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Hydrogen inhibits the proliferation and migration of gastric cancer cells by modulating lncRNA MALAT1/miR-124-3p/EZH2 axis

Baocheng Zhu^{1†}, Hengguan Cui^{1†} and Weiqiang Xu^{1,2*}

Abstract

Background: Gastric cancer is one of the most prevalent and deadly malignancies without efficient treatment option. This study aimed to investigate the effect of hydrogen gas on the behavior of gastric cancer cells.

Methods: Gastric cancer cell lines MGC-803 and BGC-823 were treated with or without H₂/O₂ gas mixture (66.7%:33.3% v/v). Proliferation and migration were assessed by MTT and scratch wound healing assays respectively. The expression of lncRNA MALAT1, miR-124-3p, and EZH2 was analyzed by real-time quantitative PCR and/or western blot. Tumor growth was estimated using xenograft mouse model.

Results: H₂ gas significantly inhibited gastric tumor growth in vivo and the proliferation, migration, and lncRNA MALAT1 and EZH2 expression of gastric cancer cells while upregulated miR-124-3p expression. lncRNA MALAT1 overexpression abolished all the aforementioned effects of H₂. lncRNA MALAT1 and miR-124-3p reciprocally inhibited the expression of each other. miR-124-3p mimics abrogated lncRNA MALAT1 promoted EZH2 expression and gastric cancer cell proliferation and migration.

Conclusions: These data demonstrated that H₂ might be developed as a therapeutics of gastric cancer and lncRNA MALAT1/miR-124-3p/EZH2 axis could be a target for intervention.

Keywords: Hydrogen, Gastric cancer, lncRNA MALAT1, miR-124-3p, EZH2, Proliferation, Migration

Background

Gastric cancer is the sixth most-common cancer with over a million new cases worldwide in 2018 and causes second most mortalities among malignancies [1]. While *Helicobacter pylori* infection is the most prominent cause of gastric cancer, many nutritional and life style factors, such as drinking, smoking, physical activity, overweight, intake of fruit and vegetable, are also significantly

associated with the development of gastric cancer [2]. Genetically, the main oncogene of gastric cancer is CDH1 (E-cadherin) as many pathogenic variants are associated with familial diffuse-type gastric cancer [3, 4]. However, many other genes including *MSH2*, *PMS2*, *BRCA1*, *PALB2*, *CTNNA1*, and *ATM*, have been identified to increase the risk of gastric cancer [5–12].

Long noncoding RNAs (lncRNAs) are transcripts larger than 200 base-pairs and similar to mRNA biochemically and structurally but do not code for protein [13]. A large number of lncRNA have been implicated in different aspects of cancer biology [14]. lncRNA MALAT1 (metastasis associated lung adenocarcinoma transcript 1)

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has been shown to promote the proliferation and migration of cancer cells, epithelial-mesenchymal transition, and metastasis of many cancer types but recently found to function as a tumor suppressor in breast and colorectal cancers [16]. LncRNA MALAT1 enhanced the stemness, proliferation, migration, invasion, and drug resistance of gastric cancer cells [17–20].

Although hydrogen gas was found effective in treating mouse squamous cell carcinoma and thought it might be used to treat other cancer [21], its clinical application was not widely explored until 2007 Ohsawa et al. demonstrated that hydrogen gas selectively eliminated the hydroxyl radical and attenuated focal ischemia and reperfusion caused oxidative stress and brain injury [22]. Increasing evidence showed the potential of hydrogen gas in preventing and relieving different cancers [23]. Drinking hydrogen-rich water for 6 weeks reduced reactive oxygen metabolites in the blood, maintained blood oxidation potential, improved the quality of life of malignant liver cancer patients after radiotherapy [24]. Daily inhalation of hydrogen for 3 months resulted in the shrinkage of metastatic gallbladder cancer and improve of quality of life [25]. This study aims to explore the effects of hydrogen gas on gastric cancer cells and the underlying molecular mechanism.

Materials and methods

Cell culture

Human gastric cancer cell lines MGC-803 and BGC-823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin (All from ThermoFisher, Shanghai, China).

Hydrogen/oxygen gas mixture (66.7%:33.3% v/v) was produced with a Hydrogen/Oxygen Generator (Asclepius Meditec, Shanghai, China). Hydrogen treatment was executed in an adjustable three gas cell culture incubator (Puhe Bio, Wuxi, China). MALAT1 overexpression vector (GenScript, Nanjing, China) and miR-124-3p mimics (GeneCopoeia, Guangzhou, China) were transfected using Lipofectamine 3000 (ThermoFisher, Shanghai, China).

MTT assay

Seeded 5000 MGC-803 or BGC-823 cells per well in 96 well plates and cultured at 37 °C overnight before treated with or without hydrogen gas for 24 h. Added 20 µl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to each well and incubated at 37 °C for 4 h. The medium was carefully removed and 100

µl of MTT solvent (4 mM HCl and 0.1% Nondet P-40 (NP40) in isopropanol) was added to every well. The plates were incubated at room temperature with orbital shaking for 15 min before read at 590 nm.

Wound healing scratch assay

After MGC-803 and BGC-823 cells in 24 well plates reached confluency, the cells were treated with 0.2 mg/ml mitomycin C for 3 min. The center of the wells were scratched with a blue tip and photographed. The plates then cultured at 37 °C for 12 h and photographed. The gaps were measured with ImageJ (NIH, Bethesda, MD). The migration rate was calculated as (gap distance at 0 h – gap distance at 24 h)/gap distance at 0 h *100.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from using MiniBEST Universal RNA Extraction Kit (TaKaRa, Beijing, China) following manufacturer's manual. First strand cDNA was synthesized from 0.5 µg total RNA using Invitrogen SuperScript III Reverse Transcriptase kit (ThermoFisher, Shanghai, China). Noncoding small RNAs were first poly (A) tailed with *E. coli* Poly(A) Polymerase (M0276, NEB, Ipswich, MA) and then reverse transcribed using Moloney Murine Leukemia Virus (M-MuLV, MMLV) Reverse Transcriptase (M0253, NEB) with GTCGCAGTGCAG GGTCCGAGGTGGCGATTTTTTTTTTTTTTTTTTTT TTTTT(A/G/C)(A/G/T/C) as primer. Real-time RT-PCR amplification was carried on an ABI StepOne Plus (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq™ kit (Takara, Beijing, China). The PCR primers were TGCTGTGTGCCAATGTTTCG and CAGCTGCCTGCTGTTTCTG for MALAT1, CTG CTCCTACATCGTAAGTGCAA and TTGCTCCCT CCAAATGCTGGT for enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), CTGAGGAGC AGCTTCAGTCC and GAGTAGCCATTGTCCACGCT for β-catenin (CTNNB1), and CCGAGAATGGGAAGC TTGTC and AAGCACCAACGAGAGGAGAA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TAA GGCACGCGGTGAATGC for miR-124-3p and CGC AAGGATGACACGCAAATTC for U6 with universal reverse primer GTGCAGGGTCCGAGGT. The relative gene expression was calculated using 2^{-ΔΔCt} method with GAPDH as internal control for lncRNA MALAT1 and EZH2 while U6 for miR-124-3p.

Western blot

MGC-803 and BGC-823 cells were lysed with RIPA lysis buffer (1% v/v NP-40, 20 mM Tris-HCL pH 7.4, 5 mM sodium pyrophosphate, 5 mM EDTA) supplemented with protease and phosphatase inhibitor cocktail (Millipore

Sigma, Burlington, MA). Total protein samples (40 µg) were resolved on 8% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked in 5% nonfat milk in TBST (50 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Tween 20) for 30 min, incubated with primary antibodies at 4 °C overnight, washed and incubated with proper horseradish peroxidase conjugated goat against mouse or rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA) at room temperature for 60 min before visualized with enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL). The primary antibodies used were anti-EZH2 (05-1319, Millipore Sigma) and anti-β-actin (A5441, Millipore Sigma).

Mouse xenograft gastric cancer models

Animal protocol was approved by Institutional Animal Care and Usage Committee of Zhongshan Hospital Affiliated to Fudan University (ZHFU20200374). Female BALB/c nude mice (5 weeks old) were purchased from Cavens Laboratory Animal Inc (Changzhou, China) and acclimatized for a week. BGC-823 cells (1×10^6) stably transfected with lncRNA MALAT1 or miR-124-3p were subcutaneously injected into the flank of a nude mouse (n = 7). Three days later, mice were exposed to hydrogen/oxygen mixture (2:1 vol:vol) for 2 h daily. All mice were sacrificed 5 weeks after inoculation.

Statistical analyses

All experiments were performed 3 times with triplicates. Statistical analyses were performed using GraphPad Prism 6 (San Diego, CA). The differences among treatments were assessed with analysis of variance followed by Bonferroni tests. A p value less than 0.05 was considered statistical significant.

Results

Hydrogen inhibited the proliferation and migration of gastric cancer cells

As hydrogen gas has been shown to inhibit cancer progression [25] and lung cancer cell proliferation [26], we first examined whether it could impact the proliferation of gastric cancer cells. After 24 h exposure to hydrogen gas, the viable MGC-803 and BGC-823 cells were reduced more than 20% compared to normally cultured ones (Fig. 1a). Moreover, H₂ gas drastically reduced the motility of MGC-803 cells by about 60% and BGC-823 cells by more than 50% (Fig. 1b, c).

Hydrogen gas inhibited the expression of MALAT1 and EZH2

Microarray analysis identified that H₂ gas significantly inhibited the expression of lncRNA MALAT1 and transcription factor EZH2 of MGC-823 cells

(Fig. 2a). Real-time quantitative PCR confirmed that 24 h H₂ exposure reduced the RNA transcript levels of MALAT1 and EZH2 about 50% in both MGC-803 and BGC-823 cells (Fig. 2b). The protein levels of EZH2 of MGC-803 and BGC-823 cells treated with H₂ were reduced more than 50% compared to control cells (Fig. 2c).

H₂ inhibited EZH2 expression through downregulating lncRNA MALAT1

Overexpressing MALAT1 upregulated the mRNA (Fig. 3a) and protein (Fig. 3b) levels of EZH2 in MGC-803 and BGC-823 cells. Furthermore, lncRNA MALAT1 abrogated the inhibition of EZH2 expression by H₂ (Fig. 3a, b).

lncRNA MALAT1 relieved the inhibitory effect of H₂ on gastric cancer cell proliferation and migration

As previously showed that H₂ gas inhibited the proliferation and migration of MGC-803 and BGC-823 cells, we next examined the roles of lncRNA MALAT1 on the effects of H₂. lncRNA MALAT1 overexpression resulted in about 25% increase of MGC-803 and BGC-823 cell proliferation (p < 0.01 MALAT1 vs. Ctrl) and abolished the inhibition of proliferation imposed by H₂ (p < 0.01 for MALAT1 + H₂ vs. MALAT1 and MALAT1 + H₂ vs. H₂) (Fig. 3c). Overexpression of lncRNA MALAT1 increased MGC-803 and BGC-823 cell migration by about 40% (p < 0.01 MALAT1 vs. Ctrl) (Fig. 3d). Moreover, MALAT1 abrogated the inhibitory effect of H₂ gas on the migration of MGC-803 gastric cancer cells (p < 0.01 for MALAT1 + H₂ vs. MALAT1 and MALAT1 + H₂ vs. H₂) (Fig. 3d).

miR-124-3p expression in gastric cancer cells was regulated by H₂ and lncRNA MALAT1

Using lncBase Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools), has-miR-124-3p was identified as a regulator and target of lncRNA MALAT1. Four strong miR-124-3p binding sites (Fig. 4a) and many other imperfect binding sites of miR-124-3p were identified in MALAT1. The miR-124-3p level of MGC-803 and BGC-823 cells was increased more than 100% by H₂ treatment (p < 0.01, H₂ vs. Ctrl) (Fig. 4b). On the other hand, lncRNA MALAT1 overexpression reduced miR-124-3p level more than 40% (p < 0.01 MALAT1 vs. Ctrl). Moreover, lncRNA MALAT1 and H₂ gas antagonized the effect of each other on the expression of miR-124-3p (p < 0.01 for MALAT1 + H₂ vs. MALAT1 and MALAT1 + H₂ vs. H₂) (Fig. 4b).

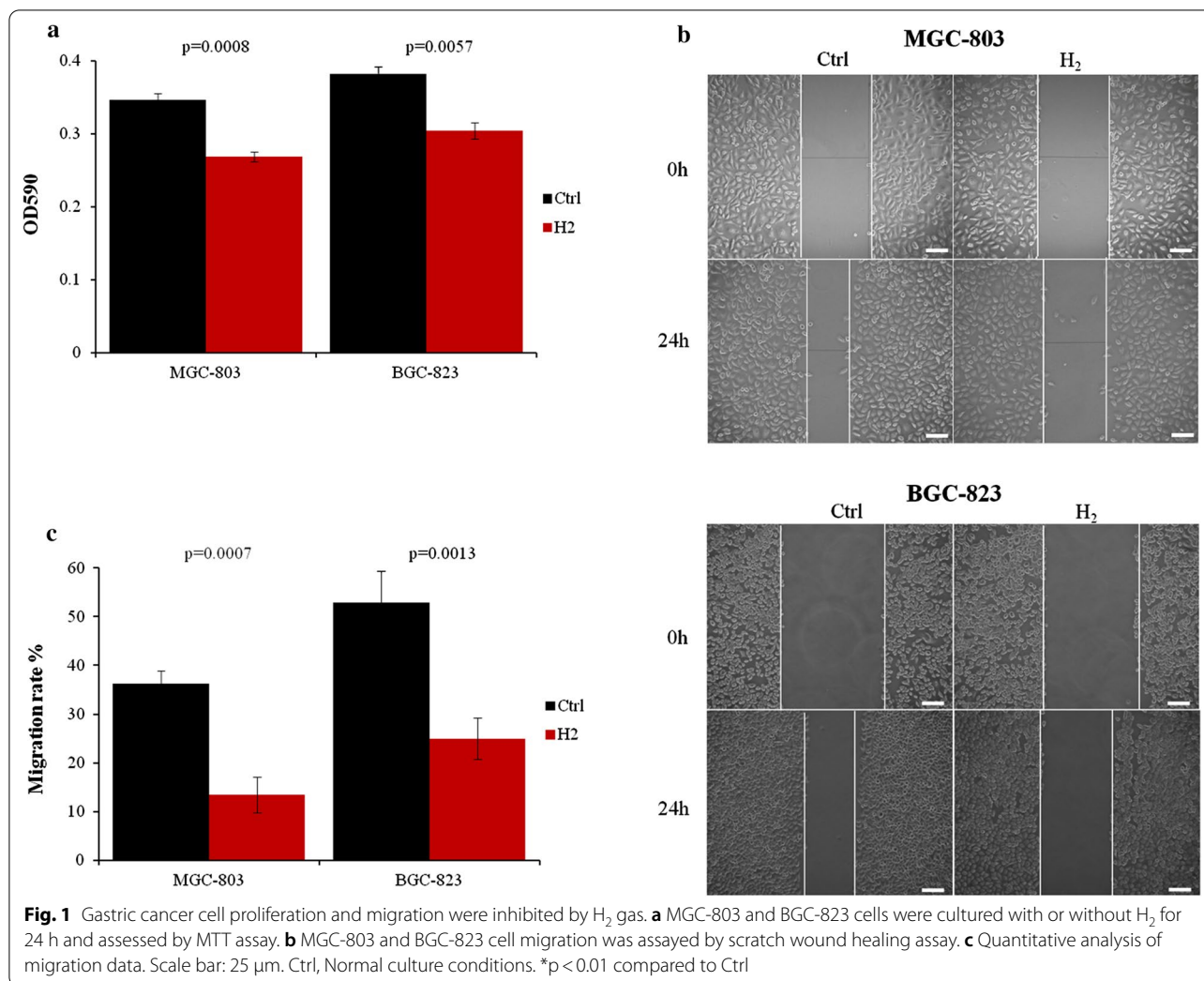


Fig. 1 Gastric cancer cell proliferation and migration were inhibited by H₂ gas. **a** MGC-803 and BGC-823 cells were cultured with or without H₂ for 24 h and assessed by MTT assay. **b** MGC-803 and BGC-823 cell migration was assayed by scratch wound healing assay. **c** Quantitative analysis of migration data. Scale bar: 25 μ m. Ctrl, Normal culture conditions. * $p < 0.01$ compared to Ctrl

lncRNA MALAT1 upregulated EZH2 expression through inhibiting miR-124-3p

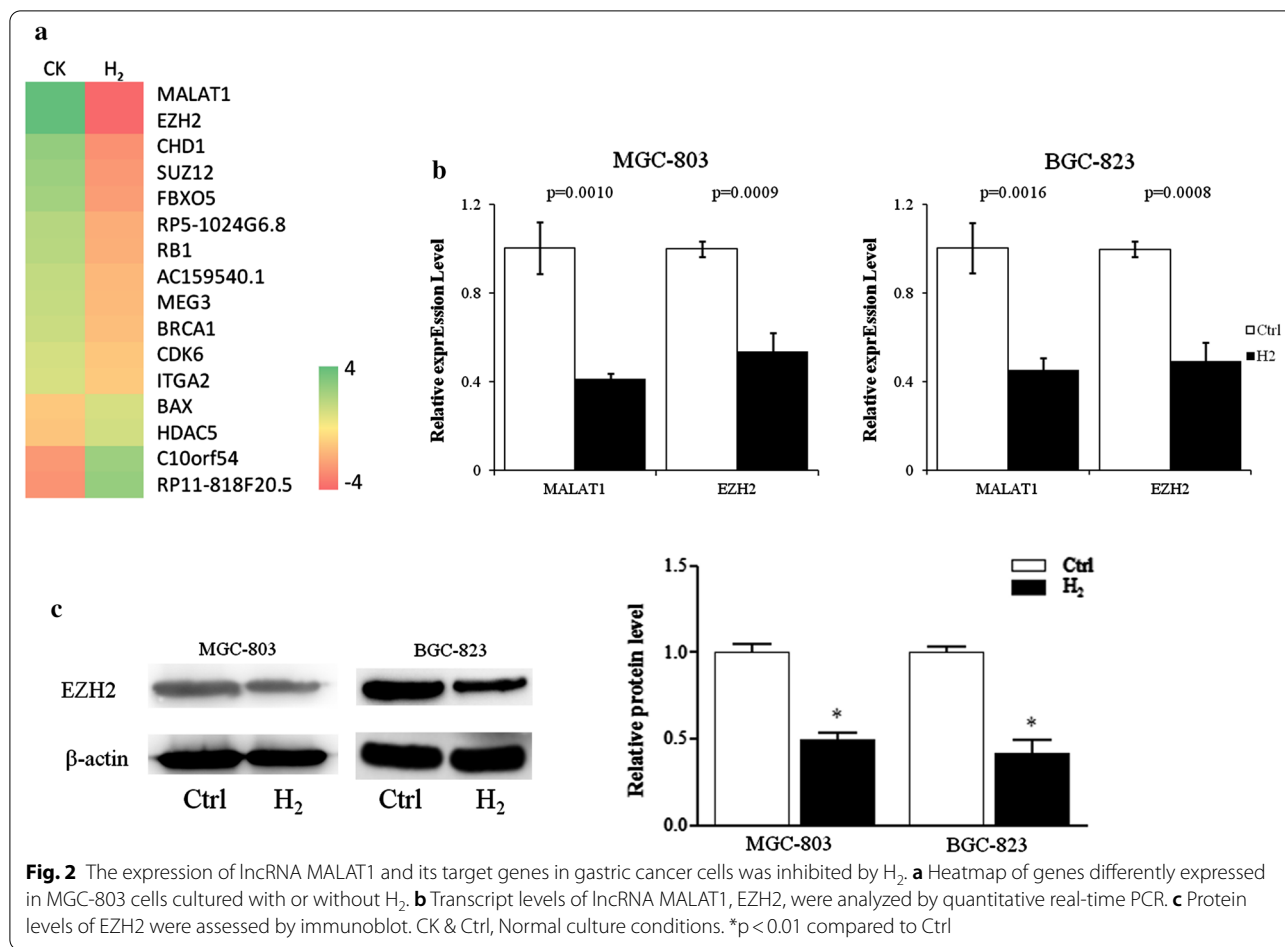
As miR-124-3p was regulated by lncRNA MALAT1 and EZH2 was identified as a direct target of miR-124-3p (Fig. 5a), the effect of miR-124-3p on the regulation of EZH2 expression by lncRNA MALAT1 was assessed. Transfection of miR-124-3p mimics reduced EZH2 mRNA level about 50% ($p < 0.01$, miR-124-3p vs. Ctrl) and suppressed the upregulation of EZH2 by lncRNA MALAT1 ($p < 0.01$ for miR-124-3p + MALAT1 vs. MALAT1 and miR-124-3p + MALAT1 vs. miR-124-3p) (Fig. 5b). Moreover, lncRNA MALAT1 and miR-124-3p reciprocally inhibited the expression of one another (Fig. 5b). The protein level of EZH2 was reduced about 60% by miR-124-3p ($p < 0.01$, miR-124-3p vs. Ctrl) and miR-124-3p abrogated the lncRNA MALAT1 induced EZH2 upregulation ($p < 0.01$ for miR-124-3p + MALAT1 vs. MALAT1 and miR-124-3p + MALAT1 vs. miR-124-3p) (Fig. 5c).

miR-124-3p abolished lncRNA MALAT1 induced proliferation and migration of gastric cancer cells

Viable MGC-803 and BGC-823 cells were reduced more than 20% by miR-124-3p mimics ($p < 0.01$, miR-124-3p vs. Ctrl) and the promotion of MGC-803 and BGC-823 cell proliferation by lncRNA MALAT1 was inhibited by miR-124-3p ($p < 0.01$ for miR-124-3p + MALAT1 vs. MALAT1, and miR-124-3p + MALAT1 vs. miR-124-3p) (Fig. 6a). miR-124-3p also inhibited the migration rate of MGC-803 and BGC-823 cells by about 50% ($p < 0.01$, miR-124-3p vs. Ctrl) and abolished lncRNA MALAT1 induced increase of cell motility ($p < 0.01$ for miR-124-3p + MALAT1 vs. MALAT1 and miR-124-3p + MALAT1 vs. miR-124-3p) (Fig. 6b).

lncRNA MALAT1 and miR-124-3p mediated the inhibition of tumor growth by H₂

Inhalation of H₂ drastically inhibited tumor growth in mouse xenografted with BGC-823 cells ($p < 0.01$, H₂ vs.



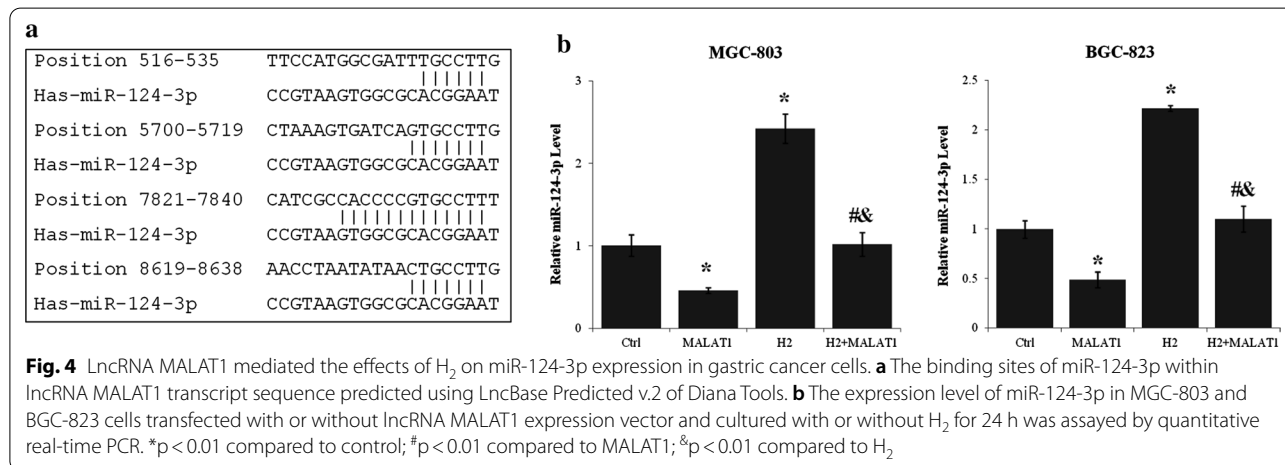
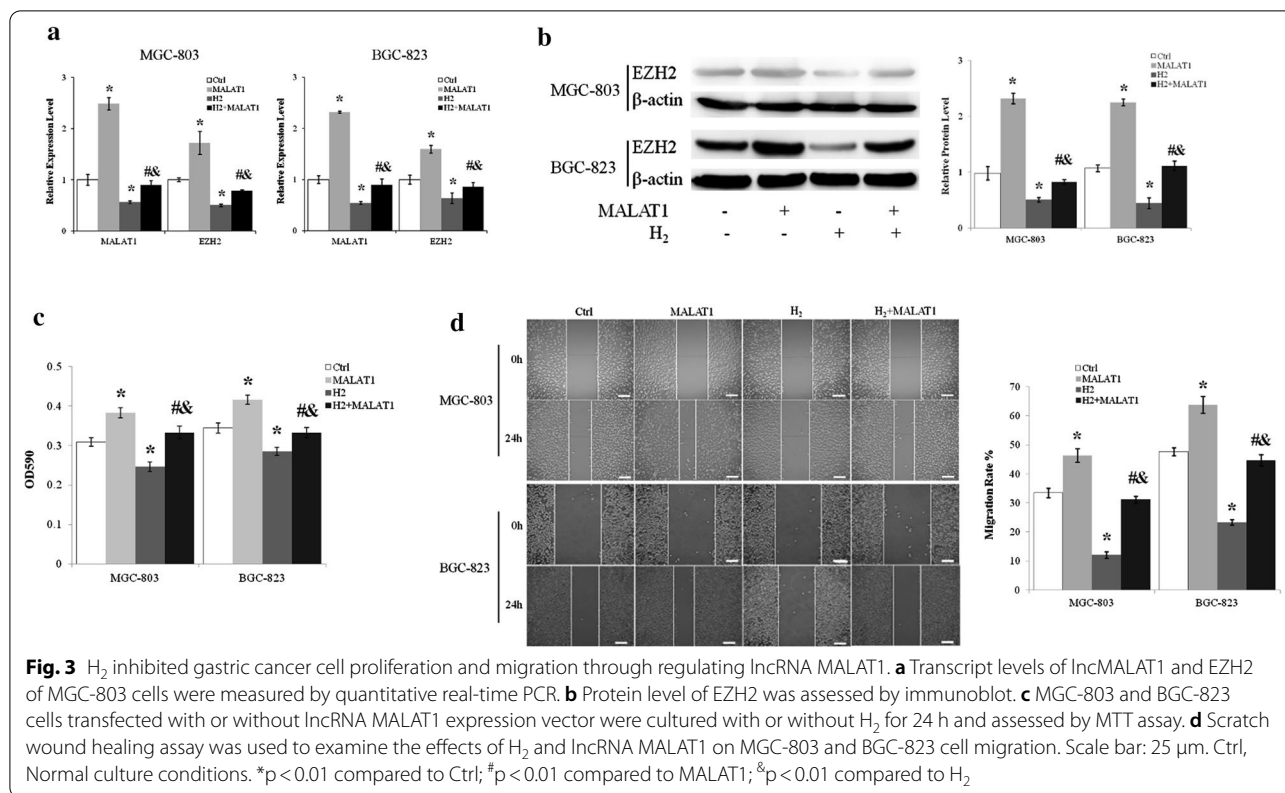
Ctrl) (Fig. 7), which was consistent with clinical observation of different cancers [23, 25]. Overexpression of lncRNA MALAT1 partially suppressed H₂ caused inhibition of tumor growth whereas miR-124-3p counteracted the effect of lncR MALAT1 (Fig. 7a). EZH2 protein level of gastric tumor tissue was reduced more than 50% by H₂ exposure, which was enhanced by miR-124-3p and suppressed by lncRNA MALAT1 (Fig. 7b).

Discussion

H₂ gas inhibited the proliferation and migration of MGC-803 and BGC-823 gastric cancer cells and downregulated the expression of lncRNA MALAT1 and EZH2. The inhibition of EZH2 by H₂ was relieved by lncRNA MALAT1 overexpression. LncRNA MALAT1 also alleviated H₂ caused inhibition of the proliferation and migration of MGC-803 and BGC-823 gastric cancer cells. MicroRNA hsa-miR-124-3p was identified as a target and regulator of lncRNA MALAT1 and suppressed the expression of EZH2. H₂ upregulated miR-124-3p expression, which was abrogated by lncRNA MALAT1 overexpression. LncRNA MALAT1 promoted proliferation and migration

of MGC-803 and BGC-823 cells were abolished by miR-124-3p mimics. H₂ exposure drastically reduced gastric cancer growth in xenograft mouse model and inhibited EZH2 expression in cancer tissue, which was enhanced by miR-124-3p and suppressed by lncRNA MALAT1.

H₂ gas has been shown to improve many conditions by clearing detrimental ROS [27]. Inhalation of high concentration of hydrogen gas protected rat retinal ganglion cells from retinal ischemia/reperfusion (I/R) injury caused oxidative stress, inflammation, and apoptosis [28]. H₂ inhalation ameliorated middle cerebral artery occlusion induced cerebral I/R injury evidenced by reduced 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine production, tampered inflammation, and reduced neural apoptosis [29]. PI3K/AKT1 signaling was shown to mediate the protection of H₂ on I/R induced cardiac injuries [30]. Malignant liver cancer patients drinking hydrogen-rich water during radiotherapy significantly reduced blood hydroperoxide level and increased biological antioxidant activities, leading to improved quality of life [24]. Daily H₂ inhalation stopped the growth of primary and metastatic tumors in a metastatic



gallbladder cancer patient with the levels of tumor biomarkers returning to normal [25]. H₂ treatment inhibited CD47 expression and induced apoptosis of A549 lung cancer cells [31]. Another study showed that SMC3 mediated the inhibition of lung cancer progression by

H₂ in in vitro study and xenograft mouse model [26]. The current study showed that H₂ could inhibit the progression and migration of gastric cancer cells. Further studies about the effects of H₂ on gastric cancer progression in animal models are warranted.

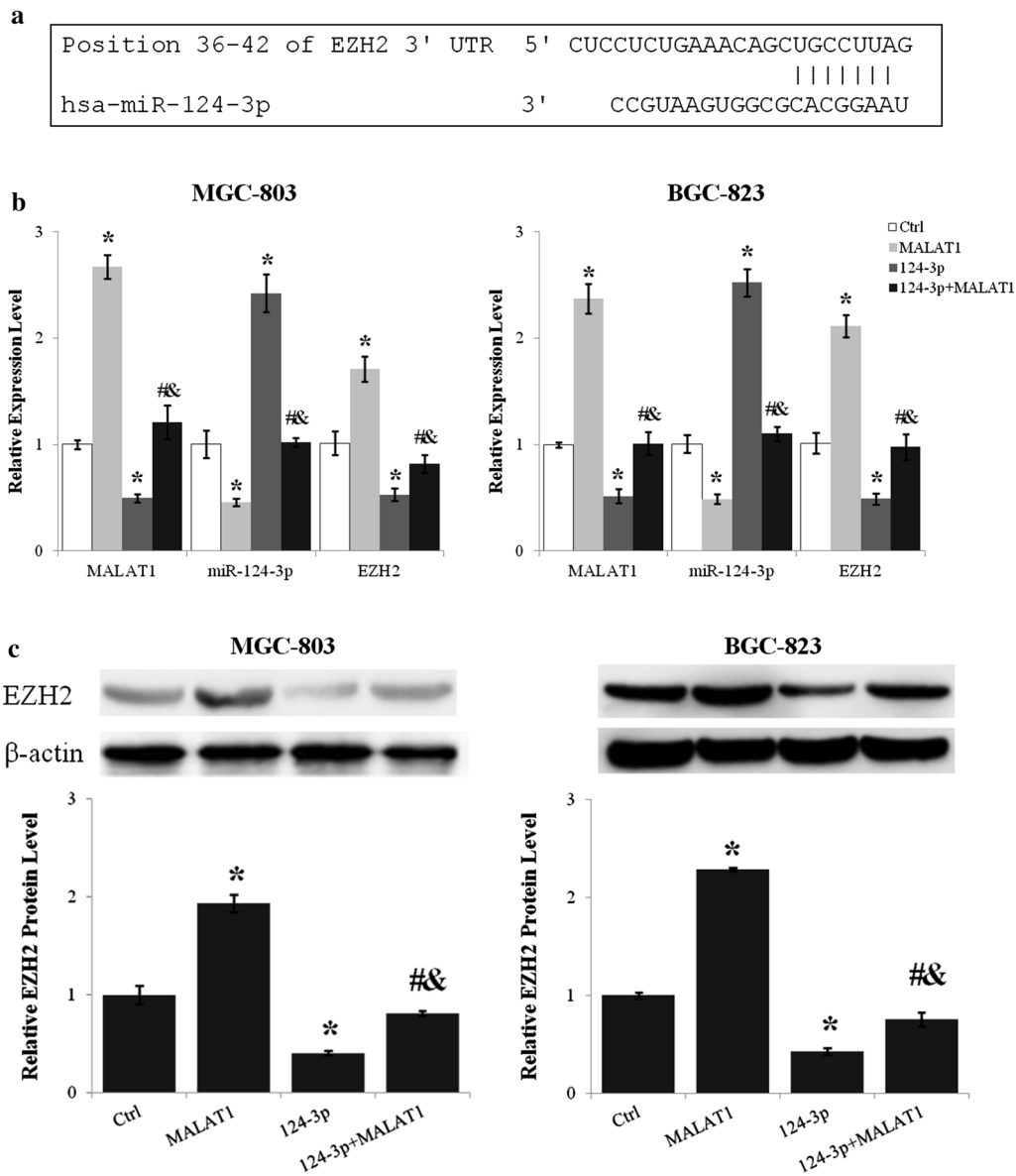
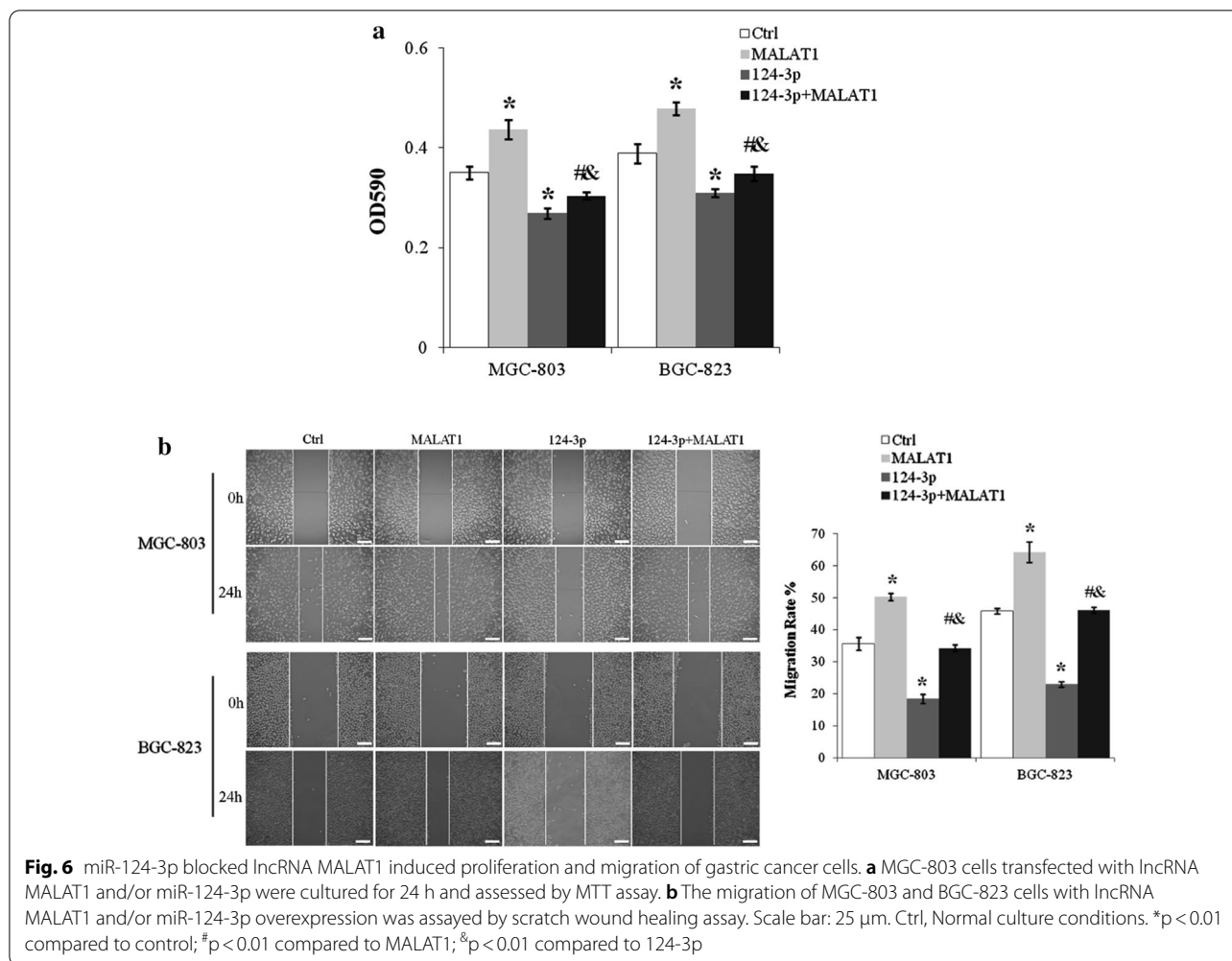


Fig. 5 The upregulation of EZH2 by lncRNA MALAT1 was antagonized by miR-124-3p. **a** The miR-124-3p binding site in EZH2 3'UTR predicted using TargetScan. **b** The expression levels of lncRNA MALAT1, miR-124-3p, and EZH2 after lncRNA MALAT1 and/or miR-124-3p overexpression were analyzed by quantitative real-time PCR. **c** The effects of lncRNA MALAT1 and miR-124-3p on EZH2 protein level were examined by western blot. Ctrl, Normal culture conditions. * $p < 0.01$ compared to control; # $p < 0.01$ compared to MALAT1; & $p < 0.01$ compared to 124-3p

Long noncoding RNA MALAT1 was identified as a factor associated with metastasis of non-small cell lung cancer [32] and regulated gene expression through different mechanism [16]. LncRNA MALAT1 was overexpressed in gastric cancers compared to adjacent

normal tissue and promoted gastric cancer cells proliferation, migration, and resistance to cisplatin through activating PI3K/AKT pathway [19, 33]. LncRNA MALAT1 regulated miR-30e/ATG5 expression to modulate autophagy and apoptosis of SGC7901 gastric



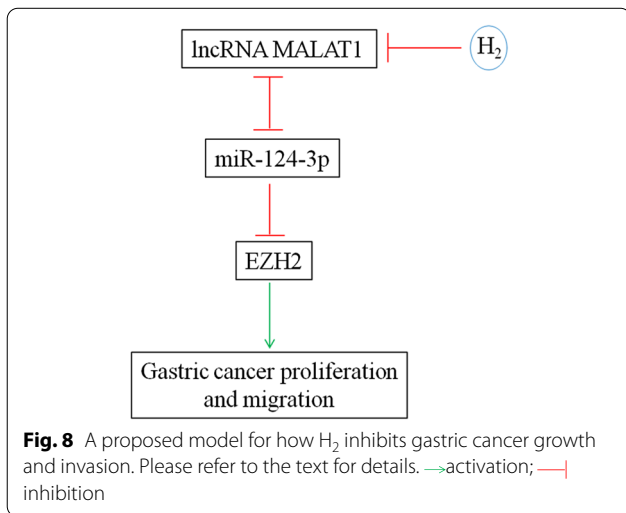
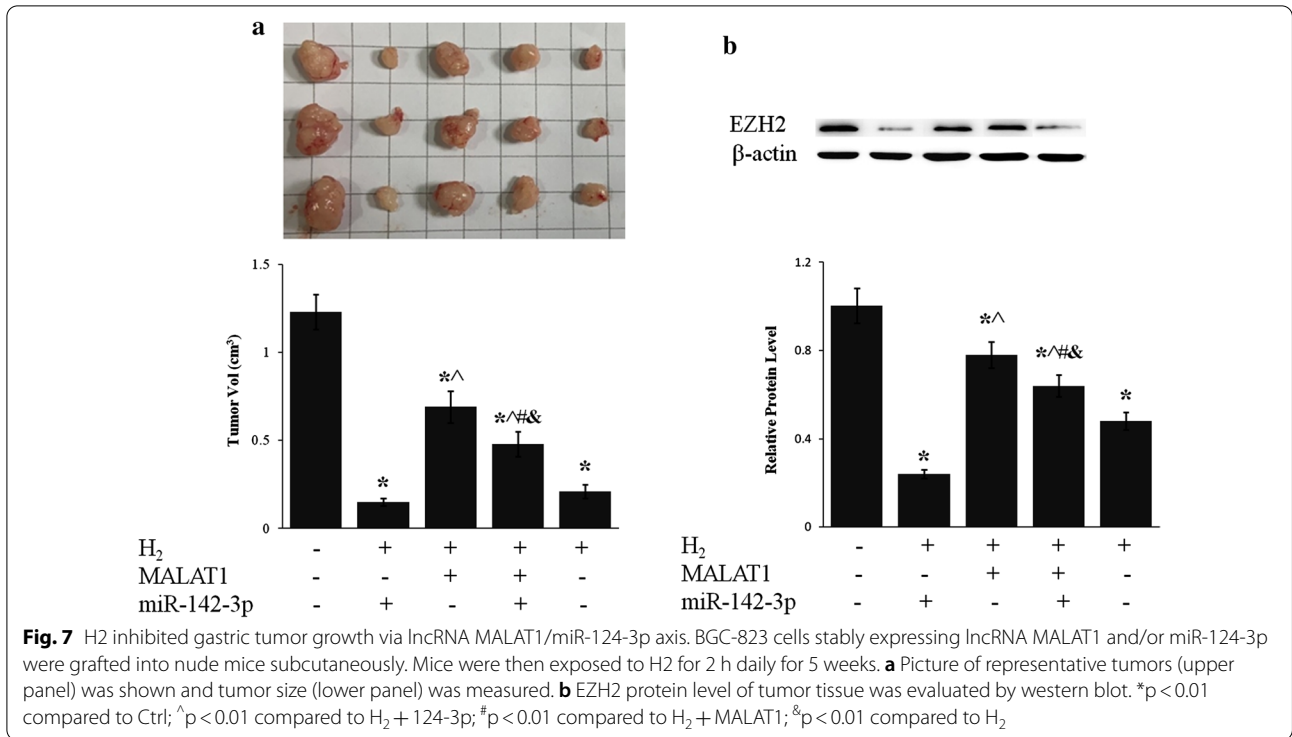
cancer cells and conferred resistance to cisplatin [34]. MALAT1 was shown to directly bind to and stabilize Sox2 mRNA, which led to the increase of stemness and chemo- and radioresistance of gastric cancer cells [20]. The current study demonstrated that lncRNA MALAT1 promoted gastric cancer proliferation and migration through modulating miR-124-3p/EZH2 axis.

MicroRNA miR-124-3p was significantly downregulated in gastric cancer tissues and its level was inversely associated with histological grade, TNM stage and lymph node metastasis. Moreover, low miR-124-3p expression was correlated with lower overall and disease-free survival rates [35]. ITGB3 [36] and ZEB1 [37] were found to be targeted by miR-124-3p in promoting gastric cancer proliferation and invasion. Moreover, Circular RNA circ-PVT1 upregulated ZEB1 expression by sponging miR-124-3p and enhanced paclitaxel resistance of gastric

tumor and gastric cancer cells [37]. lncRNA XIST and miR-124-3p competitively regulated EZH2 expression and modulated the proliferation, migration, and invasion of laryngeal squamous cancerous cells [38]. miR-124-3p was antagonized by lncRNA MALAT1 in inhibiting the expression of Slug and metastasis of hepatocellular carcinoma [39]. This study demonstrated that H₂ gas curbed gastric cancer growth in vivo and gastric cancer cell proliferation and migration in vitro by reducing lncRNA MALAT1 level, which in turn upregulated miR-124-3p and downregulated EZH2 expression (Fig. 8).

Conclusions

This study demonstrated the efficacy of molecular hydrogen in inhibiting the growth of gastric cancer by reducing the proliferation and migration of gastric cancer cells, which was through downregulating lncRNA



MALAT1 and polycomb-group family member EZH2 and upregulating miR-124-3p. Moreover, miR-124-3p inhibited EZH2 expression and it reciprocally repressed the expression of each other with lncRNA MALAT1. These data indicated that H₂ should be further studied for treating gastric cancers and lncRNA MALAT1/miR-124-3p/EZH2 axis would be a novel intervention target.

Abbreviations

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; lncRNA: Long noncoding RNA; miRNA: MicroRNA; MALAT1: Metastasis associated lung adenocarcinoma transcript 1; EZH2: Enhancer of zeste 2 polycomb repressive complex 2 subunit; GAPDH: Glycerinaldehyde 3-phosphate dehydrogenase.

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Authors' contributions

WX conceived, designed the study and wrote the manuscript. BZ and HC carried out the study and data analysis. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflict of interest.

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