human Lung Adenocarcinoma Cells in Intermittent Hypoxia

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Abstract

Metastasis is a prerequisite for cancer development, which requires both proliferation and migration/invasion of the cancer cells. In this study, we reported that severe hypoxic conditions could inhibit the expression of HIF-1α and Claudin-7 and up-regulate the expression of p18 in human lung adenocarcinoma cells (A-549 cells). We demonstrated that hypoxic conditions can promote the metastasis, while inhibit the viability and proliferation of A-549 cells by down-regulating HIF-1α/Claudin-7 and up-regulating p18. However, intermittent hypoxic condition could inhibit A-549 cells invasion and promote cell proliferation by affecting the expression of Claudin-7 and p18. We also confirm, using a small interfering RNA transfection technique (siRNA), that Claudin-7 could act as an anti-cancer biomarker of metastasis in A-549 cells. Silencing HIF-1α in A-549 cells could down-regulate the expression of Claudin-7.

Our results suggest that we could use intermittent fluctuations of O_2 level in tumor center to inhibit metastasis by controlling the timing and pattern of intermittent hypoxia. It may potentially be used as a new treatment for solid tumors, especially those that are not sensitive to radiotherapy.

Keywords: Intermittent hypoxia; Metastasis; Claudin-7 (CLDN7); A-549 cells

Introduction

Claudins (CLDNs), the major integral membrane proteins at tight junctions, play critical roles in apical cell-to-cell adhesion, maintenance of epithelial polarity, and formation of impermeable barriers between epithelial cells [1]. Some CLDNs can also form strands in other non-epithelial cells or be found outside of tight junctions [2-4], although their functions are still disputed. Several papers define the ultra-structural anatomy of the tight junction, and even suggest a single tight junction with differences in protein composition and structure in different subdomains [5,6]. The function of Claudin-7 (CLDN7) has obvious tissue specificity in the progression of tumors [7-12]. CLDN7 has been predicted to act as a tumor suppressor gene in lung carcinomas: the expression of Claudin-7 was remarkably decreased in squamous cell carcinoma and adenocarcinoma tissues [13], and its expression level was closely correlated with differentiation grade and lymphatic metastasis [13]. CLDN7 inhibits cell migration and invasion through ERK/MAPK signaling pathways in response to growth factor stimulation in human lung cancer cells [14]. A reduced expression of CLDN7 was associated with the poor outcome in non-small cell lung cancer [15]. Hypoxia and Intermittent (cycling) hypoxia are ubiquitous in solid carcinomas, which may affect the development and progression of cancer through different mechanisms of signalling regulation [16-18]. We have found that HIF-1α could regulate the expression of CLDN7 in nasopharyngeal carcinoma (NPC) cells at both mRNA and protein levels, and by which can further affect the migration and invasion ability of the cells [19]. In this study, by mimicking severe hypoxic conditions using hypoxia incubator, we demonstrated for the first time that hypoxia and intermittent hypoxia could affect the expression of CLDN7 in A-549 cells, which was interactively regulated between HIF-1α and CLDN7. Our data suggests that adjusting oxygen perfusion time and concentration may be used as a new treatment method to inhibit the progression of solid cancer.

Results

Hypoxia inhibits the proliferation and viability of A-549 cells

A-549 cells were incubated in a hypoxic atmosphere of 0.1 percent O_2, 5 percent CO_2 and 94.9 percent N_2 at 37°C for 8, 16, 24 and 48 hours respectively. HIF-1α expression was investigated using real-time PCR and western blotting (Figures 1O-Q). Cell proliferation and viability were then assessed using EdU and MTT assays (Figures 1A-N). As a control, the cells were incubated at normoxic conditions of 20 percent O_2, 5 percent CO_2, and 75 percent N_2. The expression levels of HIF-1α and mRNA proteins in A-549 were down-regulated under hypoxic conditions at 16 hours, 24 hours and 48 hours respectively. Hypoxic conditions inhibited cell proliferation and viability.

Hypoxia down-regulates the expression of CLDN7 and promotes the invasion and migration ability of A-549 cells

Hypoxic conditions can suppress the expression of CLDN7 in affected mRNA and protein levels (Figures 2G-I). By using transwell analysis, we further analyzed the migration and invasion ability of A-549 cells (Figures 2A-F). Hypoxia can accelerate the migration and invasion ability of A-549 cells.

Hypoxia stimulates changes in p18 which prolongs the G1 phase in A-549 cells

Hypoxic conditions significantly promoted p18 expression in A549

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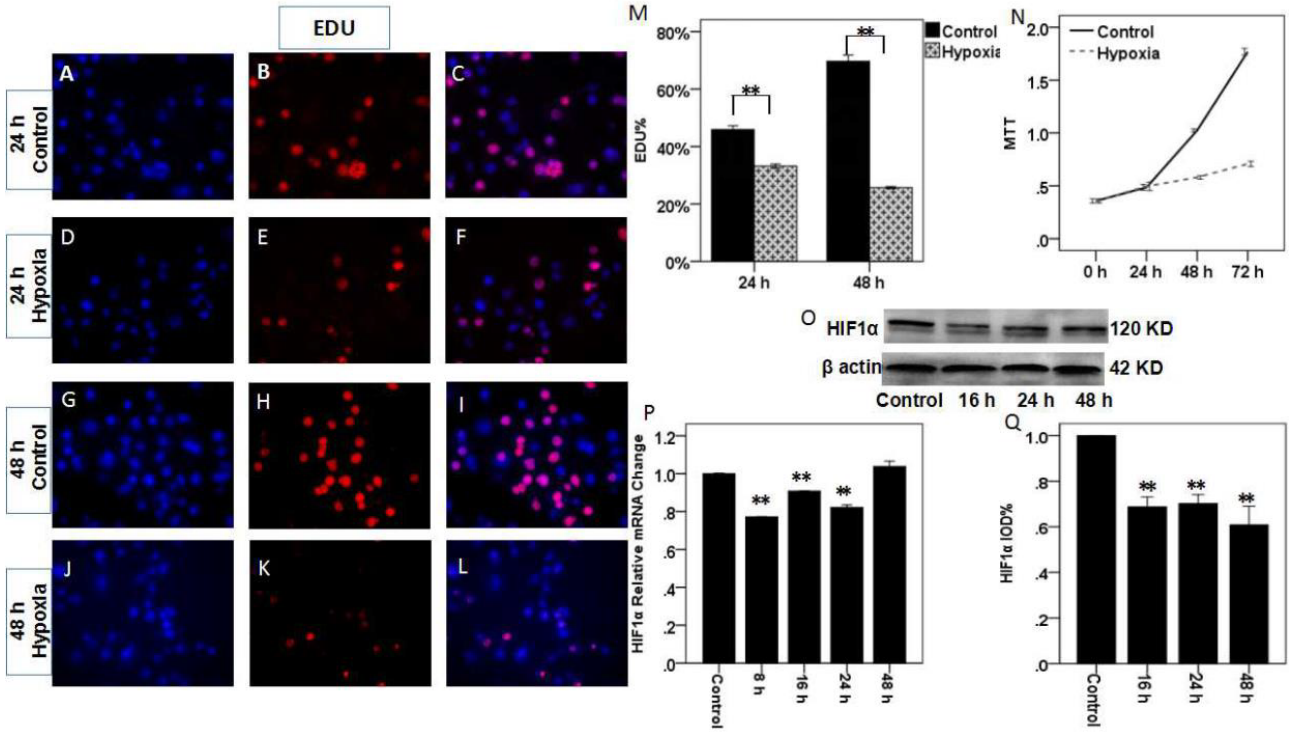

Figure 1: A-549 cells incubated in a hypoxic atmosphere of 0.1%, 5% CO_2, and 94.9% at 37°C for different amounts of time; (A to L, M) EdU of A-549 cells shows that hypoxic conditions inhibited the proliferation of the cells; (N) The MTT assay of A-549 cells showed that hypoxic conditions can significantly inhibit cell viability; (P) RT-PCR showed the HIF-1α relative mRNA change in A-549 cells, which experienced a down-regulation and then increased and reached a peak at 48 hours; (O, Q) The HIF-1α protein change in A-549 cells, which decreased significantly; Although the mRNA was increased at 48 hours, HIF-1α protein had not experienced the same change; (All positive. **: P<0.01, all data was compared to the control).

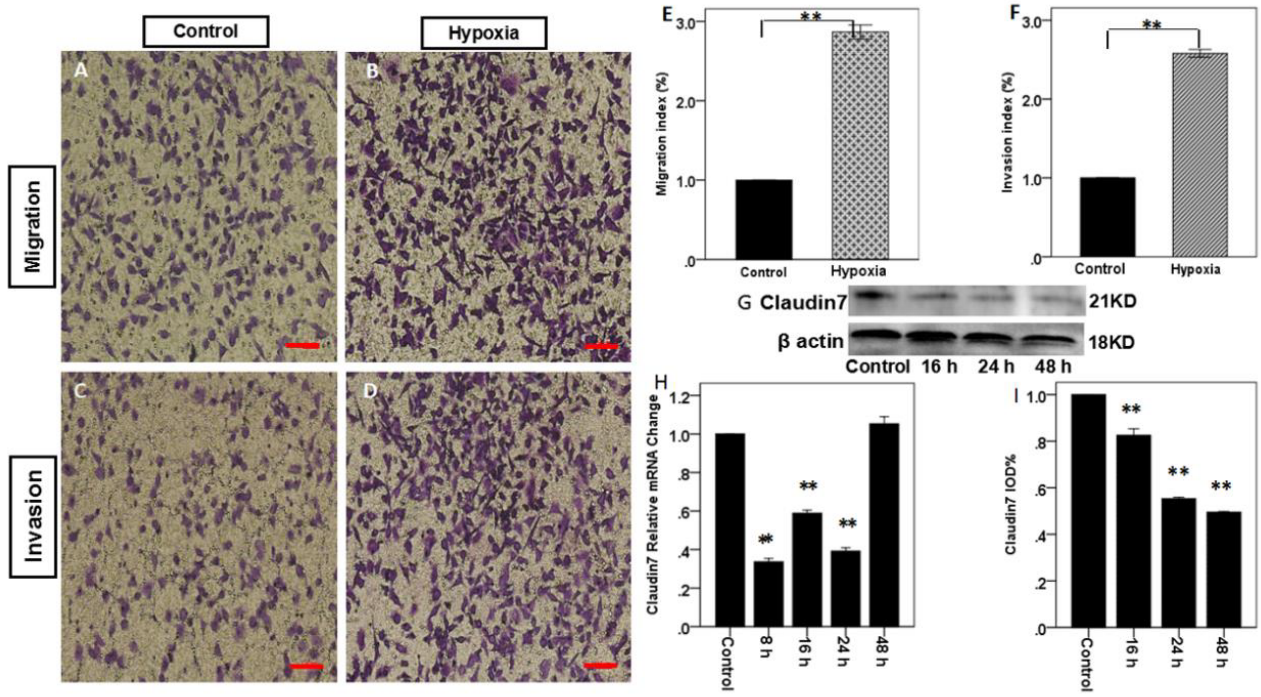

Figure 2: A-549 cells incubated in a hypoxic atmosphere of 0.1% O_2, 5% CO_2, and 94.9% at 37°C for different amounts of time; (A to D); Images of the migration and invasion experiments of the cells conducted using transwell analysis; Compared with the control, the number of cells was significantly increased in the hypoxic conditions in A-549 cells; (E,F) Bar graphs showing the transwell results of A-549 cells; (H) RT-PCR showed the CLDN7 relative mRNA change in A-549 cells with a samilar trend as HIF-1α which was down-regulated first and then increased/reached a peak at 48 hours; (G, I) The CLDN7 protein expression was decreased significantly in A-549 cells. (Scale bar =100 μm; *: P<0.05, **: P<0.01. All compared to the control group).

cells (Figures 3D-F). The cell cycle analysis was consistent with the change in p18 expression and showed significant G1 arrest (Figures 3A-C). The change was accompanied by a significant reduction of cell proliferation and viability, as demonstrated by the EdU and MTT assay (Figures 1A-N).

CLDN7 down-regulation promotes A-549 invasion and migration

Using small interfering RNA technology, we knocked down *CLDN7* in A-549 to investigate the correlation between *CLDN7* and the cell invasion capacity (for the *si-RNA* silencing gene sequences, see Table 2). The result showed that the cell invasion and migration index was significantly increased after *CLDN7* si-RNA transfection for 24-48 hours (Figures 4A-F), suggesting that *CLDN7* suppression could enhance the metastasis of A-549 cells.

HIF-1α could directly regulate the expression of CLDN7 in A-549 cells

Using siRNA transfection technique, we investigated the potential correlation between HIF-1α and CLDN7 in A-549 cells. The siRNA sequences are shown in Table 2. HIF-1α knock down inhibited CLDN7 expression in A-549 cells at both mRNA and protein levels (Figures 4G and H).

Intermittent hypoxia inhibits invasion but promotes proliferation of A-549 cells

Hypoxia can significantly promote the invasion of A-549 cells, a phenomenon does not persist in intermittent hypoxia conditions. We placed A-549 cells in a hypoxic condition as shown above, and

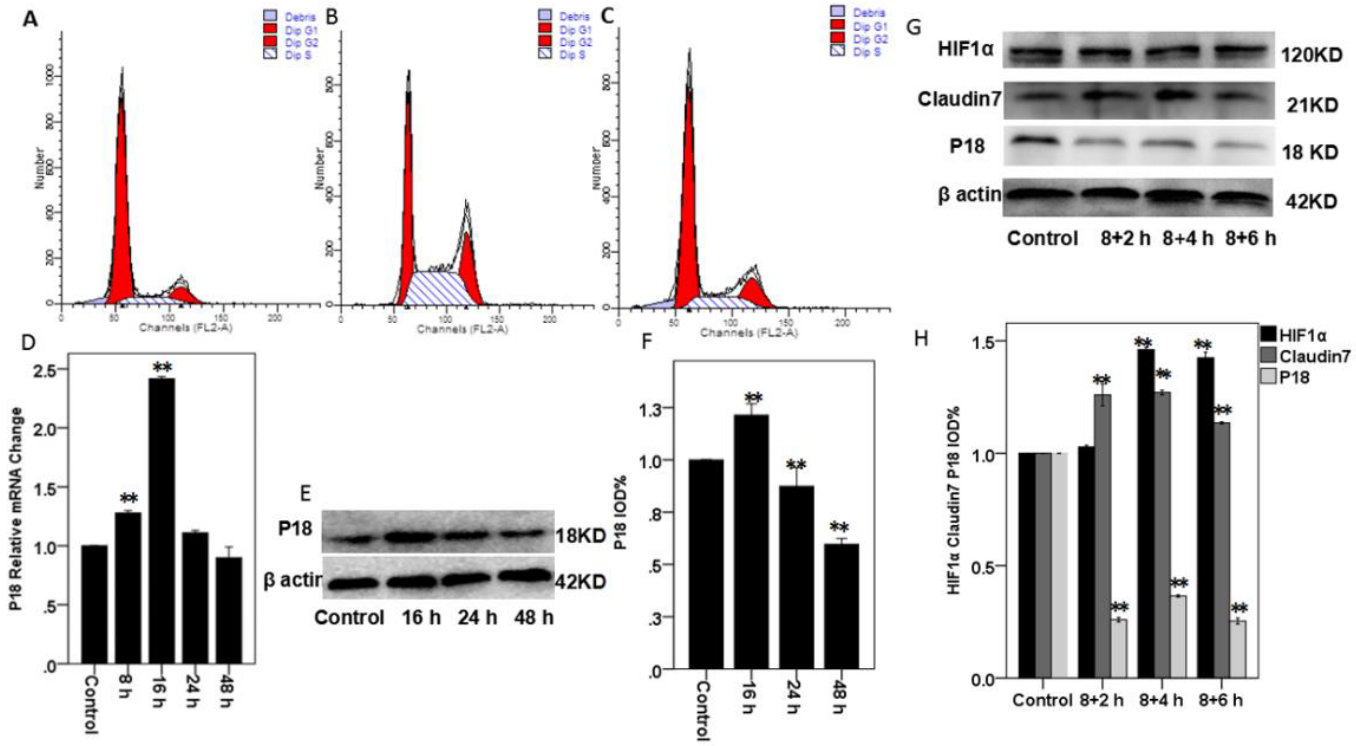

Figure 3: A to C shows the results of the cell cycle analysis; (A) Synchronized A-549 cells showed an obvious arrest at G1 phase compared with the control cells; (B) Normalcy culture condition of A-549 cells; (C) A-549 cells were arrested at G1 in hypoxic conditions, which was consistent with the pattern of p18 changes; (D, E, F) RT-PCR and western blot showed that hypoxic conditions can promote the expression of p18, whereas in both cases it reached a peak at 16 hours; (G, H) Western blot result showed the relative protein expression changes under intermittent hypoxic condition, for A-549 cells treated with 8 hours hypoxia, followed by culturing in 20% O_2 for 2, 4, and 6 hours respectively; Intermittent hypoxia promoted the expression of HIF-1α and CLDN7, and inhibited the expression of p18 dramatically. (*: P<0.05, **: P<0.01. All data were compared to the control group).

Gene	*Sense*	*Antisense*
CLDN7	*5'-AGAGCACTTTGGACAGAACCC -3'*	*5'- CTCCGGACTGGATTTCCCTC-3'*
P18	*5'-GCCGAGCCTCCTTAAAACTC-3'*	*5'- GAGGGTGCCGGTTTCTTCT -3'*
HIF1α	*5'- ACCTATGACCTGCTTGGTGC -3'*	*5'- GGCTGTGTCGACTGAGGAAA -3'*
β-actin	*5'-TGGCACCCAGCACAATGAA-3'*	*5'-CTAAGTCATAGTCCGCCTAGAAGCA-3*

Table 1: Primers used for PCR.

Gene	*Sense*	*Anti-sense*
si-HIF1α	*5'-GGCCGCUCAAUUUAUGAAUTT-3'*	*5'-AUUCAUAAAUUGAGCGGCCTT-3'*
si-CLDN7	*5'-GGCAUAAUUUUCAUCGUGGTT-3'*	*5'-CCACGAUGAAAAUUAUGCCTT-3'*
si-con	*5'-UUCUCCGAACGUGUCACGUTT-3'*	*5'-ACGUGACACGUUCGGAGAATT-3'*

Table 2: siRNA sequences.

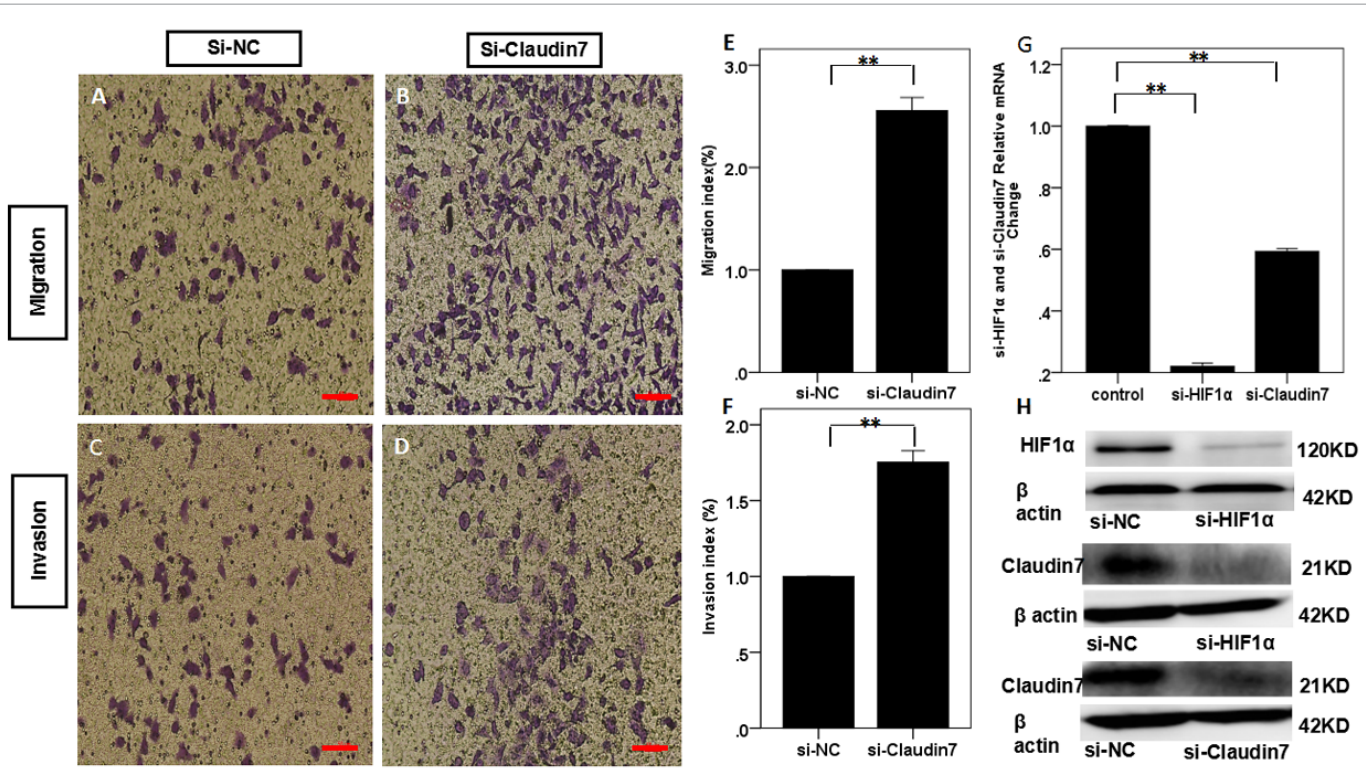

Figure 4: (A to D) Migration and invasion analysis of the A-549 cells conducted with a transwell model; si-NC and si-CLDN7 analysis in A-549 cells demonstrated that CLDN7 knock-down promoted the migration and invasion ability of A-549 cells; si-NC is the negative control of silencing the expression of CLDN7 in A-549 cells; (E, F) Quantitative analysis of the transwell study on si-NC and si-CLDN7 treatment in A-549 cells; (B) Fluorescence imaging analysis of si-HIF-1α and si-CLDN7 treatment; (G, H) RT-PCR and western blotting showed the results of HIF-1α and CLDN7 knock-down in A-549 cells; The si-RNA silencing efficiency was approximately 70% in HIF-1α and 50% in CLDN7; Western blotting result showed that silencing HIF-1α triggered down-regulation of HIF-1α and CLDN7 proteins; (Scale bars = 100μm. *: P<0.05, **: P<0.01. All data were compared to the control group).

exposed the cells to intermittent hypoxia to further investigate the expression of HIF-1α, CLDN7 and p18 at 2, 4, and 6 hours respectively, using a western blot assay (Figures 3G and H), Intermittent hypoxia significantly inhibited cell invasion by up-regulating the expression of HIF-1α and Claudin7, while promoting cell proliferation by down-regulating the expression of p18.

Discussion

Our recent study revealed a possible regulatory mechanism between HIF-1α and CLDN7 in NPCs line CNE1 and CNE2 cells [19]. We demonstrated in this study that the expression of both HIF-1α and CLDN7 was decreased in A-549 cells under extreme hypoxic conditions, while the ability of cellular invasiveness was increased. At the same time, the up-regulated expression of p18 inhibits cells transformation from G1 to S phase, thereby affecting cell proliferation.

Our study has also shown that knocking down HIF-1α in A-549 could inhibit the expression of CLDN7 at protein levels. HIF-1α may be an upstream activator regulating CLDN7, as demonstrated in NPC cells [19]. Inhibited CLDN7 by transfected siRNA could up-regulate p18 in NPC cells [19]. While we did not detect the same regulatory mechanism between CLDN7 and p18 in A-549 cells, this may be due to the low expression level of CLDN7.

Intermittent hypoxia resulting in transient fluctuations in tumor perfusion has attracted increasing attention because of the significant influence on treatment resistance displayed by tumor cells. Similarly, so has the endothelial cells of the tumor vasculature under hypoxic conditions [11,12]. By re-oxygenation of A-549 cells after hypoxic treatment, we mimicked the intermittent hypoxia present in the microenvironment of cancer cells *in vivo* and revealed that the invasion ability of cancer cells was decreased, while the proliferation was enhanced. We hypothesize that an extreme hypoxic microenvironment can inhibit the proliferation of tumor cells and promote cell migration and invasion, while removal of hypoxia by reoxygenation of the cells can inhibited cancer metastasis.

Metastasis and recurrence are the main causes of death in patients with solid tumors. According to our results, we believe that we could use the characteristic of the tumor centre and intermittent fluctuations of oxygen level to inhibit metastasis and invasion of the tumor by controlling the timing and pattern of intermittent hypoxia. It may potentially be used as a new treatment for solid tumors, especially those that are not sensitive to radiotherapy.

Materials and Methods

Antibodies and hypoxic condition

The following antibodies (Ab) were used: rabbit polyclonal Ab for CLDN7 (ab27487, Abcam, Cambridge, MA, USA), and mouse monoclonal Ab for HIF-1α (sc-53546, Santa Cruz Biotechnology, INC), mouse monoclonal Ab p18 (ab80625, Abcam Cambridge, MA, USA). Hypoxic conditions were generated and maintained using a hypoxia workstation (Ruskinn Technologies, UK).

Cells and Cell culture

A-549 cells was kindly provided by the Institute of Respiratory Disease (The First Affiliated Hospital of China Medical University, Shenyang). Cells were cultured in RPMI-1640 medium (GE Healthcare Life Sciences, South Logan, UT, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA) 10% fetal bovine serum (FBS) at 37˚C and 5.0% CO_2. To mimic severe hypoxic conditions, cells were cultured at 37°C in 5% CO_2, 94.9 % N_2, and 0.1% O_2 atmosphere in a hypoxia incubator (Ruskinn Technologies, UK).

Cell viability assay

Cell viability was assessed with a MTT assay according to the instructions from the manufacturer. Cells in each group were transferred into 96-well plates at a density of 2×10^3/well and incubated for 24, 48 and 72 hours. After the addition of 10 μg/well of the MTT reagent, the cells were further incubated for 4-6 hours in a 37°C incubator and then agitated for 10 minutes on a shaker. The absorbance was read at 490 nm using a microplate read.

Cell proliferation assay

Cells were seeded at 2×10^3 cells / well in poly-D-lysine coated 96-well chamber slides. Two hours prior to the end of the culture, 20-μm EdU was added from a 100 mM 1:10 DMSO/H_2O stock. Cells were then fixed for 20 min in 4%PFA in PBS, and EdU was detected as the protocol (KGA 337-100, KeyGEN BioTECH, Nanjing, China). The cell proliferation was quantified as the percentage of EdU incorporating cells against the total number of cells as determined by DAPI nuclear staining.

Transwell migration and Invasion assay

An invasion assay was performed using a BD BioCoat Growth Factor Reduced Matrigel Invasion Chamber (354483, BD Biosciences, San Jose, CA, USA) according to the instructions from the manufacturer. Cells were starved in 1640 medium without FBS for 24 hours, plated on the upper insert at 2.5×10^4 cells / well, and incubated in 1640 medium with FBS in 20% or 0.1% oxygen chambers, respectively. After 24 hours incubation, cells that had penetrated through to the under surfaces of the membranes were fixed in 100% methanol and stained with 0.05% Toluidine Blue Solution (206-14555, Wako Pure Chemical Industries Ltd.). For each experiment, the number of cells in seven randomly chosen fields of each filter was quantified, and these experiments were independently performed for a minimum of three times. The cell migration assay was conducted using a cell migration assay system (CBA-100, Cell Biolabs, Inc, San Diego, CA, USA) equipped with 8-μm pore size migration chambers. The methods used in this assay were similar to the Matrigel invasion assay, except that the transwell insert was not coated with Matrigel.

Cell cycle assay

Flow cytometry was performed to analyze the cell cycle in A-549 cells, fixed with 70% ethanol and stored at 4˚C for 12 h. The cells were incubated in a 100 μl solution containing 200 ng/ml RNase at 37˚C for 30 min. 5 μg/ml Propidium iodide (PI) (P3566, Molecular Probes, Eugene, OR, USA) (400 μl) was added. The cells were incubated at ambient temperature for 30 min in the dark. The results were analyzed with a FACS flow cytometer (BD FACS CalburTM).

Quantitative RT-PCR

RNA extraction and quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). RNAs were extracted from tissue samples and cells using TRIzol (Invitrogen, USA). The cDNA was synthesized from 1,000 ng RNA samples using Prime Script RT TM reagent reverse Kit (Takara Bio, Inc., Tokyo, Japan). SYBR Premix Ex Taq TM II (Takara, Japan) was used to amplify cDNA expression with β-actin (mRNA) as internal standards. The PCR reaction conditions and the primer sequences of HIF-1α, CLDN7, p18 and β-actin are shown in Table 1.

Transfection

Selective targeting of HIF-1α and CLDN7 was performed using specific siRNAs. The siRNAs were synthesized commercially and were purchased from Gene Jima (Su Zhou, Jiang Su, China). The sequences are shown in Table 2. Transfection of the siRNA (final concentration 100 nM) was performed with Lipofectamine 2000. After 24 h of transfection, the cells were cultured under normal conditions. The cells were then harvested and analyzed.

Western blotting

Cells were washed with ice-cold PBS, harvested and immediately lysed with phospho-protein extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EGTA, 1 mM NaF, supplemented with protease-phosphatase cocktail inhibitor (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration of the supernatant was determined with a BCA protein assay kit according to the manufacturer's instructions. Equivalent amounts of total cell lysates were separated by 7.5% SdS-PAGE under denaturated condition and transferred onto nitrocellulose membrane. Membranes were incubated overnight with the following primary antibodies. Horseradish peroxidase-linked secondary antibodies were employed and the signal was revealed by ECL western blotting detection reagents.

Statistical analyses

All values are reported as the mean ± SEM from minimum three independent experiments, unless otherwise stated. Two-sided Student's unpaired t test was used for the statistical analyses. ** $p<0.01$; * $p<0.05$.

Author Contributions

H.L. designed and performed experiments, analysed data and wrote the paper. Wang Y, Yang HA, Sun XY Hu Y and Guo H provided technical support, Li JY contributed with the experiment design, Yan AH provided financial support for the project.

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