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ORIGINAL ARTICLE

An inhibitor of HIF-α subunit expression suppresses hypoxia-induced dedifferentiation of human NSCLC into cancer stem cell-like cells

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Abstract

AIM: To investigate whether hypoxia induces dedifferentiation of non-small cell lung cancer (NSCLC) cells and whether a hypoxia-inducible factor (HIF) inhibitor is able to suppress the process.

METHODS: Human lung adenocarcinoma A549 cells and squamous carcinoma QG56 cells were cultured under normoxic (21% O_2) or hypoxic (4% or 1% O_2) conditions. The expression of the following genes were examined by reverse transcription-polymerase chain reaction, Western blotting and/or immunofluorescence: HIF-1α and HIF-2α subunits; differentiation marker genes, namely surfactant protein C (*SP-C*) (type Ⅱ alveolar cell marker), *CC10* (type Ⅰ alveolar cell marker) and aquaporin 5 (*AQP5*) (Clara cell marker); and stem cell-associated genes, namely *CD133*, *OCT4*, and *Musashi-1* (*MSI1*). The tumor sphere-forming ability of the cells was evaluated by culturing them in serum-free growth factor-rich medium containing epidermal growth factor (EGF) and fibroblast growth factor (FGF). CD133 expression in hypoxic regions in A549 tumors was examined by double-immunostaining of tissue cryosections with an anti-2-nitroimidazole EF5 antibody and an anti-CD133 antibody. The metastatic ability of A549 cells was examined macroscopically and histologically after injecting them into the tail vein of immunocompromised mice.

RESULTS: A549 cells primarily expressed *SP-C*, and QG56 cells expressed *CC10* and *AQP5*. Exposure of A549 cells to hypoxia resulted in a marked down-regulation of *SP-C* and upregulation of *CD133*, *OCT4*, and *MSI1* in a time-dependent manner. Moreover, hypoxia mimetics, namely desferrioxamine and cobalt chloride, elicited similar effects. Ectopic expression of the constitutively active HIF-1α subunit also caused the downregulation of *SP-C* and upregulation of *CD133* and *MSI1* but not *OCT4*, which is a direct target of HIF-2. Hypoxia enhanced the sphere-forming activity of A549 cells in serum-free medium containing EGF and FGF. Similarly, hypoxia downregulated the expression of *CC10* and *AQP5* genes and upregulated *CD133*, *OCT4*, and *MSI1* genes in QG56 cells. TX-402 (3-amino-2-quinoxalinecarbonitrile 1, 4-dioxide), which is a small molecule inhibitor of the expression of HIF-1α and HIF-2α subunits under hypoxic conditions, inhibited the upregulation of *SP-C* and hypoxia-induced down-regulation of *CD133*, *OCT4*, and *MSI1*. Notably, TX-402 significantly suppressed the hypoxia-enhanced lung-colonizing abil-

ity of A549 cells.

CONCLUSION: Hypoxia induces the de-differentiation of NSCLC cells into cancer stem cell-like cells, and HIF inhibitors are promising agents to prevent this process.

Key words: Non-small cell lung cancer; Tumor microenvironment; Hypoxia; Hypoxia-inducible factor; Differentiation; Cancer stem cells; Hypoxia-inducible factor inhibitor

Core tip: Hypoxia induces the de-differentiation of human non-small cell lung cancer cells into cancer stem cell-like cells, and TX-402, a small-molecule inhibitor of hypoxia-inducible factor (HIF)-1α and HIF-2α expression, suppresses this hypoxia-induced process and, importantly, the metastatic ability of the cells.

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INTRODUCTION

In most solid tumors, hypoxic regions are generated because of a shortage in oxygen supply[1-3]. Hypoxia influences many aspects of cancer cell biology, including neoangiogenesis, energy metabolism, cell survival, radiosensitivity, chemosensitivity, differentiation and invasion/metastasis[1-4]. Thus, hypoxia correlates with poor patient outcomes[5]. Recent preclinical studies have also demonstrated that anti-angiogenic therapies generate intratumoral hypoxia and, thereby, elicit increases in invasiveness and metastasis[6-8].

Hypoxia activates the expression of hundreds of genes in each cell[1-3]. Many of these genes are regulated by hypoxia-inducible factors (HIFs) such as HIF-1 and HIF-2. Under normoxic conditions, the α subunit of HIF-1 (HIF-1α) is hydroxylated at the proline-402 and proline-564 residues by specific Fe^{2+}, 2-oxoglutarate, and O_2-dependent prolyl hydroxylases. The subunit is then recognized and ubiquitinated by the von Hippel-Lindau tumor suppressor protein complex, leading to degradation *via* the ubiquitin-proteasome pathway. Under hypoxic conditions, the HIF-α subunit stabilizes and dimerizes with the HIF-β subunit. In the nucleus, HIF-1 binds to the hypoxia response element (HRE) of hypoxia-inducible genes and transactivates their transcription[9]. Chelating or substituting Fe^{2+} with desferrioxamine (DFO) or $CoCl_2$, respectively, increases the levels of HIF-1α and HIF-2α[1-3]. Although there are many similarities between HIF-1 and HIF-2, each has common as well as different HRE target genes.

Hypoxia influences stem cell self-renewal and multipotency[10]. HIF-2 but not HIF-1 is reported to regulate the expression of OCT4, a POU transcription factor that is associated with the self-renewal and pluripotency of stem cells[11]. Hypoxia is also known to induce the dedifferentiation of neuroblastoma and breast cancer cells[12]. These data indicate that hypoxia is involved not only in the de-differentiation of tumor cells but also in the maintenance of cancer stem cells (CSCs) with high metastatic potential.

The lung is composed of multiple types of cells such as Clara, alveolar type I (AT1), alveolar type Ⅱ (AT2), and pulmonary neuroendocrine cells[13]. Each cell type expresses lineage-specific differentiation marker gene products. For example, CC10 (also known as CCA or CCSP), aquaporin 5 (AQP5), and pro-surfactant protein C (SP-C) are the specific markers for Clara, AT1 and AT2 cells, respectively[14-16]. A rare population of progenitor cells, referred to as the bronchoalveolar stem cells (BASCs), exists in the bronchoalveolar duct junction of mouse normal lung[17]. Lung adenocarcinoma is thought to originate from BASCs, which frequently co-express SP-C and CC10[17-19]. Several recent studies have demonstrated the existence of a rare population of CD133-positive undifferentiated cells in small cell and non-small cell lung cancer (NSCLC) that exhibit CSC characteristics[20-23]. The metabolic enzyme, glycine decarboxylase (GLDC), has also been reported to be a key characteristic of CSCs in NSCLC[24]. In contrast, it is largely unknown how tumor hypoxia affects the differentiation- and stem cell-related gene expression in NSCLC cells.

Many small molecule HIF-1 inhibitors have been identified or developed to inhibit angiogenesis and suppress the growth of a variety of tumor cells[25]. However, the influence of such HIF-1 inhibitors on the hypoxia-induced dedifferentiation of human lung cancer cells to CSC-like cells remains unknown. Assuming that HIF-1α and HIF-2α are necessary for inducing and maintaining CSC characteristics under hypoxic conditions, inhibitors of their expression or activity would have a profound inhibitory effect on such hypoxia-induced processes.

TX-402 is a member of a group of hypoxia-selective cytotoxins, which includes tirapazamine (TPZ), that are activated by bioreduction to selectively exhibit cytotoxicity under severe hypoxia (< 1% O_2). TX-402 and its analogs inhibit HIF-1α protein synthesis under hypoxia without affecting the HIF-1α steady-state mRNA level and the protein degradation rate, and they also reduce the hypoxia-inducible expression of vascular endothelial growth factor (VEGF) and angiogenesis[26-28]. Although the mechanism by which TX-402 inhibits HIF-1α protein synthesis is still unclear, TPZ and other hypoxic cytotoxins have recently been reported to inhibit HIF-1α translation through the phosphorylation of translation initiation factor 2α (eIF2α) and/or the dephosphorylation of mTOR and 4E-BP1[29,30]. Of interest, TPZ has been shown to selectively deplete primitive hematopoietic stem cell subsets in bone marrow[31]. Therefore, this class of bioreductive agents could have a major impact on cancer therapy if developed appropriately. Against this background,

Table 1 Primers used for reverse transcription-polymerase chain reaction analyses

Gene	Forward primer	Reverse primer	Accession #
CC10	CTTTCAGCGTGTCATCGAAA	TTGAAGAGAGCAAGGCTGGT	U01101
SP-C	TCCACATGAGCCAGAAACAC	CTGGCCCAGCTTAGACGTAG	NM_003018
AQP5	CTACTTCACTGGCTGCTCCA	GTGGTCAGCTCCATGGTCTT	NM_001651
CD133	TGGAGTGCAGCTAACATGAG	TGCACATGAAAAGACCTGGG	NM_006017
4-Oct	GAGGAGTCCCAGGACATCAA	CTCCAGGTTGCCTCTCACTC	NM_002701
MSI1	GTTCAGAGCGTTGGACCTTC	AAACCCAAAACACGAACAGC	NM_002442
GLDC	ATTTCTCGTTGATCCCCGTTGC	GCGATGTCTACCCCAAATTCTC	NM_000170
NANOG	ACCAGACCCAGAACATCCAG	TTCACACGTCTTCAGGTTGC	NM_024865
NES	AACAGCGACGGAGGTCTCTA	TTCTCTTGTCCCGCAGACTT	NM_006617
BMI1	AGAGCTGGAAGTCGAGTGT	GCACCTTCACATTCCTCTC	NM_005180
EGFR	GCACGAGTAACAAGCTCACG	TTCCTCTGATGATCTGCAGG	NM_005228
ERBB2	AGCAGAGGATGGAACACAGCGG	CTCCTGGATATTGGCACTGG	NM_004448
NOTCH1	TGCTGGACGAGTACAACCTG	CGCATTGACCATTCAAACTG	AF308602
NOTCH2	ACCCTTGTGAGAATGCTGCT	CCATACCACTGAAGCCTGGT	NM_024408
CD117	CTATGCTCTCGCACCTTTCC	CAATGAAGTGCCCCTGAAGT	X06182
MET	GGTTTTTCCTGTGGCTGAAA	GGCATGAACCGTTCTGAGAT	NM_001127500
CD34	ACAACACGTGGTGGCTGATA	GAGTTTACCTGCCCCTCCTC	NM_001773
CD44	TGGAGCAAACACAACCTCTG	TCCACTTGGCTTTCTGTCCT	NM_000610
CD45	AGATGCCCAGTGTTCCAC	AGGGTTGAGTTTTGCATTGG	NM_002838
PCGF2	TTGCAGTGGAAACTTTGTGC	AGGTGAGACTCCACCACCAG	NM_007144
CDH1	GGTTATTCCTCCCATCAGCT	CTTGGCTGAGGATGGTGTA	L08599
SOX2	CCCCCCTGTGGTTACCTCTTC	TTCTCCCCCCTCC AGTTCG-3	NM_003106
RUNX3	GCTGTTATGCGTATTCCCGTAG	TGAAGTGGCTTGTGGTGCTGAGTGA	NM_001031680
ACTB	TGACGGGGTCACCCACACTGTGCCCATCTA	CTAGAAGCATTTGCGGTGGACGATGGAGGG	NM_001101

GLCD: Glycine decarboxylase.

in the present study, we primarily employed human lung adenocarcinoma A549 cells and investigated the effects of hypoxia and TX-402 on differentiation- and stem cell-related gene expression and metastasis.

MATERIALS AND METHODS

Cells and cell culture

Human lung adenocarcinoma cells (A549, PC3, PC9, and PC14), squamous cell carcinoma cells (PC1, PC10, and QG56), bronchoalveolar carcinoma cells (H358), and small cell carcinoma cells (PC6 and QG90) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere with 21% O_2/5% CO_2 (normoxia) or 1% O_2/5% CO_2 (hypoxia) unless otherwise stated. In some experiments, the cells were cultured in 4% O_2/5% CO_2. The cells were provided by the Chiba Cancer Center Research Institute[32].

Reagents

TX-402 (3-amino-2-quinoxalinecarbonitrile 1,4-dioxide) was synthesized according to a previous report[33]. DFO and cobalt chloride were obtained from Sigma-Aldrich (St. Louis, MO, United States). EF5 was provided by the National Cancer Institute (CTEP).

Antibodies

Mouse monoclonal anti-β-actin antibody was obtained from Sigma-Aldrich. Mouse monoclonal anti-HIF-1α and rabbit polyclonal anti-HIF-2α antibodies were purchased from Novus Biologicals (Littleton, CO, United States). The rabbit polyclonal anti-CD133 antibody, goat polyclonal anti-OCT4 antibody and rabbit polyclonal anti-E2F-1 antibody were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States), and the rabbit polyclonal anti-pro-SP-C antibody and phycoerythrin-conjugated mouse monoclonal CD133/2 antibody were obtained from Merck Millipore (Billerica, MA, United States) and Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), respectively. The goat anti-Musashi-1 (MSI1) antibody was obtained from R and D Systems (McKinley Place, MN, United States). The Cy3-labeled mouse monoclonal anti-EF5 antibody (ELK3-51) was kindly provided by Dr. Koch at Pennsylvania State University.

Semiquantitative reverse transcription polymerase chain reaction

Reverse transcription (RT) was performed in a 10 μL reaction mixture containing 1 μg of total RNA, which was extracted with guanidinium thiocyanate, 250 ng of oligo(dT), and 100 units of murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, United States) for 1 h at 37 ℃. The resulting cDNA was used to amplify target cDNAs using *Gō*Taq DNA polymerase (Agilent Technologies, Santa Clara, CA, United States). The sense and antisense primers used for polymerase chain reaction (PCR) are listed in Table 1. The PCR conditions were as follows: 95 ℃ for 5 min; 25-35 cycles at 95 ℃ for 30 s, 59 ℃ for 30 s, and 72 ℃ for 30 s; and 72 ℃ for 7 min. β-Actin was used as a loading control, which was run for each sample under the same conditions as those used for the other genes, except that the

number of cycles was 23-25. PCR products were separated on a 1.2% agarose gel. Quantification of the density of each band was performed using ImageJ version 1.45 software (National Institutes of Health).

Transfection of the plasmid encoding constitutively active HIF-1α

To express constitutively active HIF-1α, A549 cells were transfected with pcDNA3.1 as a control and pcDNA3.1/HIF-1α$^{P402A/P564A}$ using Lipofectamine 2000 (Life Technologies). Two days after transfection, total RNA was prepared as described above and used for RT-PCR analysis.

Western blotting analyses

Cells were lysed in 1% NP-40.1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L PMSF, and protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were centrifuged at 15000 *g* for 10 min at 4 ℃, and the supernatants were used for detecting SP-C and CD133. Nuclear extracts were prepared by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, United States) and were then used for detecting HIFs, OCT4 and MSI1. Proteins were resolved by 10% or 15% SDS-PAGE gels and transferred to an Immobilon-P membrane (Merck Millipore). The membrane was blocked with 5% dry milk in TBS-T and incubated with anti-HIF-1α, anti-HIF-2α, anti-SP-C, anti-CD133, anti-OCT4, anti-MSI1 or anti-E2F-1 antibodies followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected using ECL Western blotting detection reagents (GE Healthcare, Waukesha, WI, United States).

Tumor spheroid (pneumosphere) formation

To evaluate the effect of hypoxia on pneumosphere formation, cells pre-cultured under 4% O_2 for 4 d were suspended at a density of 2000 cells/mL in BEBM (bronchial epithelial cell growth) medium (Lonza, Walkersville, MD, United States) supplemented with SingleQuots, which contains retinoic acid, bovine pituitary extract, insulin, hydrocortisone, transferrin, triiodothyronine, epinephrine, gentamicin, and amphotericin B (Lonza), as well as 10 ng/mL human epidermal growth factor (EGF) (PeproTech Inc., NJ, United States), and 10 ng/mL human basic fibroblast growth factor (bFGF) (PeproTech)[34]. Cells were incubated in PrimeSurface culture dishes (35 mm) (Sumitomo Bakelite Co., Tokyo, Japan) under normoxic or hypoxic (4% O_2) conditions for 10 d. EGF and bFGF were added every 3 d. The size of the spheres was calculated by the following equation: size (μm) = (a + b)/2; where a and b are the larger and smaller diameters, respectively.

Colony formation assay

The survival of A549 cells treated with TX-402 under normoxic or hypoxic conditions was determined in a colony formation assay. For this assay, A549 cells dissociating to single cells were seeded in a 6-well plate at a density of 5 × 10^4 cells per well in 2 mL of medium. After attachment, the cells were cultured under normoxic or hypoxic conditions in the presence of DMSO (0.1%) or TX-402 (final concentration of 20 μmol/L) for 3 d. After the treatment, viable cells were counted and seeded in a 10-cm culture dish at a concentration of 100 cells/dish. The cells were further cultured for 14 d under normoxic conditions, and the colonies that were formed were fixed with methanol, stained with 0.05% crystal violet, and counted.

Immunocytochemistry

A549 cells on cover slips were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed for 30 min with 4% formaldehyde and 5% sucrose in DPBS. After washing with DPBS, the cells were permeabilized in 0.5% Triton X-100 in DPBS for 10 min followed by washing three times with DPBS. In some experiments, this permeabilization step was omitted. The cells were then treated with 3% bovine serum albumin (BSA) in DPBS containing 0.1% glycine for 1 h to block nonspecific binding sites. After extensive washing, the cells were incubated with anti-SP-C, anti-CD133, anti-OCT4 or anti-MSI1 antibodies in DPBS containing 1 mg/mL BSA and 0.1% (v/v) normal rabbit serum, normal goat serum or normal chicken serum depending on the secondary antibody at 4 ℃ overnight. Primary antibodies were omitted for negative control studies. After washing with DPBS, the first antibodies were localized with the appropriate secondary antibodies. The secondary antibodies used were FITC-goat anti-rabbit IgG, TRITC-goat anti-rabbit IgG, Alexa Fluor 488-goat anti-rabbit IgG, and Alexa Fluor 488-chicken anti-goat IgG. After rinsing, the cells were counterstained with DAPI (1 μg/mL) in DPBS, and the coverslips were mounted in 50% glycerol in DPBS containing 1 mg/mL *p*-phenylenediamine to inhibit photobleaching. The cells were observed under a confocal laser microscope (Fluoview, Olympus, Tokyo, Japan).

Immunohistochemistry

All animal experiments were performed in compliance with the institutional guidelines. A549 cells (5 × 10^6 cells) were inoculated subcutaneously into Balb/c nude mice. When an estimated tumor volume reached approximately 500 mm^3, 300 μL of EF5 solution (3 mg/mL) was administered intraperitoneally into the mice. Two hours later, subcutaneous tumors were surgically removed and frozen in optimum cutting temperature compound. Cryostat sections cut at a thickness of 10 μm were fixed with 4% paraformaldehyde and washed with DPBS. For the detection of CD133, tissue samples were treated with 5% donkey serum in DPBS/1% (w/v) BSA/20% (w/v) dry milk for 1 h to block nonspecific binding sites. Sections were then rinsed with DPBS and incubated with an anti-CD133 antibody overnight at 4 ℃. After extensive washing with DPBS, the sections were incubated with a FITC-labeled donkey anti-goat secondary antibody for 1 h. After fixation with 4% formaldehyde, the sections were washed with DPBS, treated with 5% mouse serum

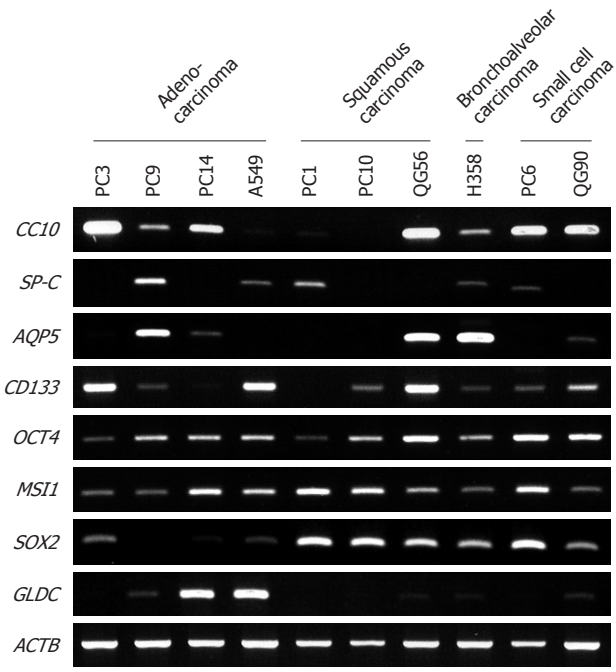

Figure 1 Expression of lineage-specific differentiation- and stem cell-associated genes in various lung cancer cell lines. Total RNA isolated from the cells was subjected to reverse transcription-polymerase chain reaction analyses; *GLDC*: Glycine decarboxylase; *MSI1*: Musashi-1; *ACTB*: β-Actin.

in DPBS/20% (w/v) dry milk/0.3% (v/v) Tween 20 overnight at 4 ℃, rinsed with 0.3% Tween 20 in DPBS, and then incubated with Cy3-labeled anti-EF5 antibody (ELK3-51) at 4 ℃ overnight to detect hypoxic cells. Tissue sections were counterstained with DAPI and observed under a confocal laser microscope (Fluoview, Olympus).

Analysis of lung-colonizing potential

For the lung-colonizing assay, A549 cells (2×10^5 cells) cultured under normoxic or hypoxic (1% O_2) conditions for 3 or 5 d were injected into the tail vein of 6-wk-old Balb/c nude mice (CLEA Japan, Inc.). The lungs were removed 40 d later and fixed in Bouin's solution, and the parietal nodules were counted. For histology, formalin-fixed, paraffin-embedded lung tissues were sectioned at a thickness of 5 μm, mounted, and stained with hematoxylin and eosin.

Statistical analysis

The Mann-Whitney *U* test and Student's *t*-test were used to determine statistical significance in metastasis assays and other assays, respectively. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression of lineage-specific differentiation marker genes and stem cell-related genes in different types of human lung cancer cell lines

We examined the expression of lineage-specific differentiation marker genes for Clara (*CC10*), AT1 (*AQP5*), and AT2 (*SP-C*) cells as well as several stem cell-related genes (*CD133*, *OCT4*, *MSI1*, *SOX2* and *GLDC*) in various types of lung cancer cell lines (Figure 1). Among the adenocarcinoma cell lines, PC3 and PC14 cells expressed *CC10*, and A549 cells primarily expressed *SP-C*. PC9 cells expressed *CC10*, *SP-C*, and *AQP5*. Among the squamous carcinoma cell lines, *SP-C* was detected only in PC1 cells. QG56 cells were positive for *CC10* and *AQP5*, but PC10 cells expressed none of the markers. By comparison, bronchoalveolar carcinoma H358 cells expressed all of the lineage-specific differentiation marker genes. Small cell carcinoma PC6 and QG90 cells expressed high levels of *CC10*. The stem cell-related gene, CD133, was expressed at higher levels in PC3, A549 and QG56 cells compared to the other cells. All cell lines were *OCT4*-positive/*MSI1*-positive. The expression of *SOX2* was detected in all cell lines except PC9 cells. *GLDC* was highly expressed in PC14 and A549 cells compared with other cell lines. Taken together, these results suggest that there is not a simple causal relationship between the expression of the differentiation marker genes and the expression of the stem cell-related genes. For subsequent analyses, we mostly used A549 and QG56 cells.

Hypoxia suppresses the expression of lineage-specific marker genes

We first confirmed the expression of HIF-1α and HIF-2α in A549 and QG56 cells at the mRNA level (Figure 2A). We then cultured these cells under normoxic or hypoxic conditions for up to 5 d and monitored cell growth and viability. The growth of A549 cells under hypoxic conditions was slightly inhibited by 4 d after the onset of hypoxia compared with their growth under normoxic conditions (Figure 2B). No cell death was observed in hypoxic conditions (Figure 2D). Hypoxia also slightly inhibited the growth of QG56 cells but had no detectable effect on viability (Figure 2C, D). Because these hypoxic conditions did not appear to be cytotoxic, we cultured A549 and QG56 cells under hypoxia, and we then examined the expression levels of *SP-C*, *CC10*, and *AQP5*. As described above, normoxic A549 cells primarily expressed *SP-C* and small amounts of *CC10* and *AQP5* (Figure 3A). Upon hypoxic exposure, the expression level of these genes was greatly reduced in a time-dependent manner (Figure 3A, B, D). QG56 cells mainly expressed *CC10* and *AQP5* (Figure 3A), and the expression level of these genes was greatly reduced in a time-dependent manner after exposure to hypoxia (Figure 3A, C, E).

Hypoxia influences the expression of stem cell-related genes

The above results prompted us to compare in detail the expression of a panel of stem cell-related genes between normoxic and hypoxic cells. Among the genes tested, the expression levels of *CD133*, *OCT4*, and *MSI1* were increased by hypoxia in A549 cells (Figure 4A left, B, C) in a time-dependent manner (Figure 4F). *NANOG* and

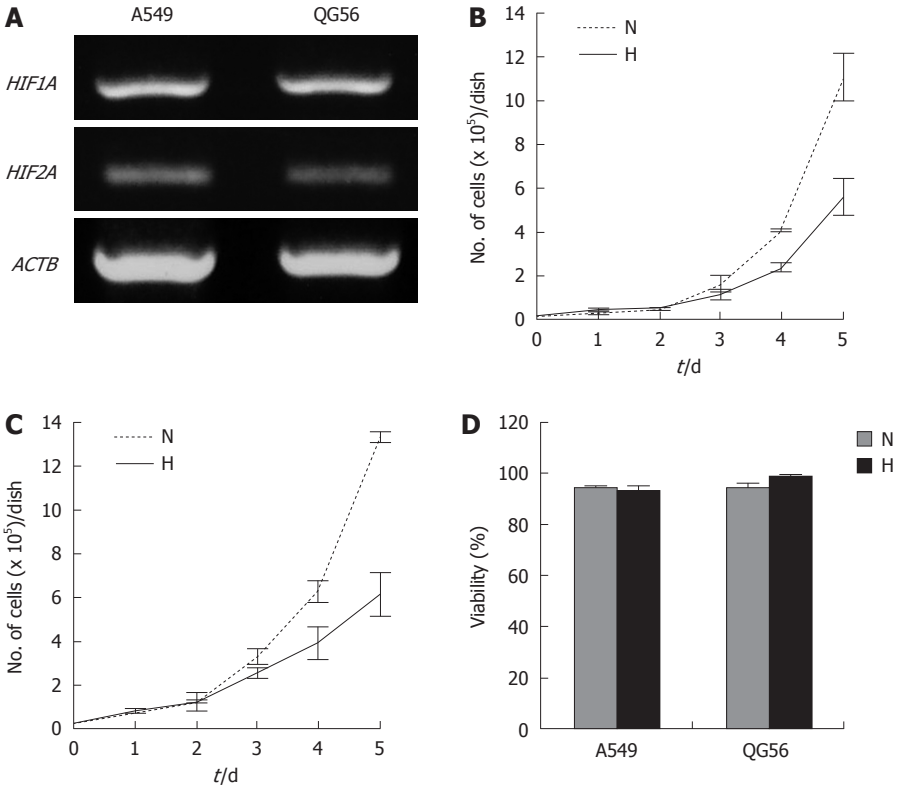

Figure 2 Expression of hypoxia-inducible factor α subunits and cell growth in A549 and QG56 cells under hypoxic conditions. The cells were cultured under normoxic (N) or hypoxic (H) conditions. A: Expression of hypoxia-inducible factor (HIF)-1α and HIF-2α mRNA; **B: Cell growth of A549; C: Cell growth of QG56 cells; D:** Cell viability; **The cells were cultured for 5 d; Bars, SD (***n* = 3).

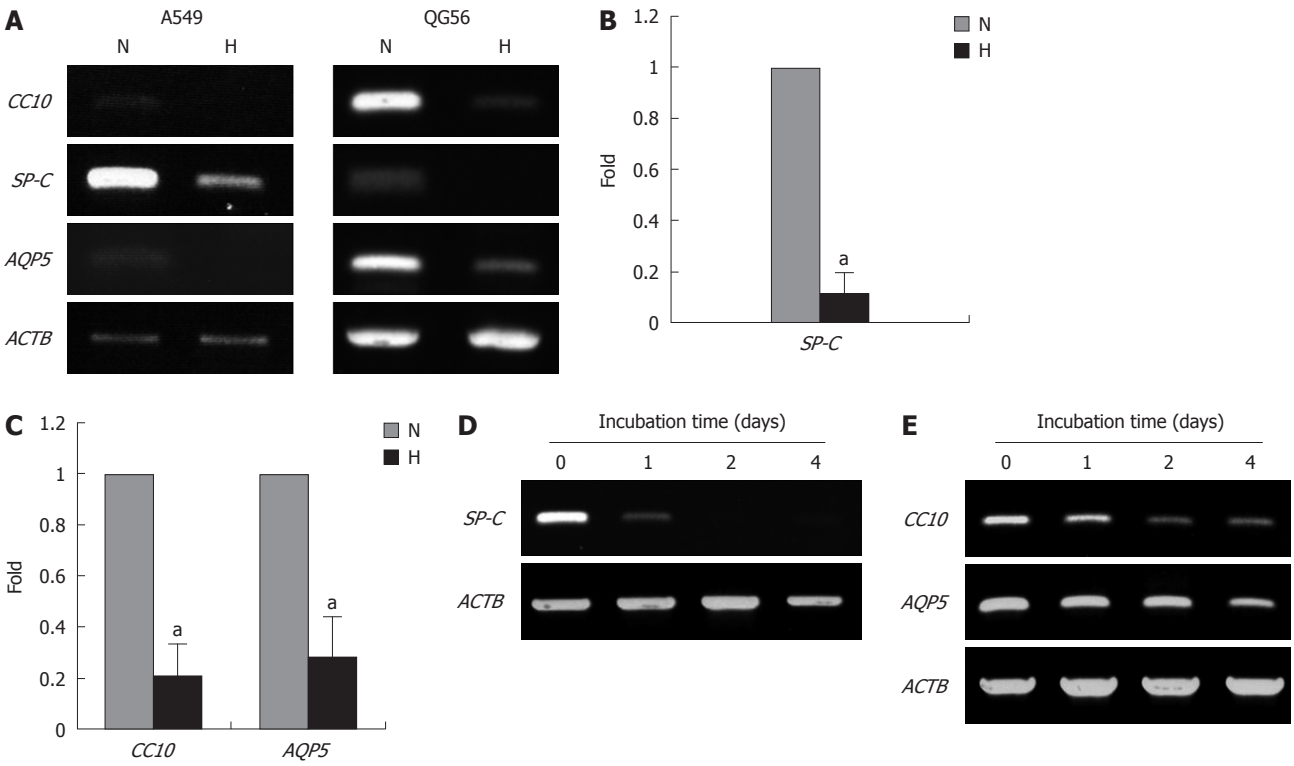

Figure 3 Effect of hypoxia on the expression of differentiation-associated genes. The cells were cultured under normoxic (N) or hypoxic (H) conditions for 5 d. A: Expression of differentiation-associated genes in A549 and QG56 cells; **B: Normalized expression levels of the genes in A549; C: Normalized expression levels of** the genes in QG56 cells; **D: Time-course of the expression of SP-C in A549 cells; E: The expression of CC10 and AQP5 in QG56 cells; The expression level of each** gene was normalized to that of β-*Actin* (*ACTB*); **Data are shown as fold-change relative to normoxia (normoxia values set to equal 1),** $^{a}P < 0.05$ (*n* = 3); **The cells were** cultured under hypoxic conditions for the indicated periods.

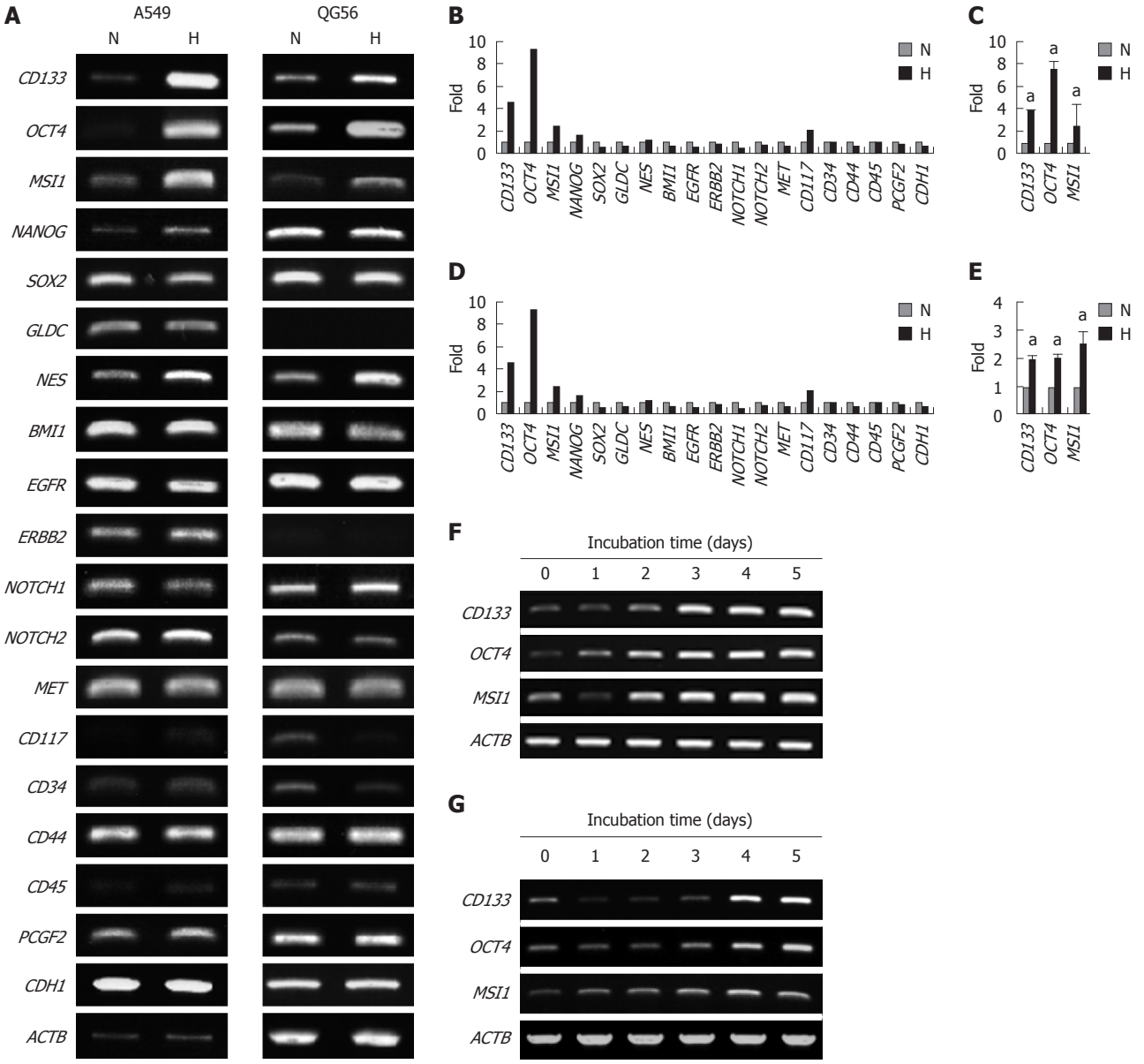

Figure 4 Effect of hypoxia on the expression of stem cell-associated genes. The cells were cultured under normoxic (N) or hypoxic (H) conditions. **A: Expression of stem cell-associated genes in A549 and QG56 cells; B and C: Normalized expression levels of the genes in A549 cells; Cells were cultured for 5 d; D and E:** Normalized expression levels of the genes in QG56 cells; **F: Time-course of the expression of** *CD133*, *OCT4*, and *MSI1* in A549; **G: Time-course of the expression of** CD133, OCT4, and MSI1 in QG56 cells (G). **The expression level of each gene was normalized to that of** *β-Actin* (*ACTB*); **Data are shown as fold-change relative to** normoxia (normoxia values set to equal 1). $^{a}P < 0.05$ ($n = 3$); **The cells were cultured for the indicated periods.**

CD117 were also slightly upregulated by hypoxia. The expression of other genes, including *SOX2*, *GLDC*, *NES*, *BMI1*, *EGFR*, *ERBB2*, *NOTCH1*, *NOTCH2*, *CD34*, *CD44*, *CD45*, *PCGF2* (*MEL18*), and *CDH1* (*E-cadherin*), was detected in A549 cells and was only marginally affected by hypoxia (Figure 4A, B). Hypoxia also enhanced the expression of *CD133*, *OCT4*, and *MSI1* in QG56 cells, but to a lesser extent than in A549 cells (Figure 4A, D, E). Time-course experiments revealed that the expression of *OCT4*, *CD133*, and *MSI1* gradually increased under hypoxia (Figure 4G). Unlike in A549 cells, *NANOG* expression was already high in QG56 cells and was not affected by hypoxia. The expression of *NES* was slightly upregulated by hypoxia, but the expression of *SOX2*, *BMI1*, *EGFR*, *ERBB2*, *NOTCH1*, *NOTCH2*, *CD34*, *CD44*, *CD45*, *PCGF2*, and *CDH1* was unaffected. The expression level of *GLDC* was quite low in these cells (Figure 4A, D).

Pneumosphere formation under normoxic and hypoxic conditions

The ability to form spheres in serum-free culture conditions has been considered to be an important marker that represents the subset population of CSC-like cells[35]. We tested the ability of A549 and QG56 cells to form spheres under normoxic and hypoxic conditions. In this experiment, we allowed the cells to form spheres in 4% O_2 to minimize the inhibitory effect of hypoxia (1% O_2)

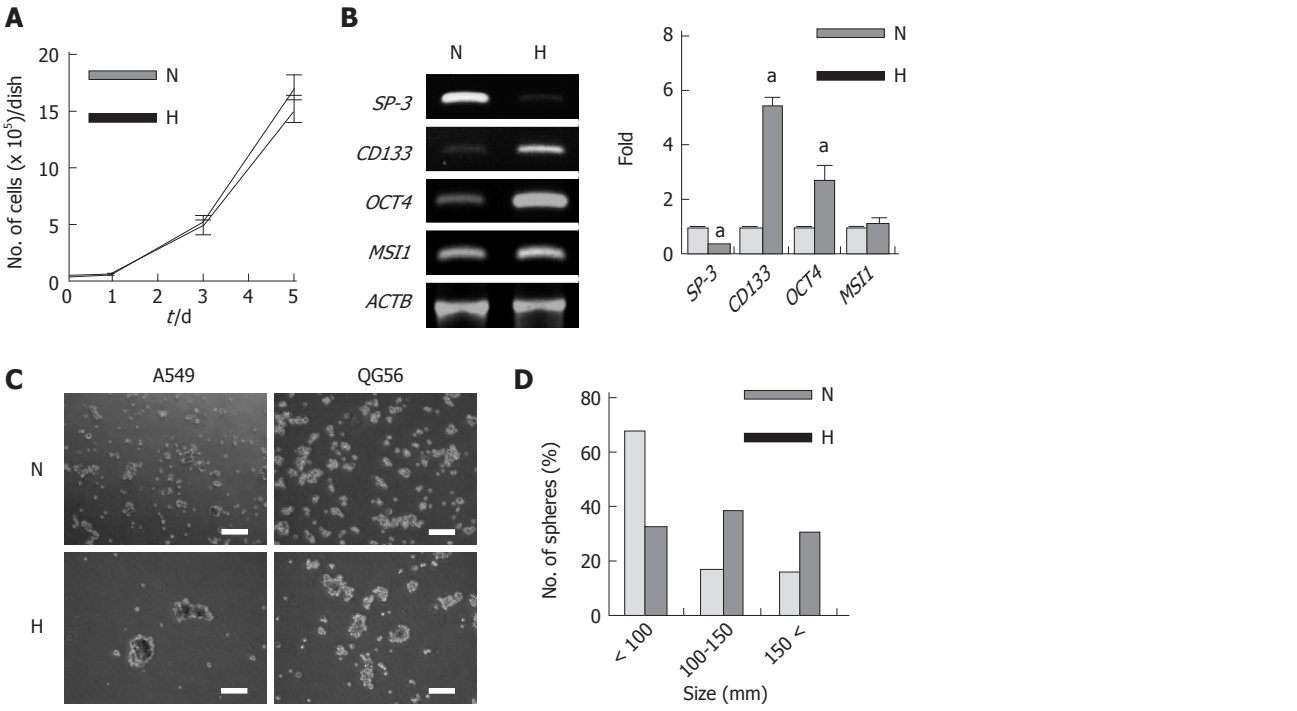

Figure 5 Changes in gene expression and sphere-forming activity of A549 and QG56 cells under hypoxic conditions. The cells were cultured under normoxic (N) or hypoxic (4% O_2) (H) conditions. **A: Growth of A549 cells. Bars, SD (n = 3); B: Differentiation- and stem cell-related gene expression in A549 cells, the cells were** cultured for 5 d, **data are shown as fold-change relative to normoxia (normoxia values set to equal 1),** $^aP < 0.05$ (n = 3); **C: Sphere formation; A549 or QG56 cells pre-**cultured under normoxic or hypoxic (1% O_2) conditions for 2 d were reseeded in BEBM supplemented with various additives (see Materials and Methods) and then further cultured for 7 d; **Left panels represent A549 cells and right panels represent QG56 cells. Scale bars = 200** μm; **D: Size distribution of the spheres formed by** A549 cells.

on cell growth. Cell growth in complete medium was not inhibited in 4% O_2 (Figure 5A) and the changes in the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1* were similar to those observed in 1% O_2 (Figure 5B). The ability of A549 cells to form spheres was markedly increased under hypoxic conditions. Hypoxic cells formed larger spheres than normoxic cells (Figure 5C left, D). QG56 cells formed only loosely attached cell aggregates under normoxic conditions, but they formed more compact aggregates under hypoxic conditions (Figure 5C right). Taken together with the gene expression results, these data suggest that hypoxia can strongly induce the dedifferentiation of A549 cells but can only weakly induce the dedifferentiation of QG56 cells.

Effect of hypoxia on SP-C and CD133 protein expression in A549 cells

We examined SP-C and CD133 protein expression in A549 cells to examine the effect of hypoxia on a representative differentiation marker and a stem cell-associated marker, respectively. Immunofluorescence studies revealed that SP-C expression in normoxic A549 cells (some of which exhibited plasma membrane staining as indicated by arrowheads in Figure 6A), was significantly reduced in hypoxic A549 cells (Figure 6A). In contrast, CD133 expression was enhanced in hypoxic cells compared to normoxic cells (Figure 6B). These results were also confirmed by immunoblot analysis (Figure 6C, D). To examine whether the up-regulation of CD133 was located in hypoxic regions in tumors, we immunostained cryosections prepared from A549 subcutaneous tumors with an anti-CD133 antibody. To detect hypoxic cells in tumors, we injected EF5 intraperitoneally into tumor-bearing mice 2 h before the surgical removal of the tumor masses. Double-immunostaining of the sections with an anti-CD133 antibody and an anti-EF5 antibody revealed that CD133 expression was upregulated in some, but not all, hypoxic (EF5-positive) cells compared to normoxic (EF5-negative) cells (Figure 6E).

HIF-1 induces changes in SP-C and stem cell-related gene expression in A549 cells

To investigate whether the hypoxia-induced changes in the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1* were mediated by HIFs, we treated A549 cells with DFO, a hypoxia mimetic. DFO treatment induced a decrease in the expression of *SP-C* and an increase in the expression of *CD133*, *OCT4*, and *MSI1* (Figure 7A, B). Cobalt chloride, which is another hypoxia mimetic, induced similar changes in A549 cells (Figure 7C, D). To obtain more direct evidence of the importance of HIFs in hypoxia-induced changes, we transfected A549 cells with plasmids constitutively expressing CA-HIF-1α (pcDNA3.1/HIF-$1\alpha^{P402A/P564A}$). CA-HIF-1α overexpression resulted in a decrease in *SP-C* expression and an increase in *CD133* and *MSI1* expression (Figure 7E, F). The expression of *OCT4* did not change, which was consistent with the report that *OCT4* is a direct target of HIF-2α but not HIF-

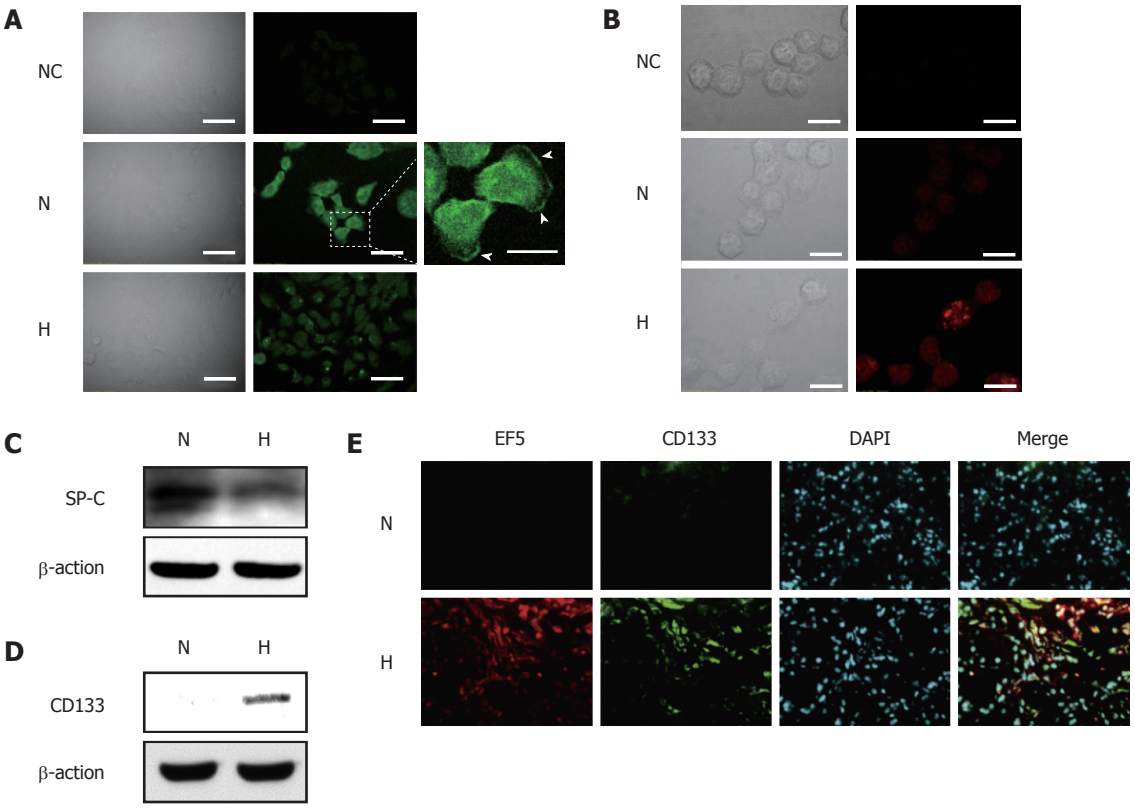

Figure 6 Expression of SP-C and CD133 proteins in hypoxic A549 cells *in vitro* and *in vivo*. A549 cells were cultured under normoxic (N) or hypoxic (H) conditions for 5 d; **A: Immunostaining for SP-C, arrowheads indicate the localization of SP-C proteins at cell membranes, NC indicates negative control (normal rabbit serum), Scale bars = 50** μm (white bars) and 20 μm (yellow bars); **B: Immunostaining for CD133. Formaldehyde-fixed, nonpermeabilized cells were immunostained with** phycoerythrin-conjugated monoclonal anti-CD133 antibody, **NC indicates negative control, scale bars = 20** μm; **C: Western blot analysis of SP-C protein expression;** D: **Western blot analysis of CD133 protein expression, C and D total cell lysates were subjected to immunoblot analysis for SP-C and CD133,** β-Actin was used as a loading control; **E: CD133 expression in hypoxic cells in A549 subcutaneous tumors, tissue sections were double-stained with an anti-CD133 antibody (green) and** an anti-EF5 antibody (red). Nuclei were stained with DAPI. **Upper panels represent the EF-5-negative (normoxic) area and bottom panels represent the EF-5-positive** (hypoxic) area, **scale bars = 100** μm.

1α[9]. These results indicate that HIF-1 induces changes in the expression of SP-C, CD133, and MSI1.

To gain some insight into the mechanism underlying the HIF-mediated suppression of *SP-C* expression in A549 cells, we examined the effects of trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, and 5-azacytidine, an inhibitor of DNA methyltransferase. We showed that TSA but not 5-azacytidine efficiently diminished the suppressive effect of hypoxia on *SP-C* expression (Figure 7G, H), thereby suggesting that HDAC is involved in hypoxia-induced gene expression changes in A549 cells.

TX-402 inhibits the expression of HIF-1α and HIF-2α and restores the hypoxia-induced gene expression changes in A549 cells

TX-402 has been shown to inhibit the expression of HIF-1α protein[26-28], but its effect on HIF-2α protein expression remains to be tested. Thus we examined whether TX-402 inhibited the expression of HIF-1α and HIF-2α in A549 cells under hypoxic conditions. HIF-1α and HIF-2α accumulated after incubating the cells under hypoxic conditions for 9 h. Treatment with 20 μmol/L TX-402 significantly suppressed the accumulation of both subunits, but the levels of HIF-1α and HIF-2α mRNA were unaffected (Figure 8A, B).

The proliferation of A549 cells was inhibited by TX-402 (IC50 value of approximately 20 μmol/L) without any discernible cell killing under hypoxic conditions over a culture period of 3 d (Figure 8C). We then treated the cells under hypoxic conditions for 3 d in the presence of 20 μmol/L TX-402 and examined the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1*. We found that TX-402 restored the expression levels of *SP-C*, *CD133*, *OCT4*, and *MSI1* in hypoxia to the normoxic levels (Figure 8D). These results were also corroborated by an immunofluorescence study (Figure 8E). We detected nuclear OCT4 and MSI1 using nuclear extracts and found that TX-402 restored the levels of OCT4 and MSI1 in hypoxia to their normoxic levels (Figure 8F).

TX-402 abrogates the hypoxia-induced lung-colonizing potential of A549 cells

We examined the lung-colonizing potential of A549 cells that were cultured under hypoxic conditions after injecting them into the tail veins of nude mice. As evidenced by macroscopic and histological observations, hypoxic A549 cells formed a larger number of metastatic foci in

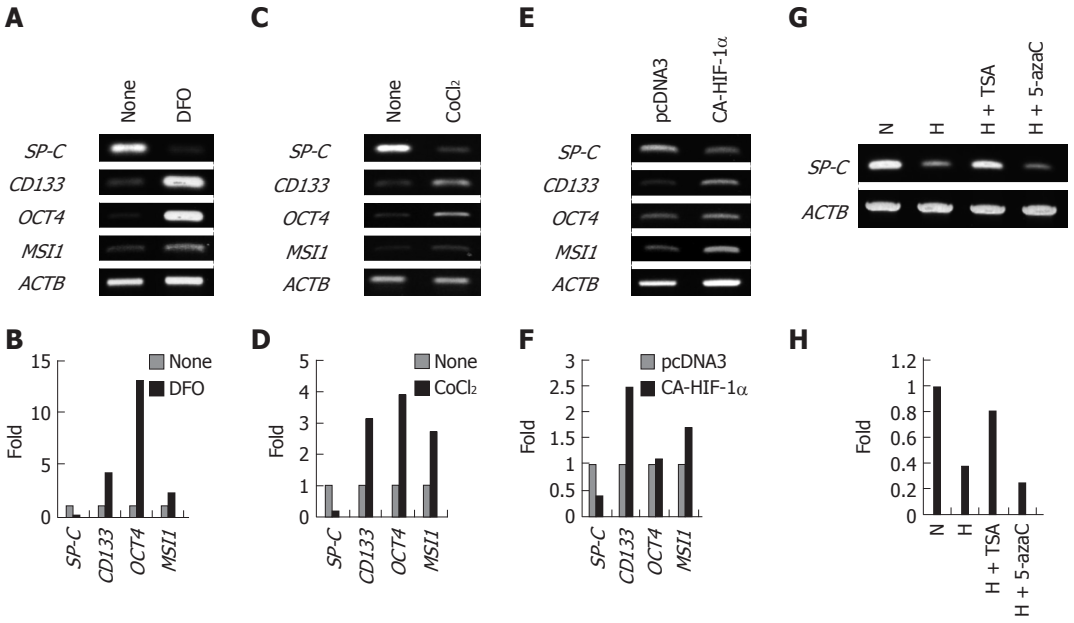

Figure 7 Hypoxia-inducible factor mediates the expression of differentiation- and stem cell-related genes in A549 cells. A and B: Effect of DFO, the cells were cultured with or without 100 μmol/L DFO for 3 d; C and D: Effect of $CoCl_2$, the cells were cultured in the presence or absence of 200 μmol/L $CoCl_2$ for 3 d; E and F: Effect of the ectopic expression of HIF-1α$^{P402A/P564A}$, the cells were transfected with pcDNA3.1 or pcDNA3.1/HIF-1α$^{P402A/P564A}$ and allowed to grow for 2 d; G and H: Effects of TSA and 5-azacytidine on the hypoxia-induced repression of *SP-C* expression, the cells were cultured under normoxic (N) or hypoxic (H) conditions in the presence or absence of TSA (300 nmol/L) or 5-azacytidine (5-azaC) (4 μmol/L) for 3 d. B, D, F, and H: The expression level of each gene was normalized to that of *β-Actin* (*ACTB*), data are shown as fold-change relative to normoxia (normoxia values set to equal 1).

the lungs than did normoxic A549 cells (Figure 9A, B). The lung-colonizing ability of hypoxic A549 cells was abolished by TX-402 (Figure 9C, D). To exclude the possibility that this suppressing effect of TX-402 was due to impaired colony-forming ability and enhanced senescence induction, we examined the effect of TX-402 on the colony-forming ability of A549 cells. Although the colony-forming ability of the cells that were treated with 20 μmol/L TX-402 under hypoxic conditions for 3 d was slightly but significantly inhibited (Figure 9E, F), the inhibitory effect of TX-402 on the lung-colonizing ability was more profound.

DISCUSSION

Here we show that A549 cells primarily express *SP-C*, which indicates that most of these cells are in the AT2 cell lineage. These cells also express the *CD133*, *OCT4*, *MSI1*, *SOX2*, and *GLDC* stem cell markers, suggesting that A549 cells comprise subpopulations of stem and progenitor cells. The exposure of A549 cells to hypoxia had the following effects without any sign of cell death: suppression of the expression of *SP-C*; upregulation of the expression of *CD133*, *OCT4* and *MSI1*; and slight upregulation of the expression of *NANOG*. A549 cells have been shown to form iPSC-like colonies when introduced to Oct4, Sox2, Nanog and Lin28 together with a non-degradable form of HIFs[35]. Our results were in agreement with these data and further provide important information that tumor hypoxia itself can render CSC-like NSCLC cells.

Recent studies have shown that CD133-positive tumor cells exhibit higher tumorigenicity, clonogenicity, and metastatic ability than CD133-negative cells in different types of cancers, including primary non-small cell and small cell lung cancers[36-41]. Therefore, the hypoxia-induced expression of CD133 might partly contribute to the CSC-like phenotype of hypoxic A549 cells. OCT4 is overexpressed in bladder cancer, and ectopic expression of OCT4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues[42,43]. Furthermore, recent reports have demonstrated that OCT4 expression is associated with the differentiation state of various cancer cells[44,45], and that it is essential for the successful reprogramming of somatic cells to induced pluripotent stem cells[46]. NANOG functions to maintain the pluripotency and co-expression of OCT4, and it is necessary for inducing the CSC-like properties of A549 cells[47]. MSI1 is an RNA-binding protein that is linked to asymmetric cell division[48]. Based on these reports, our results suggest that hypoxia induces the dedifferentiation of A549 cells. However, although we found that A549 cells express *GLDC*, its expression was not influenced by hypoxia. Because murine BASCs are SP-C/CC10 double-positive[17,18,48] and the recently identified putative mouse lung stem/progenitor cell population is CC10-positive/SP-C-negative[49], the properties of hypoxic A549 cells do not closely conform to those known to be characteristic of stem/progenitor cells.

The mechanism underlying the hypoxia-induced suppression of *SP-C* involves HIF-1α at least in part, because the overexpression of CA-HIF-1α suppressed *SP-C* ex-

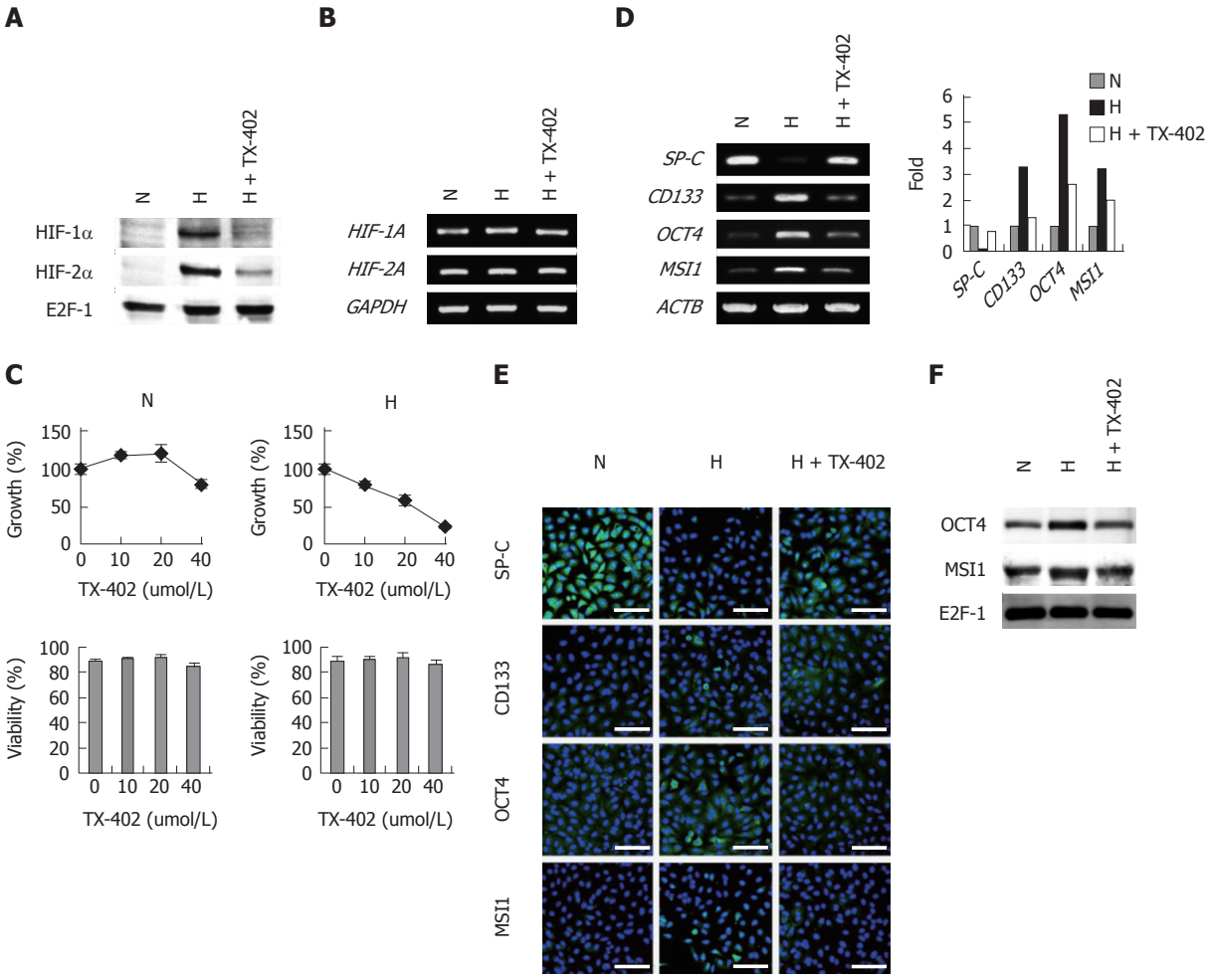

Figure 8 Effects of TX-402 on hypoxia-inducible factor-α expression, cell proliferation, viability and gene expression levels of A549 cells. The cells were cultured under normoxic (N) or hypoxic (H) conditions in the presence or absence of 20 μmol/L TX-402 for 3 d; **A: Expression of HIF-α subunits, Nuclear extracts and** total RNA were subjected to Western blot; **B: Expression of HIF-α subunits, Nuclear extracts and total RNA were subjected to reverse transcription-polymerase chain** reaction (RT-PCR) analysis; **C: Cell growth and viability, bars, SD (*n* = 3); D: RT-PCR analysis of the expression of** *SP-C*, *CD133*, *OCT4* and *MSI1* Mrna, **the expression level of each gene was normalized to that of** *β-Actin* (*ACTB*), **data are shown as fold-change relative to normoxia (normoxia values set to equal 1); E: Immuno-**fluorescence analysis of the expression of SP-C, CD133, OCT4 and MSI1 protein, **Nuclei were stained with DAPI, and the merged images are shown, Perinuclear and** nuclear localization of OCT4 and MSI1 was observed, **Scale bars = 50 μm; F: Western blot analysis of the nuclear localization of OCT4 and MSI1, Nuclear extracts of** the cells were subjected to the analysis, **E2F-1 was used as a loading control.**

pression. Because TSA reversed the suppressive effect of hypoxia, HDAC is also likely to be involved in the mechanism. Alternatively, because HIF-1α is post-translationally modified by acetylation of lysine residues within the N terminus leading to the stabilization of the protein[50], it is possible that TSA reduces the level of HIF-1α by directly acting at the protein level, which in turn restores hypoxia-induced *SP-C* repression to normal levels. In contrast, the upregulation of *CD133*, *OCT4*, and *MSI1* in hypoxic A549 cells is undoubtedly mediated by HIFs because treatment with the hypoxia mimetics, namely DFO and $CoCl_2$ (both of which stabilize HIF-α subunits) upregulated all of them. Furthermore, we showed that CA-HIF-1α overexpression up-regulated *CD133* and *MSI1* but not *OCT4*, which is an HIF-2α-specific target gene[11].

It is thought that CSCs display self-renewing ability, a high capacity for tumor initiation, and a high metastatic potential. We used sphere formation and lung-colonizing assays to examine whether hypoxic A549 cells also have these functional phenotypes. The lung-colonizing assay can examine the tumor-initiating capacity and growth of a single cell or a small mass of cells in orthotopic sites that mimic the *in vivo* niche conditions of CSCs in NSCLC. In the present study, we showed that hypoxic A549 cells formed larger spheres and more lung nodules after intravenous implantation compared to normoxic A549 cells. This result suggests that hypoxic A549 cells have high self-renewing activity, tumor-initiating capability, and/or metastatic ability. Thus, based on the gene expression data and functional studies, we conclude that hypoxia is able to induce subpopulations of A549 cells with CSC-like phenotypes.

To determine whether our findings could be generalized to other cell types, we also investigated the effect of hypoxia on QG56 cells, and we observed that the expression of the *CC10* and *AQP5* differentiation marker

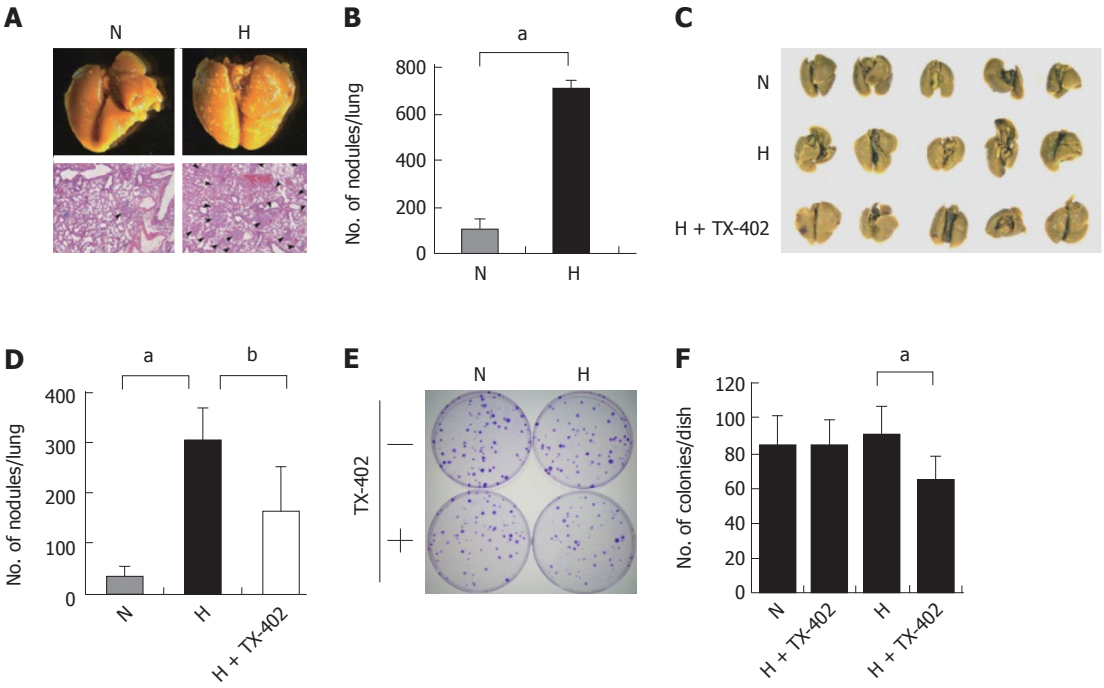

Figure 9 Effect of TX-402 on the lung-colonizing capability of A549 cells cultured under hypoxic conditions. A, B: Macroscopic and histological observations of the lungs. A549 cells cultured under normoxic (N) or hypoxic (H) conditions for 5 d were injected into the tail vein of Balb/c nude mice (n = 5), the lungs were processed for macroscopic and histological (hematoxylin and eosin staining) observations, and the number of metastatic foci was counted, data are presented as the mean ± SE, $^{b}P < 0.01$; C,D: Effect of TX-402 on the hypoxia-induced lung-colonizing ability of A549 cells. A549 cells cultured under normoxic (N) or hypoxic (H) conditions with vehicle or 20 μmol/L TX-402 for 3 d were injected into the tail vein of BALB/c nude mice (n = 5), The lungs were fixed, and the number of nodules per lung was then counted. Data are presented as the mean ± SE, $^{b}P < 0.01$ and $^{a}P < 0.05$; E, F: Colony-forming ability of TX-402-treated A549 cells, A549 cells (100 cells/dish) cultured under normoxic (N) or hypoxic (H) conditions in the presence of solvent (DMSO) or 20 μmol/L TX-402 for 3 d were seeded and cultured for an additional 14 d, the colonies were stained with crystal violet, data are presented as the mean ± SD (n = 6), $^{b}P < 0.01$.

genes was markedly suppressed under hypoxic conditions. Inversely, *CD133*, *OCT4*, and *MSI1* were up-regulated in hypoxic QG56 cells, and hypoxia weakly enhanced the sphere-forming capacity of QG56 cells. These results suggest that hypoxia also induces dedifferentiation of the cells.

An important finding of our study is that TX-402 blocked hypoxia-induced changes in the expression of the stem cell-related genes and an increase in the lung-colonizing ability of A549 cells, which most likely occurred *via* inhibition of the expression of HIF-α subunits. Although we observed a slight reduction in the survival of cells that were treated with TX-402 under hypoxic conditions, TX-402 was not cytotoxic at the concentration used. Therefore, it is likely that in addition to its growth-inhibitory effect in mild hypoxia, TX-402 inhibited the lung-colonizing ability of A549 cells by repressing cell dedifferentiation. Further studies using other NSCLC cell lines or primary patient samples are required to generalize the effects of TX-402 on dedifferentiation and lung-colonizing ability.

In conclusion, our results suggest that hypoxia induces the dedifferentiation of NSCLC cells into CSC-like cells with high metastatic potential and that HIF inhibitors, such as TX-402, may prevent this process. Recent studies have demonstrated that hypoxia in tumors can be induced by the administration of antiangiogenic agents, such as bevacizumab and VEGF receptor tyrosine kinase inhibitors, and that intermittent use of these drugs accelerates tumor growth and metastasis presumably by increasing the CSC population[6-8]. Therefore, combination therapy where antiangiogenic agents are combined with HIF-targeting drugs could be effective in improving patient outcomes. Thus, further studies on HIF-targeting drugs are warranted to determine their full potential in the treatment of disease.

ACKNOWLEDGMENTS

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COMMENTS

Background

Hypoxia influences many aspects of cancer cell biology, including neoangiogenesis, energy metabolism, cell survival, radiosensitivity, chemosensitivity, differentiation and invasion/metastasis, *via* hypoxia-inducible factors (HIFs). Hypoxia also induces the dedifferentiation of some tumor cells, rendering them more cancer stem cell (CSC)-like and metastatic.

Research frontiers

The effects of hypoxia on the dedifferentiation and maintenance of CSC phenotypes of non-small cell lung cancer (NSCLC) is largely unknown. Furthermore, it remains to be examined whether HIF inhibitors can suppress the hypoxia-induced process.

Innovations and breakthroughs

In this study, the authors demonstrate that hypoxia induces the dedifferentiation of NSCLC and that TX-402, a small-molecule inhibitor of HIF-1α and HIF-2α expression, can suppress the hypoxia-induced process and, importantly, metastatic ability.

Applications

By understanding how tumor hypoxia induces the dedifferentiation of NSCLC, this study could represent a future strategy for therapeutic intervention in the treatment of patients with NSCLC.

Terminology

Tumor hypoxia is generated in most solid tumors because of a shortage in oxygen supply. HIF is a transcription factor that is composed of HIF-α and HIF-β subunits, and it plays a central role in hypoxia-induced biological processes. CSCs are defined as those cells within a tumor that can self-renew, drive tumorigenesis, exhibit chemoresistance, exhibit radio-resistance, and have high metastatic potential.

Peer review

The authors describe an inhibitor of the HIF-α subunit expression that suppresses the hypoxia-induced dedifferentiation of human NSCLC cells into CSC-like cells. This article was highly evaluated because the authors examined the hypoxic effect on NSCLC cells in detail.

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