Hypoxia exposure upregulates MALAT-1 and regulates the transcriptional activity of PTB-associated splicing factor in A549 lung adenocarcinoma cells

LING HU, JING TANG, XIAOHUAN HUANG, TAO ZHANG and XIAOLING FENG

Department of Pathology, Chongqing Three Gorges Medical College, Chongqing 400000, P.R. China

Received December 12, 2016; Accepted February 15, 2018

DOI: 10.3892/ol.2018.8637

Abstract. Hypoxia has been reported to be a critical microenvironmental factor that induces cancer metastasis and proliferation in gastric, liver and hepatic cancers; however, the underlying mechanisms of this are largely unknown. Long noncoding RNAs (lncRNAs) have emerged as crucial factors of several aspects of tumor malignancy, including tumorigenesis, metastasis and chemoresistance. However, the potential association of lncRNAs with hypoxia-induced cancer malignancy remains to be determined. In the present study, the differential expression of lncRNAs following the induction of hypoxia in A549 lung adenocarcinoma cells was analyzed reverse transcription-quantitative polymerase chain reaction. It was identified that the lncRNA metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1) was upregulated significantly by hypoxia in A549 cells. By considering its promotive effects on malignant tumor behaviors, in the present study, it was identified that upregulated MALAT-1 released the binding of PTB-associated splicing factor (PSF) to its target gene, GAGE6, and thus promoted proliferation, migration and invasion of A549 cells following hypoxia exposure. These results advance the overall understanding of the mechanism of hypoxia-induced lung cancer metastasis and may assist in the development of novel therapeutics.

Introduction

As the second most common cancer type, lung cancer has long been the leading cause of cancer-related mortality worldwide. Despite advancements and improvements in early-stage diagnosis, immune therapy, surgical and medical treatments, the 5-year survival rate of lung cancer patients remains poor (1). With regard to the treatment of early-stage non-small lung cancer cell (NSCLC), great improvements in local control have been made over the last decade in operable and inoperable patients (2,3); however, ~20% of patients continue to develop distant metastasis (4,5). This has encouraged further research focusing on the molecular mechanisms of NSCLC invasion leading to metastasis, and further understanding in this regard could to be utilized to refine patient selection for already existing therapies.

Long noncoding RNAs (lncRNAs) comprise a class of noncoding RNAs that are >200 nucleotides in length, of which the majority of biological functions are unknown (6). Previous research has identified lncRNAs to be involved in crucial processes of cancer malignancy, including metastasis and tumorigenesis (7). One of the most well-studied lncRNAs, metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), is a highly conserved nuclear lncRNA. It functions as a molecular decoy, serving as a structural link between ribonucleoproteins (7). Through the development of a MALAT-1 knockout model in human lung tumor cells, Ji *et al* (8) discovered that MALAT-1 significantly regulates a set of metastasis-associated genes, rather than regulating alternative splicing. Consequently, migration was dramatically impaired in murine xenografts of MALAT-1-deficient cells. This was further demonstrated by blocking MALAT-1 using antisense oligonucleotides after tumor implantation.

Hypoxia represents a major microenvironmental condition that directly impacts the regulation of hundreds of protein-coding genes involved in dealing with the limitation of oxygen supply (9). Not only protein-coding genes, lncRNAs are not presented to be tightly regulated by hypoxia. Lelli *et al* (9) identified a strong induction of MALAT-1 in the organs (including the kidneys, testes and lungs) of mice exposed to inspiratory hypoxia. This raised the question of whether hypoxia induces lncRNA in tumor cells and thus regulates malignant processes.

PTB-associated splicing factor protein (PSF) contains a DNA-binding domain that is responsible for epigenetically regulating its target genes, such as the proto-oncogene G antigen 6 (GAGE6), and two RNA-binding domains (RBDs) that bind to lncRNAs, such as MALAT-1, to release PSF from its downstream proto-oncogene and activate transcription (10-12). MALAT-1 was demonstrated to epigenetically bind to the RBD of PSF and release it from the promoter region of GAGE6, thus inhibiting its proto-oncogene activity

Correspondence to: Professor Xiaoling Feng, Department of Pathology, Chongqing Three Gorges Medical College, Bai'an Tianxing Road, Wanzhou, Chongqing 400000, P.R. China
E-mail: 1628983284@qq.com

Key words: hypoxia, MALAT-1, metastasis, PTB-associated splicing factor, G antigen 6

in melanoma cells (13). In lung cancer cells, MALAT-1 was reported to be positively associated with tumor malignant behaviors including proliferation, colony formation and migration (14). In A549 cells, the regulatory role of hypoxia-induced MALAT-1 in the processes of epigenetic regulation of PSF remains unknown.

In the present study, the differential expression of MALAT-1 the exposure to hypoxic conditions of A549 cells was detected. Subsequent analysis of the regulatory role of MALAT-1 upregulation in cell proliferation, migration and invasion via epigenetic regulation of the transcriptional activity of PSF on its downstream target gene, GAGE6, was performed. The present study aimed to provide a novel therapeutic target for metastasis in lung cancer.

Materials and methods

Cell culture and in vitro hypoxia exposure. The lung adenocarcinoma cell line A549 was obtained from ATCC (Manassas, VA, USA) and cultured routinely in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum (NBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% CO_2 incubator at 37˚C. In order to avoid possible interference with serum steroids, A549 cells were pre-incubated with 10% dextran-coated charcoal (DCC). Cells were then placed in an anaerobic system (Forma Scientific, Marietta, OH, USA) filled with 1% O_2, 5% CO_2, and 94% N_2 for hypoxic exposure. Cells were incubated in 5% CO_2 incubator were considered as normoxia control. Following hypoxic exposure, the medium was replaced with fresh DMEM supplemented with 10% NBS; and 24 h later, cells were suspended by trypsin and collected for further analysis.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were lysed using TRIzol reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol, and 0.5 μg total RNA was reverse transcribed using Ribo™ mRNA/lncRNA qRT-PCR Starter kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. qPCR was performed in triplicate using 2X SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) with an ABI7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: MALAT-1, 5'-GACTTCAGGTCTGTCTGTTCT-3' (forward) and 5'-CAACAATCACTACTCCAAGC-3' (reverse); nuclear paraspeckle assembly transcript 1 (NEAT1), 5'-ATGCCACAACGCAGATTGAT-3' (forward) and 5'-CGAGAAACGCACAAGAAGG-3' (reverse); β-actin, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3' (forward) and 5'-CTGTCACCTTCACCGTTCCAGTTT-3' (reverse); humanin (HN), 5'-AAGAACAGGTTTCTCTCTGTCCT-3' (forward) and 5'-AGCTTTCCCTCCAATCTACTACT-3' (reverse); and HOX transcript antisense RNA (HOTAIR), 5'-GAGAGAGGGAGCCCAGAGTT-3' (forward) and 5'-GCTTGGGTGTAATTGCTGGT-3' (reverse). At the first step, cDNA was denatured at 95˚C for 5 min; for each cycle, denatured at 95˚C for 10 sec, annealing and elongation at 60˚C for 60 sec, repeat 40 cycles. In order to quantify gene expression, the $2^{-(\Delta\Delta Cq)}$ method was used (15). β-actin expression levels were used as an internal control. RT-qPCR reactions were performed in triplicate.

Semi-quantitative PCR. complementary DNA (cDNA) was synthesized using Ribo mRNA/lncRNA qRT-PCR Starter kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. The PCR for β-actin mRNA was performed using the following reaction conditions: 20 cycles of 95˚C for 10 sec, 60˚C for 60 sec. The quantification of the *MALAT-1* gene was performed using the following PCR conditions: 26 cycles of 95˚C for 10 sec, 60˚C for 60 sec. The PCR products were fractionated using 2% agarose gel and stained with ethidium bromide (EB; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). *Cell proliferation assay*. Cell proliferation was detected by using a Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. A total of $1x10^4$ cells/well were suspended in 100 μl medium, plated into 96-well plate, supplemented with 10% FBS and cultured for 1-5 days. After the incubation period, 20 μl CCK-8 solution was added to each well and incubated for a further 1 h at 37˚C. Absorbance was then measured at 450 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Wound healing assay. To detect cell migration *in vitro*, cells were allowed to form a confluent monolayer in a 6-well plate prior to scraping a conventional 10-μl pipette tip across the monolayer to form a 'wound'. To avoid noise in the results due to proliferation, medium containing 1% FBS was used for the subsequent incubation. Cell migration was imaged using under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation, Tokyo, Japan) at the same location at 0 and 24 h and the number of invading cells was recorded.

Transwell chamber invasion assay. Cells were grown to ~80% confluence and incubated in serum-free medium for 24 h. Cells were then trypsinized and $2x10^4$ cells in serum-free medium were added to the upper compartments of a Transwell chamber (Corning Inc., New York, NY, USA) pre-coated with 1 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with medium containing 10% FBS. Cells were incubated for 24 h at 37˚C, after which the non-invaded cells were removed from the upper surface. The invaded cells were then fixed in 4% paraformaldehyde and stained with 0.5% crystal violet in PBS. Stained cells were visualized under an X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at amplification of x100. Cells in five randomly selected fields were counted, and the average number was recorded.

MALAT-1 knockdown. A small interfering RNA (siRNA) targeted to MALAT-1 at position 7,211-7,229 (5'-GAAGGAGCTTCCAGTTGAA-3') or scrambled siRNA (5'-GGATACGGAGTACTATAGC-3'), which was considered as a negative control was synthesized (Sangon Biotech Co., Ltd., Shanghai, China). For RNA interference, 10 pmol siRNA duplex and 2 μl Lipofectamine RNAi MAX (Thermo Fisher Scientific, Inc.) in 200 μl Opti-MEM I were added to $3x10^4$ cells and incubated for 3 h at 37˚C with 5% CO_2. Next, the medium was replaced

by DMEM supplemented with 10% FBS and the transfected cells were incubated for another 24 h.

Chromatin immunoprecipitation (ChIP). Cells were cultured were seeded in 10-cm dishes to form an 80% confluent monolayer. For each sample, $1x10^7$ cells were harvested. ChIP for PSF was conducted using a murine monoclonal antibody against PSF (cat. no. ab11825, Abcam, Cambridge, UK) or a non-specific rabbit IgG (control, cat. no. ab171870, Abcam) conjugated to secondary Dynal magnetic beads (Thermo Fisher Scientific, Inc.). Cells were cross-linked with 1% formaldehyde for 15 min at room temperature and sonicated at 30% for 10 sec and left on ice for 1 min. The sonication step was repeated 10 times. Next, 10 μg of either antibody or control was added into 100 μl lysate for a 4-h incubation at 4˚C with 10 μl protein-A beads (Thermo Fisher Scientific, Inc.). The beads were washed three times with washing buffer (150 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 1% NP-40) and eluted in 300 μl 3 M NaCl elution buffer for 3 h at 65˚C. The ChIP products were analyzed by RT-qPCR following the aforementioned protocol. Dihydrofolate Reductase (DHFR) 5' untranslated region (UTR) was used as a negative control sequence. The primers for detecting the PSF-reacting region on the GAGE6 promoter were as follows: 5'-GCCTTCTGC AAAGAAGTCTTGCGC-3' (forward) and 5'-ATGCGAATT CGAGGCTGAGGCAGACAAT-3' (reverse); DHFR 5'UTR, 5'-CTGATGTCCAGGAGGAGAAAGG-3' (forward) and 5'-AGCCCGACAATGTCAAGGACTG-3' (reverse).

RNA immunoprecipitation (RIP). Cells ($1x10^6$) were lysed for 10 min on ice in a lysis buffer [150 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 1% NP-40, 100 U/ml RNase Inhibitor (Merck KGaA; Sigma-Aldrich)]. The cell lysate was diluted (1:4) with binding buffer containing 100 mM Tris-HCL, pH 7.4, 300 mM NaCl, 2 mM $MgCl_2$ and 100 U/ml RNase Inhibitor. Next, the PSF antibody (cat. no. ab11825; 1:200; Abcam) or control IgG (cat. no. ab171870; Abcam) was added to protein-A beads (Thermo Fisher Scientific, Inc.) and incubated for 4 h at 4˚C. The beads were washed three times with binding buffer and resuspended in 100 μl releasing buffer [100 mM Tris-HCl pH 7.4, 30 μg/ml proteinase K (Merck KGaA; Sigma-Aldrich)] to release the ribonucleoprotein complex. TRIzol reagent was used to extract RNA from the immunoprecipitates.

Western blot analysis. Proteins from total cell lysates were extracted using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and quantified using BCA protein assay kit (Merck KGaA; Sigma-Aldrich). A total of 20 μg total protein was fractionated on 8% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (Life Technologies; Thermo Fisher Scientific, Inc.). Membranes were blocked in PBS containing 0.1% Tween-20 and 5% skim milk powder. Membranes were then incubated with specific primary antibodies (1:2,000) overnight at 4˚C. The following antibodies were used: PSF antibody (cat. no. ab11825; Abcam), β-actin antibody (1:2,000; cat. no. ab8226; Abcam). Then the secondary antibody anti-mouse IgG conjugated with horseradish peroxidase (HRP) from Abcam (1:5,000; cat. no. ab97040) was incubated with membrane for 2 h at room temperature. Proteins were visualized using an Enhanced Chemiluminescence kit (SuperSignal West Pico substrate; Pierce; Thermo Fisher Scientific, Inc.). Densitometry on bands was made using the NIH Image J 1.45S software (National Institutes of Health, Bethesda, MD, USA).

Data analysis and statistics. Data are presented as the mean ± standard error of the mean (SEM). All experiments were repeated a minimum of three times. Differences were analyzed using a one-way ANOVA followed by Tukey's post-hoc test with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia induces the expression of MALAT-1 lncRNA. To examine the overall impact on the expression profiles of lncRNAs, this study established the expression levels of two lncRNAs, including MALAT-1, NEAT1, and one mRNA, HN. To exclude global expression changes, the β-actin levels in normoxia- and hypoxia-exposed A549 cells were determined by RT-qPCR after 24 h. The results showed that, without significantly altering the NEAT1, HN and HOTAIR expression levels, there was a marked upregulation of MALAT-1 in hypoxia-exposed cells when compared with normoxia-exposed cells (Fig. 1A). To investigate the hypoxia-induced upregulation of MALAT-1, MALAT-1 expression levels were detected at 0, 12, 24, 48 and 72 h following the induction of hypoxic conditions. The results demonstrated that upregulated MALAT-1 initially appeared at 0 and reached a peak at 12 h, after which the levels gradually decreased (Fig. 1B). This indicates that induction of hypoxic conditions upregulates MALAT-1 expression for a relatively long period of time in A549 cells.

Upregulated MALAT-1 lncRNA binds to PSF and releases it from the GAGE6 promoter region. MALAT-1 has been reported to be a key regulator in controlling the malignancy of lung cancer cells (13,14). Therefore, the present study was designed to investigate the regulatory roles of MALAT-1 and PSF in the effects of hypoxia in A549 cells. MALAT-1/PSF binding was detected by RIP in hypoxia-exposed A549 cells. Following confirmation of the upregulation of MALAT-1 and consistent PSF expression levels after hypoxia exposure for 24 h (Fig. 2A), it was evident that the amount of MALAT-1 bound to PSF increased significantly after exposure to hypoxic conditions (Fig. 2B). As Li *et al* (13) showed that the binding of MALAT-1 to PSF dissociates the PSF/GAGE6 promoter complex, we chose to detect whether hypoxia may be responsible for the dissociation of the PSF/GAGE6 promoter region. Hypoxia significantly released the binding of PSF to the GAGE6 promoter region, as shown by ChIP assay (Fig. 2C). In order to further confirm that upregulated MALAT-1 caused this dissociation, siMALAT-1 was introduced into hypoxia-exposed A549 cells and, 24 h later, the efficiency of MALAT-1 knockdown was detected by RT-qPCR (Fig. 2D). A ChIP assay identified increased binding of PSF to the GAGE6 promoter region (Fig. 2E).

Hypoxia promotes proliferation, migration and invasion partially via upregulating MALAT-1. The effects of hypoxia

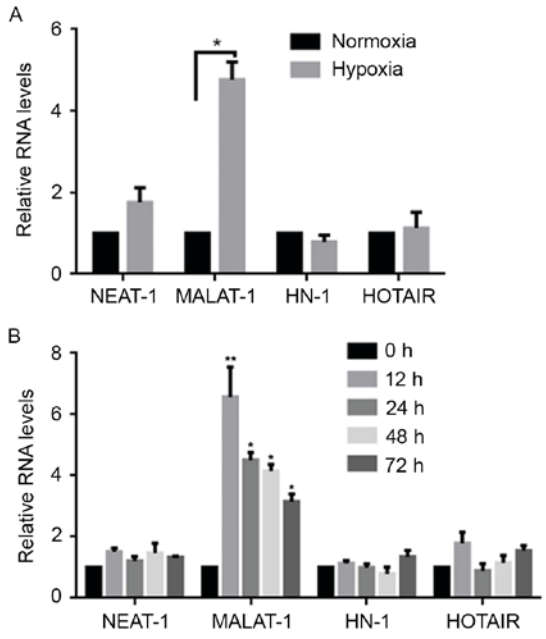

Figure 1. Hypoxia exposure induces the expression of MALAT-1. (A) Using a normoxia treatment group for comparison, the expression levels of NEAT-1, MALAT-1, HN and HOTAIR in hypoxia-treated A549 cells were detected by RT-qPCR. Data were normalized to expression of β-actin mRNA. *P<0.05. (B) Following hypoxia exposure, the expression levels of NEAT-1, MALAT-1, HN and HOTAIR were detected by RT-qPCR at 0, 12, 24, 48 and 72 h after hypoxia exposure. *P<0.05 and **P<0.01 vs. 0 h. MALAT-1, metastasis-associated lung adenocarcinoma transcript-1; NEAT-1, nuclear paraspeckle assembly transcript 1; HN; HOTAIR, HOX transcript antisense RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

exposure on the proliferation in A549 cells were detected by CCK-8 assay, which demonstrated that, following hypoxia, the proliferation rate markedly increased from day 4 onwards compared with the normoxia group at the same time-point (Fig. 3A). In order to establish whether the upregulation of MALAT-1 was involved, siMALAT-1 was introduced into A549 cells after hypoxia treatment. When the level of MALAT-1 lncRNA was downregulated by the siRNA, the proliferation rate of hypoxia-treated A549 cells decreased compared with the control siRNA-transected group (Fig. 3B); however, in normoxia-treated A549 cells, the knockdown of MALAT-1 led to only a marginal difference (P>0.05). Due to the involvement of MALAT-1 in regulating cell migration and invasion, the potential effects of hypoxia on migration and invasion were investigated. Wound healing and Transwell assays demonstrated that hypoxia exposure strongly promoted migration and invasion in A549 cells (Fig. 3C and D).

Discussion

It has been reported that hypoxia serves critical roles in various malignant behaviors of lung cancer (16-18). The present study identified that the expression level of lncRNA MALAT-1 was upregulated after hypoxia exposure in lung adenocarcinoma A549 cells, while the levels of several other lncRNAs were unaltered. Following the removal of hypoxic conditions, upregulated MALAT-1 lncRNA persisted for >72 h. Notably, MALAT-1 epigenetically binds to the RBD of PSF, which is a tightly regulated region due to its DNA-binding activity. Consequently, the present study investigated whether the upregulated MALAT-1 RNA affects the interaction between PSF and the GAGE6 promoter region. The ChIP assay results revealed that hypoxia exposure dramatically inhibited the binding of PSF to the GAGE6 promoter region and resulted in the upregulation of GAGE6 mRNA and protein. In accordance with the initial hypothesis, hypoxia promoted proliferation, migration and invasion in A549 cells. These results indicate that hypoxia regulates certain malignant characteristics of lung cancer cells through transcriptionally regulating the lncRNA MALAT-1. Taken together, the results indicated a potential molecular mechanism underlying the effects of hypoxia on the malignancy of lung cancer cells.

Previous studies have used various oxygen concentrations for hypoxia exposure, ranging from 0.5 to 5% O_2 (19-21). To define an effective concentration of oxygen for tumor proliferation and migration, A549 cells were exposed to a range of oxygen concentrations for 24 h, and proliferation was detected after 48 h. The results of the present study demonstrated that, with the exception of 0.5%, any oxygen concentration <5% significantly promoted cell proliferation. Instead of promoting cell proliferation, 0.5% oxygen exerted a fatal effect on A549 cells (data not shown).

Hypoxia exposure has been found to promote tumor progression *in vivo* in numerous studies involving different types of cancer. However the results have varied. In D12 and R-18 melanoma, hypoxia exposure was reported to increase tumor progression (19,22). Lung cancer cells pre-treated with hypoxia presented higher metastasis in human fibrosarcoma in mice. Contrarily, Büchler *et al* (23) demonstrated that hypoxia exposure influenced the number of metastatic lesions, but not the proliferation or tumor formation processes. Cairns *et al* (21) reported that acute hypoxia exposure of the cervical carcinoma cell line ME-180 significantly promoted lymph node but not lung metastasis (24). In the present study, to demonstrate the effects of hypoxia exposure on proliferation, migration and invasion, the lung adenocarcinoma cell line A549 was exposed to 1% oxygen for 24 h. Significant effects on proliferation, migration and invasion in A549 cells were discovered and thus enabled the conclusion that hypoxia was a stimulating factor for tumor progression.

Previous studies have suggested that the expression levels of many lncRNAs are altered in various human cancers and play crucial roles in tumor progression (25). lncRNA-LET, which is closely associated with hepatocellular carcinoma metastasis, was demonstrated to be attenuated in a hypoxic microenvironment (26). In an independent study, lncRNA-p21 was identified to be involved in the regulation of the physiological processes following hypoxic exposure by modulating hypoxia-enhanced glycolysis (27). However, the mechanism of lncRNAs in hypoxia in cancer has not been thoroughly elucidated.

MALAT-1 RNA is overexpressed in numerous human cancer types, such as non-small cell lung cancer (28), hepatocellular carcinoma (29) and bladder cancer (30). MALAT-1 is localized in nuclear speckles (31). Its abundance and aberrant expression in various cancers indicate its critical roles in tumor progression, including metastasis. Tian *et al* (32) transfected melanoma A-375 cells with MALAT-1 siRNA and analyzed the migration and invasion by Transwell assay with or without Matrigel. Cells with impaired expression of MALAT-1

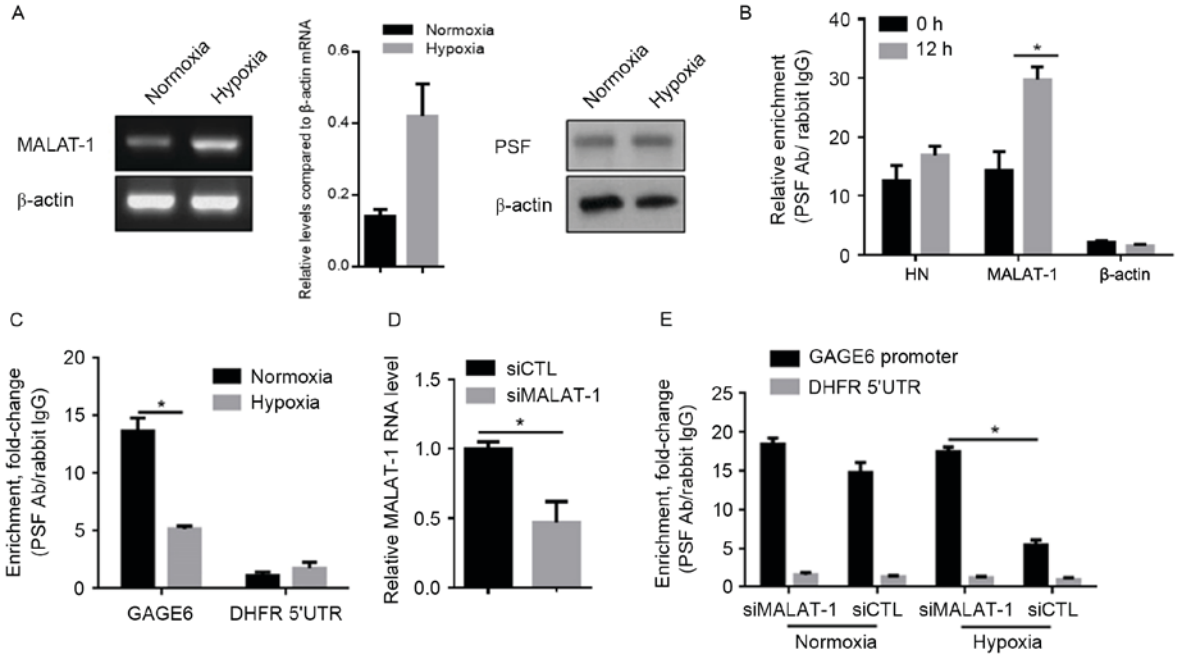

Figure 2. Hypoxia exposure promotes the interaction between MALAT-1 and PSF, and thus leads to the dissociation of PSF from the GAGE6 promoter. (A) Semi-quantitative PCR (left panel) and semi-quantitative western blot analysis (right panel) were performed to confirm the changes in the expression levels of MALAT-1 and PSF, respectively, after hypoxia exposure. (B) A RIP assay was performed to detect the binding of MALAT-1 to PSF protein after 24 h exposure. (C) After hypoxic exposure, siRNA targeted to MALAT-1 was introduced for further RIP assay. (D) Detection of MALAT-1 knockdown efficiency by RT-qPCR after siRNA transfection for 24 h. (E) A ChIP assay was performed to detect the effects of hypoxia-induced MALAT-1 expression on the binding of PSF to its target gene, GAGE6. *P<0.05. MALAT-1, metastasis-associated lung adenocarcinoma transcript-1; PSF, PTB-associated splicing factor; RIP, RNA immunoprecipitation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; Ab, antibody; GAGE6, G antigen 6; DHFR 5'UTR, Dihydrofolate reductase 5'-untranslated region; siCTL, control siRNA; siMALAT-1, siRNA targeting MALAT-1.

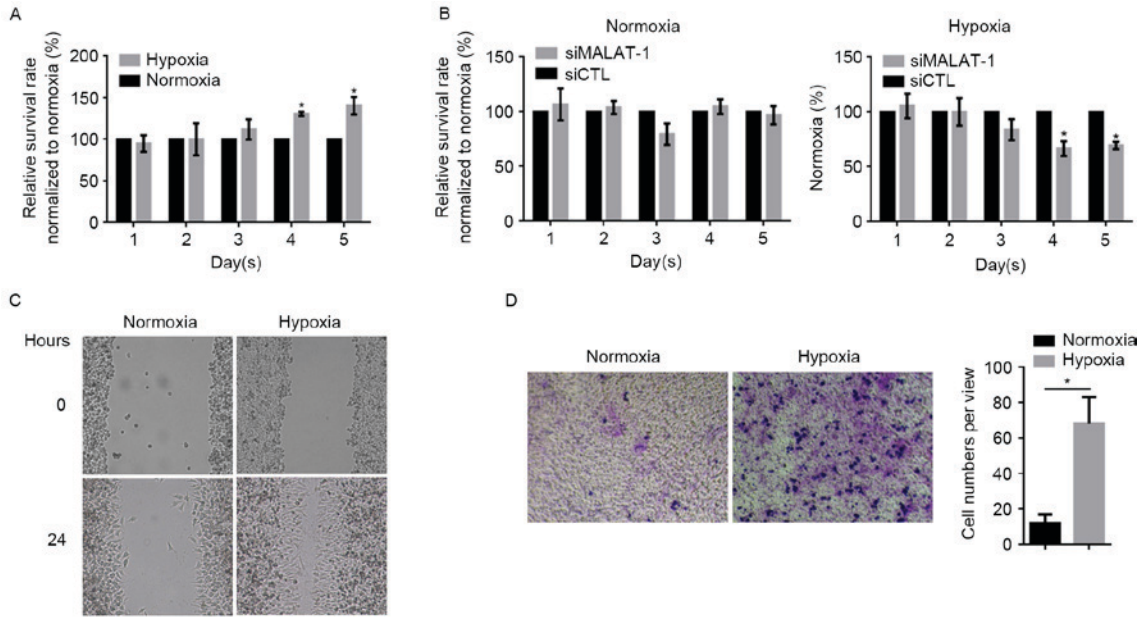

Figure 3. Effects of hypoxia exposure on proliferation, migration and invasion. (A) A Cell Counting Kit-8 assay was performed to detect the proliferation rate after hypoxia exposure. *P<0.05 vs. normoxia. (B) After siMALAT-1 transfection and exposure to hypoxia or normoxia, cell proliferation was detected. *P<0.05 vs. normoxia. (C and D) The effects of hypoxia exposure on (C) migration and (D) invasion were examined by wound-healing assay and Transwell assay, respectively. *P<0.05. MALAT-1, metastasis-associated lung adenocarcinoma transcript-1; siMALAT-1, siRNA targeting MALAT-1; siCTL, control siRNA.

migrated and invaded less effectively. Han *et al* (33) observed the upregulation of MALAT-1 in bladder urothelial carcinoma compared with the matched normal urothelial tissues, indicating that increased expression of MALAT-1 was associated with high-grade and high-stage bladder urothelial carcinoma. Furthermore, silencing MALAT-1 inhibited proliferation, migration and invasion in bladder urothelial carcinoma T24 and 5,637 cells (33).

In conclusion, the results of the present study indicate that the expression of the lncRNA MALAT-1 is significantly increased in hypoxia-exposed cells and promotes hypoxia-induced proliferation, migration and invasion in A549 cells. The transcriptional regulation of GAGE6, a proto-oncogene, by PSF is inhibited by the upregulation of MALAT-1 in an epigenetic manner, and thus promotes proliferation, migration and invasion. However, more convincing evidence from animal and clinical studies is required to support these findings. Further study will explore the exact mechanism of the upregulation of MALAT-1 by hypoxia exposure in A549 cells, and employ animal studies for testing the expression of MALAT-1 *in vivo*. In summary, these findings suggest that the hypoxia/MALAT-1/PSF pathway may contribute to the development of novel anti-cancer therapeutics directed against hypoxic tumor targets.

Acknowledgements

The authors would like to thank Dr Tao Hong of Sichuan University (Chengdu, China) for English language editing.

Funding

No funding received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LG contributed to the design of the study. LH was responsible for project design, organization and manuscript writing. JT, XH and TZ conducted the molecular experiments. XF contributed to the project design and organisation and the supervision of experimental progress and final approval of the version to be published.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin 63: 11-30, 2013.
2. Ginsberg RJ and Rubinstein LV: Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. Ann Thorac Surg 60: 615-622, 1995.
3. Timmerman R, Paulus R, Galvin J, Michalski J, Straube W, Bradley J, Fakiris A, Bezjak A, Videtic G, Johnstone D, *et al*: Stereotactic body radiation therapy for inoperable early stage lung cancer. JAMA 303: 1070-1076, 2010.
4. Chi A, Liao Z, Nguyen NP, Xu J, Stea B and Komaki R: Systemic review of the patterns of failure following stereotactic body radiation therapy in early-stage non-small-cell lung cancer: Clinical implications. Radiother Oncol 94: 1-11, 2010.
5. Martini N, Bains MS, Burt ME, Zakowski MF, McCormack P, Rusch VW and Ginsberg RJ: Incidence of local recurrence and second primary tumors in resected stage I lung cancer. J Thorac Cardiovasc Surg 109: 120-129, 1995.
6. Sarkar SP and Adshead G: Whose DNA is it anyway? European court, junk DNA and the problem with prediction. J Am Acad Psychiatry Law 38: 247-250, 2010.
7. Li G, Zhang H, Wan X, Yang X, Zhu C, Wang A, He L, Miao R, Chen S and Zhao H: Long noncoding RNA plays a key role in metastasis and prognosis of hepatocellular carcinoma. Biomed Res Int 2014: 780521, 2014.
8. Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, *et al*: MALAT-1, a novel noncoding RNA and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22: 8031-8041.
9. Lelli A, Nolan KA, Santambrogio S, Gonçalves AF, Schönenberger MJ, Guinot A, Frew IJ, Marti HH, Hoogewijs D and Wenger RH: Induction of long noncoding RNA MALAT1 in hypoxic mice. Hypoxia (Auckl) 3: 45-52, 2015.
10. Song X, Wang B, Bromberg M, Hu Z, Konigsberg W and Garen A: Retroviral-mediated transmission of a mouse VL30 RNA to human melanoma cells promotes metastasis in an immunodeficient mouse model. Proc Natl Acad Sci USA 99: 6269-6273, 2002.
11. Song X, Sui A and Garen A: Binding of mouse VL30 retrotransposon RNA to PSF protein induces genes repressed by PSF: Effects on steroidogenesis and oncogenesis. Proc Natl Acad Sci USA 101: 621-626, 2004.
12. French NS and Norton JD: Structure and functional properties of mouse VL30 retrotransposons. Biochim Biophys Acta 1352: 33-47, 1997.
13. Li L1, Feng T, Lian Y, Zhang G, Garen A and Song X: Role of human noncoding RNAs in the control of tumorigenesis. Proc Natl Acad Sci USA 106: 12956-12961, 2009.
14. Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, *et al*: MALAT-1, a novel noncoding RNA and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene 22: 8031-8041, 2003.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
16. Semenza GL: HIF-1 and tumor progression: Pathophysiology and therapeutics. Trends Mol Med 8 (4 Suppl): S62-S67, 2002.
17. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59: 5830-5835, 1999.
18. Semenza GL: Oxygen sensing, homeostasis, and disease. N Engl J Med 365: 537-547, 2011.
19. Rofstad EK, Rasmussen H, Galappathi K, Mathiesen B, Nilsen K and Graff BA: Hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor. Cancer Res 62: 1847-1853, 2002.
20. Zhang L and Hill RP: Hypoxia enhances metastatic efficiency in HT1080 fibrosarcoma cells by increasing cell survival in lungs, not cell adhesion and invasion. Cancer Res 67: 7789-7797, 2007.
21. Cairns RA, Kalliomaki T and Hill RP: Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 61: 8903-8908, 2001.
22. Rofstad EK, Galappathi K, Mathiesen B and Ruud EB: Fluctuating and diffusionlimited hypoxia in hypoxia-induced metastasis. Clin Cancer Res 13: 1971-1978, 2007.
23. Büchler P, Reber HA, Lavey RS, Tomlinson J, Büchler MW, Friess H and Hines OJ: Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res 120: 295-303, 2004.
24. Kalliomäki TM, McCallum G, Lunt SJ, Wells PG and Hill RP: Analysis of the effects of exposure to acute hypoxia on oxidative lesions and tumour progression in a transgenic mouse breast cancer model. BMC Cancer 8: 151, 2008.
25. Reis EM and Verjovski-Almeida S: Perspectives of long non-coding RNAs in cancer diagnostics. Front Genet 3: 32, 2012.

26. Yang F, Huo XS, Yuan SX, Zhang L, Zhou WP, Wang F and Sun SH: Repression of the long noncoding RNA-LET by histone deacetylase 3 contribuetes to hypoxia-mediated metastasis. Mol Cell 49: 1083-1096, 2013.
27. Yang F, Zhang H, Mei Y and Wu M: Reciprocal regulation of HIF-1α and lincRNA-p21 modulates the Warburg effect. Mol Cell 53: 88-100, 2014.
28. Ji Q, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J and Li Q: Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/b-catenin signal pathway. PLoS One 8: e78700, 2013.
29. Lai MC, Yang Z, Zhou L, Zhu QQ, Xie HY, Zhang F, Wu LM, Chen LM and Zheng SS: Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation. Med Oncol 29: 1810-1816, 2012.
30. Xu C, Yang M, Tian J, Wang X and Li Z: MALAT-1: A long non-coding RNA and its important 30 end functional motif in colorectal cancer metastasis. Int J Oncol 39: 169-175, 2011.
31. Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, Freier SM, Bennett CF, Sharma A and Bubulya PA: The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell 39: 925-938, 2010.
32. Tian Y, Zhang X, Hao Y, Fang Z and He Y: Potential roles of abnormally expressed long noncoding RNA UCA1 and Malat-1 in metastasis of melanoma. Melanoma Res 24: 335-341, 2014.
33. Han Y, Liu Y, Nie L, Gui Y and Cai Z: Inducing cell proliferation inhibition, apoptosis and motility reduction by silencing long noncoding ribonucleic acid metastasis-associated lung adenocarcinoma transcript 1 in urothelial carcinoma of the bladder. Urology 81: 209.e1-e7, 2013.