Mechanism of hydrogen on cervical cancer suppression revealed by high-throughput RNA sequencing

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Abstract. Cervical cancer is considered one of the diseases with the highest mortality among women and with limited treatment options. Hydrogen (H₂) inhalation has been reported to have a variety of tumor-suppressive effects, but the exact mechanism remains unclear. In the present study, HeLa cervical cancer cells and HaCaT keratinocytes treated with H₂, and a HeLa xenograft mouse model subjected to H₂ inhalation were established. TUNEL, Cell Counting Kit-8 and Ki67 staining assays were used to detect cell apoptosis and proliferation. Oxidative stress was determined according to the levels of reactive oxygen species, malondialdehyde and superoxide dismutase. Tumor growth was recorded every 3 days, and the excised tumors were stained with hematoxylin and eosin. High-throughput RNA sequencing and subsequent Gene Ontology (GO) enrichment analysis were performed in HeLa-treated and un-treated HeLa cells. The expression of hypoxia-inducible factor (HIF)-1α and NF-κB p65 was verified by western blotting, immunohistochemistry and reverse transcription-quantitative PCR. The results revealed an increased apoptosis rate, and reduced cell proliferation and oxidative stress in H₂-treated HeLa cells but not in HaCaT cells. Similarly, decreased tumor growth and cell proliferation, and enhanced cell apoptosis were observed in H₂-treated HeLa tumors. RNA sequencing and GO analysis suggest that downregulated HIF1A (HIF-1α mRNA) and RelA (NF-κB p65) levels, and reduced NF-κB signaling were associated with the antitumor effect of H₂. Finally, decreased HIF-1α and NF-κB p65 expression both at the transcriptional and translational levels were observed in H₂-treated HeLa cells and in HeLa-derived tumors. In conclusion, the present study reveals a novel mechanism of H₂ against cervical cancer, which may serve as a potential therapeutic target in clinical practice.

Introduction

Cervical cancer is the fourth most common type of cancer and the fourth leading cause of cancer-associated mortality among women worldwide (1). In addition, cervical cancer is the second leading cause of cancer-associated mortality in women aged 20-39 years in the USA, and it was estimated that there may be 13,800 new cases and 4,290 mortalities in 2020 (2). Radical hysterectomy with pelvic lymphadenectomy is the standard recommended surgical therapy for patients with early-stage cervical cancer (3). However, this method also increases the number of circulating tumor cells, and promotes cancer progression and metastasis (4). In addition to surgical therapy, molecular-targeted strategies have recently received attention in the field of cancer treatment. However, molecular-targeted strategies have not shown significant benefit in the majority of patients with cervical cancer, as these precision drugs are available for only 3-13% of patients (5,6). Thus, alternative treatments for patients with cervical cancer are needed.

Molecular hydrogen (H₂) biology and H₂ therapy are novel and rapidly developing areas of research (7-9). H₂ has been shown to exert antioxidant and anti-inflammatory effects (10), and to protect neurons from oxidative stress injury during cerebral ischemia-reperfusion (11). Importantly, since the tumorigenesis and progression of cancer are closely associated with the levels of peroxidation and inflammation, H₂ may play a role in tumor regulation in endometrial (12), lung (13) and breast cancer (14). A prospective follow-up study of 82 patients with stage III and IV cancer, including lung, pancreatic and gynecological cancer (including cervical cancer), revealed significant improvements in fatigue, insomnia, anorexia, pain and physical status after 4 weeks of H₂ inhalation (15). However, the potential antitumor mechanism of H₂ intervention in cervical cancer is currently unknown.

High-throughput RNA sequencing is a powerful method for revealing transcriptional and non-transcriptional signatures in cells and animals (16). It maximizes the identification of the regulatory effects of different treatments at the genetic level. The human cervical adenocarcinoma HeLa cell line expresses...
over 10,000 proteins and is considered the most commonly analyzed cells in cervical cancer research (17,18). Previous studies reported that HeLa cells can be used in vitro (19) and in vivo xenotransplant models (20) to simulate the process of cervical cancer. In addition, HeLa cells have been used to investigate the regulatory role of H2 in Wnt/β-catenin signaling (21). But the tumor inhibitory effect of H2 and its related mechanism in HeLa cells remain unknown. Thus, RNA sequencing of control and H2-treated HeLa cells may be helpful for identifying the potential antitumor mechanism of H2 in cervical cancer.

The present study demonstrated that treatment with 66.7% H2 significantly elevated the apoptosis rate, and reduced the cell proliferation and oxidative stress of HeLa cells in vitro. Furthermore, tumor growth and cell death were also observed in H2-treated HeLa tumors. Decreased hypoxia inducible factor (HIF1A) and RELA proto-oncogene, Nf-kB subunit (RelA) expression levels were detected in the H2 group through RNA sequencing. The expression of HIF1A and RelA and their encoded protein expression were further confirmed by reverse transcription-quantitative PCR (RT-qPCR), western blotting, and immunohistochemistry (IHC).

Materials and methods

Cell line and cell experiments. The human cervical cancer HeLa cell line and nontumor HaCaT keratinocytes were purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% filtered FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified 5% CO2 environment for 1 h every 12 h. Cisplatin was obtained from APeXBIO Technology LLC (cat. no. A8321). Our preliminary results suggested that the IC50 of cisplatin treatment of HeLa cells for 24 h is 12 µg/ml, which is consistent with previously reported results (22). Cisplatin was used in the present study at a concentration of 5 µg/ml. After 7 days of H2 treatment, cells were evaluated for their viability, apoptosis, reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD) levels. In addition, RNA was isolated for RNA sequencing. Cells cultured under a normal environment for 1 h every 12 h. Cisplatin was used in the present study was 1,582 mm3. All animal experiments were approved by the Ethics Committee for Animal Studies of Naval Medical University (approval no. 202027).

Cellular apoptosis and proliferation assay. TUNEL assay by immunofluorescence staining was performed to determine the cellular apoptosis rate. Briefly, 2x10⁶ HeLa or HaCaT cells were seeded into 48-well plates and, after H2 intervention, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature, the cells were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Furthermore, the In situ Cell Death Detection kit (cat. no. 11684809910; Roche Diagnostics) was used according to the manufacturer's protocol, and cells were then counterstained with DAPI (Sigma-Aldrich; Merck KGaA) for nuclear staining and observed through a fluorescence microscope (Olympus Corporation). The percentage of TUNEL-positive cells was calculated as the ratio of the number of TUNEL-positive nuclei/total number of nuclei, which were counted in three different random fields of view.Cell proliferation was evaluated using a Cell Counting Kit (CCK)-8 assay (cat. no. C0037; Beyotime Institute of Biotechnology) following the manufacturer's instructions. The absorbance at 450 nm was detected using a microplate reader (BioTek Instruments, Inc.).

Cellular oxidative stress detection. The level of ROS was determined using the Cellular ROS Assay kit (cat. no. ab113851; Abcam) according to the manufacturer's instructions. The ROS level was calculated as ROS positive area/total area x 100%. In addition, MDA and SOD levels were detected using the commercially available test kits Lipid Peroxidation (MDA) Assay kit (cat. no. ab118970; Abcam) and SOD Colorimetric Activity kit (cat. no. EIASODC; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). HeLa tumors fixed in 4% paraformaldehyde were dehydrated and then embedded in paraffin. Next, samples were sectioned at 5-µm thickness and stained with hematoxylin and eosin (H&E). Tumor histopathological changes were captured with an optical microscope (Olympus Corporation).

For IHC, the sections were deparaffinized, hydrated at 70˚C in xylene and microwaved at full power for 20 min for
RNA reads in H2 groups/mean value in controls. Fold-change (FC) was calculated as the mean value of chance for the given set of genes. Heatmaps and volcano plots followed by a Bonferroni correction for multiple comparisons late the statistical significance of gene overrepresentation, bioinformatics tool (https://david.ncifcrf.gov/tools.jsp) (24). Gene Ontology (GO) enrichment analysis was carried out using DAVID (https://github.com/fw1121/ClustVis) (23). Gene Ontology component analysis were performed by ClustVis online tool (cat. no. G7360; Promega Corporation) according to the manufacturer's instructions.

RNA sequencing. cDNA libraries were constructed in a strand-specific manner from 4 μg DNase-treated RNA using TruSeq Stranded Total RNA Library Prep kit (cat. no. 20020597; Illumina, Inc.). Total RNA, including mRNA and small RNA (circRNA, lncRNA, and miRNA) was then fragmented, subjected to two rounds of cDNA synthesis, and adapters were then ligated to double-strand cDNA. All libraries were sequenced on Illumina NovaSeq 6000 and HiSeq X Ten platforms (Illumina, Inc.) to merge the raw reads (quality control). The clean reads were then mapped to the human genome by HISAT2 software (v 2.1.0) using default parameters. Next, transcript assemblies were constructed using StringTie software (v 1.5.3; National Institutes of Health, USA), and the data were normalized relative to the cytoplasmic control GAPDH or the nuclear control lamin B1 as the integral optical density ratio.

Bioinformatic analyses. Multivariate analysis and principal component analysis were performed by ClustVis online tool (https://github.com/fw1121/ClustVis) (23). Gene Ontology (GO) enrichment analysis was carried out using DAVID bioinformatics tool (https://david.ncifcrf.gov/tools.jsp) (24). A hypergeometric test (Fisher's exact test) was used to calculate the statistical significance of gene overrepresentation, followed by a Bonferroni correction for multiple comparisons to estimate the proportion of enriched genes that may occur by chance for the given set of genes. Heatmaps and volcano plots were generated using TBtools (v 1.055; programmed by C.J. Chen). Fold-change (FC) was calculated as the mean value of RNA reads in H2 groups/mean value in controls.

Western blotting. Protein samples from HeLa cells in the control and H2 treatment groups were lysed with Tissue or Cell Total Protein Extraction kit (cat. no. C510003; Sangon Biotech Co., Ltd.) following the manufacturer's protocol. Protein concentration was determined using a BCA Protein Assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). Equal quantities of protein (20 μg per lane) from the lysate of control or H2 treatment groups were subjected to SDS-PAGE. After transferring to a PVDF membrane and blocking with 5% skimmed milk for 2 h at room temperature, the membrane was incubated overnight at 4°C with specific primary antibodies against HIF-1α (cat. no. ab51608), GAPDH (cat. no. ab8245), NF-κB (cat. no. ab16502) or lamin B1 (cat. no. ab16048) (all from Abcam) at a 1:1,000 dilution. After washing three times, the membrane was incubated with HRP-labeled goat anti-rabbit IgG (H + L) or HRP-labeled goat anti-mouse IgG (H + L) secondary antibody (1:2,000 both; cat. nos. A0208 and A0216, respectively; both from Beyotime Institute of Biotechnology). For semi-quantitative analysis, the bands were semi-quantified using ImageJ software (v 1.53; National Institutes of Health, USA), and the data were normalized relative to the cytoplasmic control GAPDH or the nuclear control lamin B1 as the integral optical density ratio.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tumors or cultured HeLa cells with TRizol® (Invitrogen; Thermo Fisher Scientific, Inc.), and its quality and quantity were assessed using NanoDrop2000c (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA was then reverse transcribed with random hexamers (cat. no. N8080127; Invitrogen; Thermo Fisher Scientific, Inc.) and SuperScript II kit (cat. no. 18064014; Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed with specific primers and SYBR-Green PCR Master Mix kit (cat. no. 4368702; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. An 8-μl (final volume) reaction system was established, which contained 300 nM primers. The thermocycling conditions were as follows: Initial denaturation start cycle at 95°C for 3 min, followed by 32 cycles at 95°C for 10 sec and then 95°C for 30 sec. The expression levels of HIF1A and RelA were calculated by the 2^ΔΔCq method (25), normalized to that of GAPDH and converted to FC values. The following pairs of primers were used: HIF1A forward, 5'-GACAGCTTTTACGTTGGAGC-3' and reverse, 5'-TCTTGAGCGCTTGGTCTG-3'; RelA forward, 5'-ACGGACAGATTTGCAAG-3' and reverse, 5'-CTTCCATGTCAGTCTTCT-3'; GAPDH forward, 5'-GGAGCGGATCCTCCACAAAT-3' and reverse, 5'-GGCTTTTGTCATCTTCTAGG-3'.

Statistical analysis. Data are expressed as the mean ± standard error of mean. Differences between two groups were compared using unpaired Student's t-test, while multiple comparisons were performed with one-way ANOVA followed by Tukey's post hoc test. In addition, multiple comparisons in two independent variables were performed with two-way ANOVA followed by Bonferroni's post hoc test. Statistical analysis was performed using SPSS v 23.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

H2 intervention reduces HeLa cell proliferation, and promotes cellular apoptosis and oxidative stress in a selective and dose-dependent manner. Firstly, the effects of H2 treatment in HeLa cells were explored in vitro. As shown in Fig. 1A and B, the ratio of TUNEL-positive cells among all H2-treated Hela cells was significantly increased compared with that
of the controls (P<0.01). Treatment with 66.7% H₂ markedly increased the apoptosis rate. Importantly, the increase in HeLa cell apoptotic rate induced by 5 μg/ml cisplatin treatment (IC₅₀=12 μg/ml) was consistent with the effect of 66.7% H₂ treatment. Notably, no significant differences in apoptosis rate were observed in HaCaT cells treated with or without H₂ gas. The CCK-8 assay revealed that cell proliferation was reduced after 33.3% H₂ treatment progressively, and 66.7% H₂ further decreased the proliferation of HeLa cells over time but not of HaCaT cells (Fig. 1C). In addition, the effect of time was significant associated with the cell proliferation in both HeLa cells and HaCaT cells. These results suggest that H₂ has a specific concentration-dependent inhibitory effect on HeLa cells rather than on non-tumor HaCaT cells. Thus, H₂ at a concentration of 66.7% was then used for the further experiments.

ROS are generated by ageing mitochondria due to overproduction of free radicals and reduced antioxidant defenses (26,27). The present study demonstrated that the ROS level in the H₂-treated HeLa cells was markedly reduced compared with that of the control group (P<0.01; Fig. 1D and E), which is consistent with the previously reported anti-oxidative stress activity of H₂ gas (10). In addition, the level of MDA, an indicator of lipid peroxidation, was also decreased in HeLa cells treated with H₂ (Fig. 1F), whereas an increased level in SOD, an important representative of antioxidant enzymes, was observed in the H₂ group compared with that of the controls (P<0.01; Fig. 1G). These results suggested...
that H₂ intervention induced HeLa cell apoptosis, inhibited cell proliferation and reduced the oxidative stress level.

Reduced tumor growth is observed in a H₂-induced xenograft in vivo model. A total of 1x10⁷ HeLa cells were subcutaneously injected into the left armpit of nude mice, and the tumor volume was recorded every 3 days starting 7 days post-injection (Fig. 2A). At days 31 and 34, the tumor volume in mice under high H₂ environment was significantly smaller than that in the control group (P<0.05 at day 31 and P<0.01 at day 34), which was consistent with the change in tumor weight on day 34 (P<0.01; Fig. 2B and C).

H&E staining revealed that the tumor cell death rate in the H₂ xenograft group was elevated, and was accompanied by cell swelling as well as nuclear fragmentation and disintegration (Fig. 2D; black arrows). In addition, H₂ treatment significantly increased the apoptosis rate and reduced the cell proliferation of HeLa cells (Fig. 2E and F). Taken together, these data suggested that a high-H₂ environment inhibited tumor growth in tumor-bearing mice.

Bulk RNA sequencing of H₂-treated HeLa cells and control cells. To investigate the potential mechanism by which H₂ inhibits tumor cell growth, whole-genome sequencing was performed (Fig. 3). As shown in Fig. S1A-C, 11 upregulated and 16 downregulated circular RNAs (circRNAs) were determined in H₂-treated HeLa cells through the HiSeq4000 platform. In addition, 2 long non-coding RNAs (lncRNAs) were detected to be upregulated, while 6 lncRNAs were downregulated in H₂-treated HeLa cells (Fig. S2A-C). A total of 12 hsa-microRNAs (miRNAs or miRs) were found to be upregulated, and 2 miRNAs to be downregulated, including hsa-miR-431-5p and hsa-miR-762 (Fig. S3A-C). Importantly, mRNA sequencing revealed that 10 mRNAs were upregulated in HeLa cells subjected to H₂ treatment, and 4 mRNAs were downregulated, including HIF1A, miR23B, miR4651 and RelA (Fig. 4A-C). miR23B and miR4651 are non-coding RNAs, whereas HIF1A and RelA are the coding genes for HIF-1α and NF-κB p65 subunit, respectively. These results suggested that the mRNA levels of HIF-1α and NF-κB p65 subunit were reduced in H₂-treated HeLa cells compared with those of the controls.

GO enrichment analysis for HeLa cells with H₂ intervention. To further confirm the aforementioned gene regulation in H₂-treated HeLa cells, GO enrichment analysis was performed. As shown in Fig. 5A, the biological process (BP), cellular component (CC) and molecular function (MF) were analyzed. ‘Translation’ and ‘NF-κB signaling’ were the two most dominant processes in BP, and gene regulation mainly occurs in the nucleus and non-membrane-bounded organelles. MF analysis revealed that ‘DNA and RNA binding’ were dominant. GO
enrichment scatter plot further indicated that the processes of ‘NF-κB signaling’, ‘protein transport’ and ‘multicellular organismal movement’ are enriched in H₂-treated HeLa cells (Fig. 5B). These results suggested that downregulation of the NF-κB signaling pathway may be an important target for H₂ intervention in HeLa cells.

HIF-1α and NF-κB p65 expression levels are downregulated in H₂-treated HeLa cells in vivo and in vitro. As the p65 subunit is a classic active type of the NF-κB family (28), the present study verified the expression levels of NF-κB p65 and HIF-1α in HeLa cells. As expected, western blot analysis demonstrated that the HIF-1α and NF-κB p65 protein levels were significantly reduced in H₂-treated HeLa cells compared with those in control HeLa cells (Fig. 6A-C). RT-qPCR analysis revealed that the mRNA expression levels of HIF-1α and NF-κB p65 (HIF1A and RelA) were elevated in HeLa cells (Fig. 6D and E).

The levels of HIF-1α and NF-κB p65 were then determined in HeLa cell-derived tumors. As shown in Fig. 6F-H, IHC demonstrated that the expression of HIF-1α and NF-κB p65 in H₂-treated HeLa cells was significantly reduced compared with that of the controls. Furthermore, the mRNA levels in vivo were also investigated, and the results revealed a significant decrease in HIF1A and RelA expression in H₂-treated tumors compared with that of control tumors (Fig. 6I and J). These results indicated that the expression levels of HIF-1α and NF-κB p65 were downregulated both in HeLa cells and in HeLa tumors.

Discussion

Cervical cancer is the fourth most common type of cancer in women worldwide, and ~70% of cervical cancer cases are caused by human papillomavirus (HPV)16 and 18 infections (29). HPVs are diverse at the level of genotype and pathogenicity, and can be classified into mucosal or cutaneous according to their epithelial tropism (30). The mucosal high-risk HPV16 and HPV18 are primarily associated with squamous intraepithelial lesions, which may progress to invasive squamous cell carcinoma such as cervical cancer (31). The pathogenic effects of HPV can be avoided by preventive measures, mainly vaccination. Although the currently available immunization methods have been shown to be effective in decreasing cervical cancer, genital warts and anogenital dysplasia, the global vaccination rates remain low, and more effective alternative medical treatments are therefore needed (32).

The present study first determined the pro-apoptotic and anti-proliferative effects of H₂ treatment in HeLa cells. A H₂ concentration of 33.3% significantly increased the cell apoptosis and reduced the cell proliferation of HeLa cells but not those of non-tumor HaCaT cells. Furthermore, a high concentration of H₂ further inhibited HeLa cell proliferation and promoted cell apoptosis. These data reveal that H₂ has a dose-dependent role on cervical cancer HeLa cell suppression. Importantly, the increase in HeLa cell apoptotic rate induced by 5 μg/ml cisplatin treatment (IC₅₀=12 μg/ml) is similar to the effect of 66.7% H₂ treatment, suggesting that H₂ therapy may
be a novel therapy for cervical cancer with less toxicity and efficacy comparable to chemotherapy.

Unexpectedly, H₂ intervention reduced the oxidative stress levels, including decreased ROS and MDA concentrations, and increased SOD levels, which appeared to have the opposite effect on tumor cell suppression. The tumor inhibitory effect of H₂ was then verified in HeLa tumor-bearing mice. The tumor volume at days 31 and 34 post-injection in tumor-bearing mice with continuous H₂ treatment was significantly reduced compared with that of controls, and decreased tumor weight and cell proliferation as well as increased tumor cell apoptosis were also observed at day 34 post-injection. To explore the specific mechanisms of H₂ on HeLa cell suppression, high-throughput RNA sequencing, including cirRNA, lncRNA, miRNA and mRNA sequencing, was performed. In cervical cancer, as well as in multiple other cancer types, short, non-coding single strands of RNAs, including circRNA, lncRNA and miRNA, play a vital role, since their deregulation has been widely reported (33-35). For example, miRNA may modulate oncogenic viral gene expression, as well as the

Figure 4. Highly deregulated mRNAs in HeLa cells with or without H₂ intervention. (A) A mRNA heatmap was generated based on the 10 maximum and 10 minimum FC values (n=3 per group). (B) Volcano plot for deregulated mRNAs [log2(FC) > 1 or < -1, adjusted P<0.05] between H₂ and control groups. The area above the gray horizontal solid line indicates P<0.05. (C) Upregulated mRNAs (red) and downregulated mRNAs (aqua blue) are listed. H₂, hydrogen; FC, fold-change.
gene expression of the host (36). Specifically, miRNA genes may be located at the susceptible sites in the amplified or deleted genome/regions in human tumors (37). Furthermore, as miRNAs are associated with the regulation of cell proliferation and apoptosis, changes in their expression may be responsible for proliferative disease, including cancer (38).
The present study revealed that 11 circRNAs, 2 lncRNAs and 12 miRNAs were upregulated, and 16 circRNAs, 6 lncRNAs and 2 miRNAs were downregulated in H2-treated HeLa cells. These observations suggest that non-coding RNAs may account for the tumor inhibitory roles of H2 on HeLa cervical cancer cells, but the specific mechanism needs to be further explored.

In addition, RNA sequencing demonstrated the changes in expression of several genes closely associated with tumor development, such as HIF1A and RelA, which encode HIF-1α and NF-κB p65 subunit, respectively. GO analysis further indicated that NF-κB signaling was involved in the inhibitory mechanism of H2-treated HeLa cells. Declined expression of HIF1A and RelA and their translated HIF-1α and NF-κB p65 proteins was then confirmed by RT-qPCR, western blotting and IHC both in vitro and in vivo. p65 subunit is a classic active type of the NF-κB family (28). It has been reported that NF-κB p65 is a molecular lynchpin that links chronic infection and inflammation with elevated cancer risk (39). NF-κB is persistently activated in multiple types of cancer and exerts a variety of pro-tumorigenic functions. For example, NF-κB activation in cancer cells usually leads to the elevation of anti-apoptotic genes to provide a cell survival mechanism for resisting the physiological stress that induces inflammation (40). Furthermore, NF-κB stimulates cytokines that regulate the immune response and inflammation, including TNF-α, IL-1, IL-6 and IL-8, as well as adhesion molecules, which result in the recruitment of leukocytes to inflammatory sites (28,40). In addition, NF-κB signaling was shown to modulate various other conserved cellular processes, including...
cell proliferation (41,42) and apoptosis (43). H2 intervention in HeLa cells and tumors was demonstrated to reduce both NF-kB p65 protein and mRNA levels in the RNA-sequencing and molecular biology experiments of the present study, suggesting a decrease in inflammatory response and tumor growth.

In cancer, hypoxia is considered as a vital characteristic of the tumor microenvironment that drives tumor progression (44,45). Hypoxic adaptation is largely modulated by a group of transcriptional regulators named hypoxia-inducible factors (HIFs) (46). HIF is a heterotrimeric complex consisting of an O2-regulated α-subunit and an O2-independent β-subunit (also called aryl hydrocarbon receptor nuclear translocator) (47). There are three HIF-α proteins recognized in humans: HIF-1α, -2α and -3α. Under normal O2 conditions, HIF-α subunits are tightly modulated by a series of enzymes called HIF prolyl hydroxylases (PHDs). PHD is a non-heme Fe (II)- and 2-oxoglutarate-dependent dioxygenase that hydroxylates HIF-α subunits at specific prolyl residues. The hydroxylated HIF-α subunits are identified by the von-Hippel Lindau tumor-suppressor E3 ligase and degraded via the proteasome pathway (46,47). Intratumoral hypoxia induces both HIF-1 and HIF-2 activation, and overexpression of HIF-1α is strongly associated with elevated metastasis and mortality in numerous human cancer types (45,48), including cervical cancer (49). Upregulated HIF-1α in these cancer types results in a series of regulatory processes leading to tumor progression, including migration, invasion, angiogenesis, glucose metabolism and pH regulation (44,45,50,51).

The present study demonstrated that both HIF1A (HIF-1α gene) and HIF-1α protein levels were reduced in H2-treated HeLa cells and HeLa tumors, which is important for elucidating the potential mechanisms of H2 gas on cervical cancer treatment. Notably, the reduced oxidative stress observed in HeLa cells may contradict the antitumor effects of H2. However, since ROS are essential in the stabilization of HIF that maintains the transcription of genes involved in tumor development (52), this downward trend of oxidative stress may be considered as an antitumor effect in general. Additionally, since HeLa cells are infected by HPV18 and its mechanism is associated with the regulation of oxidative stress (53), it could be hypothesized that H2 intervention may also regulate the HPV18 genome in HeLa cells, thereby exerting a tumor-suppressor effect. Future research on this topic needs to be conducted.

The present study also has several limitations. As one of numerous cervical cancer cell lines, experiments solely on HeLa cell lines cannot determine whether all types of cervical cancer cells exhibit a consistent effect after H2 intervention, and therefore additional cell types need to be further evaluated. Furthermore, the growth characteristics of cervical cancer cells is affected in a subcutaneous tumor model in the present study, therefore the effects of H2 treatment are different from what would be seen in human cervical cancer. The establishment of an in situ cervical cancer model in follow-up experiments is therefore needed. In addition, the specific mechanism of the inhibitory effect of H2 on HIF-1α and NF-kB (such as the regulation of the transcriptional levels of HIF1A and RelA) needs to be verified in further research. Also, the lack of negative and positive controls in this study may lead to a deviation in IHC results to a certain extent.

In conclusion, the present study suggests a novel H2-induced tumor suppression target towards HIF-1α and NF-kB. Inhibition of HIF-1α and NF-kB reduces cervical cancer HeLa cell proliferation and oxidative stress level, and decreases tumor growth, which makes H2 therapy a potential target in the treatment of cervical cancer.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive repository (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA718006), with accession number: PRJNA718006.

Authors’ contributions
JC, XL, and ZJ conceived and designed the research. JC, JG, JW, LL and GC performed the in vitro and in vivo experiments. JD and ZW performed the RNA sequencing and analyzed the data. JC, JG, and XL were responsible for data analysis and writing of the manuscript. All the authors listed have approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Ethics Committee for Animal Studies of Naval Medical University (approval number: 202027).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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