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Original Article

Long noncoding RNA MIR210HG is induced by hypoxia-inducible factor 1α and promotes cervical cancer progression

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Abstract: Increasing evidence has indicated that long noncoding RNAs (lncRNAs) play essential roles in various types of cancer, especially the ability of tumor cells to adapt to hypoxia conditions. However, only a few of them have been experimentally validated in cervical squamous cell carcinoma (CSCC). In the current study, we identified a hypoxia-induced lncRNA MIR210HG was excessively expressed in CSCC tissues and regulated by human papillomavirus (HPV) type 16 E6 and E7 via hypoxia-inducible factor 1α (HIF-1α). Functional assays revealed the role of MIR210HG in promoting proliferation, migration and invasion of CSCC cells in vitro under normoxia as well as hypoxia conditions. Meanwhile, stable MIR210HG silencing dramatically repressed tumor growth and pulmonary metastasis in vivo. Mechanistically, the depletion of MIR210HG or HIF-1α decreased each other's expression level, while silencing MIR210HG or HIF-1α respectively downregulated the expression levels of phosphoglycerate kinase 1 (PGK1), one of key metabolic enzymes in the glycolysis pathway. Furthermore, decreased expression of PGK1 by HIF-1α knockdown was reversed through the overexpression of MIR210HG. Also, we demonstrated HIF-1α can activate the transcription of MIR210HG via binding its promoter. Taken together, these results expand our understanding of the cancer-associated functions of hypoxia-induced lncRNAs, and highlight MIR210HG forms a feedback loop with HIF-1α contributing to cervical carcinogenesis, with potential implications for therapeutic targeting.

Keywords: Cervical squamous cell carcinoma, long noncoding RNA, MIR210HG, hypoxia, HIF-1α

Introduction

Cervical cancer is one of the main causes of death in females. Although screening and vaccination programs have been expanded, the number of new cases of cervical cancer has continued to increase, which means that cervical cancer is still a major public health concern [1]. Mortality rates in low-income developing countries and regions are vastly different from those in developed countries, with an 18-fold difference in mortality and 85% of deaths occurring in underdeveloped countries due to limited treatment options and economic and cultural factors [2]. At present, the conventional treatment of cervical cancer includes radiotherapy, chemotherapy and surgery, but patients at advanced stages are prone to developing radiotherapy and chemotherapy resistance [3]. Therefore, it is urgently necessary to identify new therapeutic targets for cervical cancer.

Hypoxia is a hallmark of the solid tumor microenvironment. It induces genomic instability, which in turn helps cancer cells respond adaptively to meet the needs of carcinogenesis, cancer progression and relapse [4]. Considering that hundreds of protein-coding genes are transactivated by HIF-1 under hypoxic conditions, whether lncRNAs could be responsible for hypoxia and their regulatory functions are

far from clear [5]. Choudhry et al. [6] performed the integrated genomic analyses of both non-coding and coding transcripts in hypoxic cells, and indicated that noncoding RNA was also hypoxia-inducible. Since then, dysregulated lncRNAs targeted by HIF-1 in several types of cancers have been investigated [7, 8]. However, there are still some unknown lncRNAs which may be the potential molecules in response to hypoxia and their functional mechanism in tumorigenesis need to be further elucidated.

Interestingly, increasing evidence suggests that lncRNAs are closely implicated in regulating HIF-1α activity. For example, Xiang et al. [9] proposed that c-Myc-mediated repression of lncRNA IDH1-AS1 sustains activation of the Warburg effect by HIF-1α under normoxic conditions. LncRNA FEZF1-AS1 promotes pancreatic cancer cell proliferation and invasion, through miR-142/HIF-1α axis under hypoxic condition while through miR-133a/EGFR axis under normoxic condition [10]. Furthermore, Hua et al. [11] found that lncRNA AC020978 promote the nuclear translocation of PKM2 and regulate PKM2-enhanced HIF-1α transcription activity. However, few studies have explored the link between hypoxia and MIR210HG in CSCC.

In the current study, we identified a hypoxia-inducible lncRNA, MIR210HG, which contributes to cervical cancer progression. Further study demonstrated that HPV16 E6 and E7 could regulate the expression level of MIR210HG by modulating transcription factor HIF-1α. Meanwhile, we found that HIF-1α knockdown could inhibit the transcription of MIR210HG and vice versa, silencing HIF-1α or MIR210HG could respectively downregulate PGK1 expression. In addition, HIF-1α can activate the transcription of MIR210HG via binding its promoter. Taken together, these findings indicate the functions of MIR210HG in CSCC progression and uncover the positive feedback loop of HIF-1α/MIR210HG which subsequently regulate the expression of PGK1 in CSCC, and this axis may serve as a potential target for cancer therapy.

Materials and methods

Tissue specimens

Total of twenty-one CSCC and eighteen normal cervical epithelial (NCE) tissues used in this study were collected at Nanfang Hospital from September 2019 to September 2020. Informed consent was obtained from all patients. No patients had been treated with radiotherapy or chemotherapy before surgery and all specimens preserved in sample protector for RNA/DNA (Takara, Japan) at -80°C. This study was approved by the Ethics Committee of Southern Medical University.

Cell culture

The human cervical squamous cancer cell lines CaSki, SiHa and HEK293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L D-glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) and incubated at 37°C in the humidified incubator with 5% CO_2.

Cell transfection

The small interfering RNAs (siRNAs) separately targeting HPV16 E6 (si-E6), HPV16 E7 (si-E7), MIR210HG (si-MIR210HG), HIF-1α (si-HIF-1α), PGK1 (si-PGK1) and a scrambled oligonucleotide control were purchased from GenePharma (Shanghai, China). The sequences of siRNA used for RNA interference are listed in Table S1. The short hairpin RNA (shRNA) vector specific to MIR210HG (sh-MIR210HG) and a scrambled negative control vector were obtained from Genechem (Shanghai, China). To construct an overexpression plasmid, the full-length of MIR210HG (NR_038262) was synthesized and cloned into the pcDNA3.1 vector by Genechem (Shanghai, China).

Cell transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). The stably shRNA transfected cells were screened under G418 (Genview) pressure. Cells were harvested for further analyses 24-72 h after transfection.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from the cells or tissues using RNAiso Plus reagent (Takara) following the manufacture's protocol. The concentration and purity of extracted RNA were assessed by NanoDrop 2000 (Thermo). The reverse transcription reactions were performed by using a PrimeScript RT reagent kit (RR047A, TaKaRa). qRT-PCRs were performed with a SYBR Premix

Ex Taq kit (RR420A, TaKaRa) on a ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Gene expression levels relative to β-actin were calculated by $2^{-\Delta\Delta CT}$ method. All the primer sequences used in this study are listed in Table S2.

CCK-8 proliferation assay

After transfection, CaSki and SiHa cells in the logarithmic growth phase were seeded into 96-well plates at a density of 3×10^3 cells per well and incubated for 1.5 h at 37°C and 5% CO_2 before the optical density (OD) at 450 nm was detected. Proliferation was measured by the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) every 24 h after transfection for 4 days.

Transwell assay

For migration and invasion assays, transwell assay was conducted using 24-well transwell chamber pre-coated with or without Matrigel (BD). Treated CaSki and SiHa cells (4×10^4/well) in serum-free DMEM medium were added to the upper chamber and 20% fetal bovine serum medium was added in the lower chamber. Cells were cultured at 37°C with 5% CO_2 for 24 h and then those on the lower surface were fixed with methanol and stained with 0.1% crystal violet. Cells were counted in five randomly selected areas under microscope field.

Western blot

Total cell lysates were prepared using immunoprecipitation assay lysis buffer containing protease and phosphorylase inhibitors and clarified by centrifugation (12000 g for 15 min at 4°C). The protein concentration was detected by a BCA Protein Assay Kit (Beyotime). Proteins were mixed with loading buffer, then cell lysates were separated by sodium dodecyl sulphate SDS-PAGE and transferred onto polyvinylidene difluoride membranes, which were subsequently blocked in 5% skimmed milk for 2 h. Afterwards, primary antibodies against E6 (ab70, Abcam), E7 (ab20191, Abcam), HIF-1α (36169S, CST), PGK1 (17811-1-AP, Proteintech) were incubated overnight at 4°C. The next day, the membrane was incubated with second antibody at room temperature for 2 h, washed with TBST and then developed with the ECL system and normalized to the gray value of β-actin.

Isolation of nuclear and cytoplasmic RNA

Cells were partitioned into nuclear and cytoplasmic fractions using the PARIS™ Kit (Invitrogen) according to the manufacturer's instructions. RNA isolated from each of the fractions was analyzed by qRT-PCR to determine the expression levels of MIR210HG, nuclear control transcript (MALAT1) and cytoplasmic control transcript (GAPDH).

Luciferase reporter assay

The length of MIR210HG promoter region containing two kilobase (kb) was constructed into pGL3-based vectors. To determine the effect of HIF-1α on MIR210HG promoter, pGL3-based construct containing MIR210HG promoter sequences and renilla luciferase reporter plasmid were cotransfected into HEK293T cells with or without HIF-1α knockdown. Cultured under normoxia or 250 μM $CoCl_2$ 24 h after transfection, firefly and renilla luciferase activity were measured by dual-luciferase reporter assay kits (Beyotime). The ratio of firefly luciferase to renilla activity was calculated for each sample.

Xenografted tumor and pulmonary metastasis model in vivo

All animal experiments were approved by the Institutional Committee on Animal Care and Use of Southern Medical University. The female nude mice between 4 and 5 weeks were purchased from Beijing HFK Bioscience Co., Ltd. SiHa cells transfected with the negative control vector or the sh-MIR210HG vector were paired, 1×10^7 cells were inoculated subcutaneously into either side of flank of the same female nude mouse and 1×10^6 cells were injected intravenously into tail. The tumor volumes were measured every week and calculated as Length × Width^2 × 0.5. After 5 weeks, the mice were euthanized after injection and the tumors were fixed for hematoxylin-eosin (HE) and immunohistochemistry (IHC) staining.

Statistical analysis

For the cell functional analyses, results are presented as mean ± standard deviation (SD). The comparison of means between two groups was conducted using Student's t test, while one-way analysis of variance was used for multiple comparisons. Correlation was calculated according to Pearson correlation. $P < 0.05$ is considered

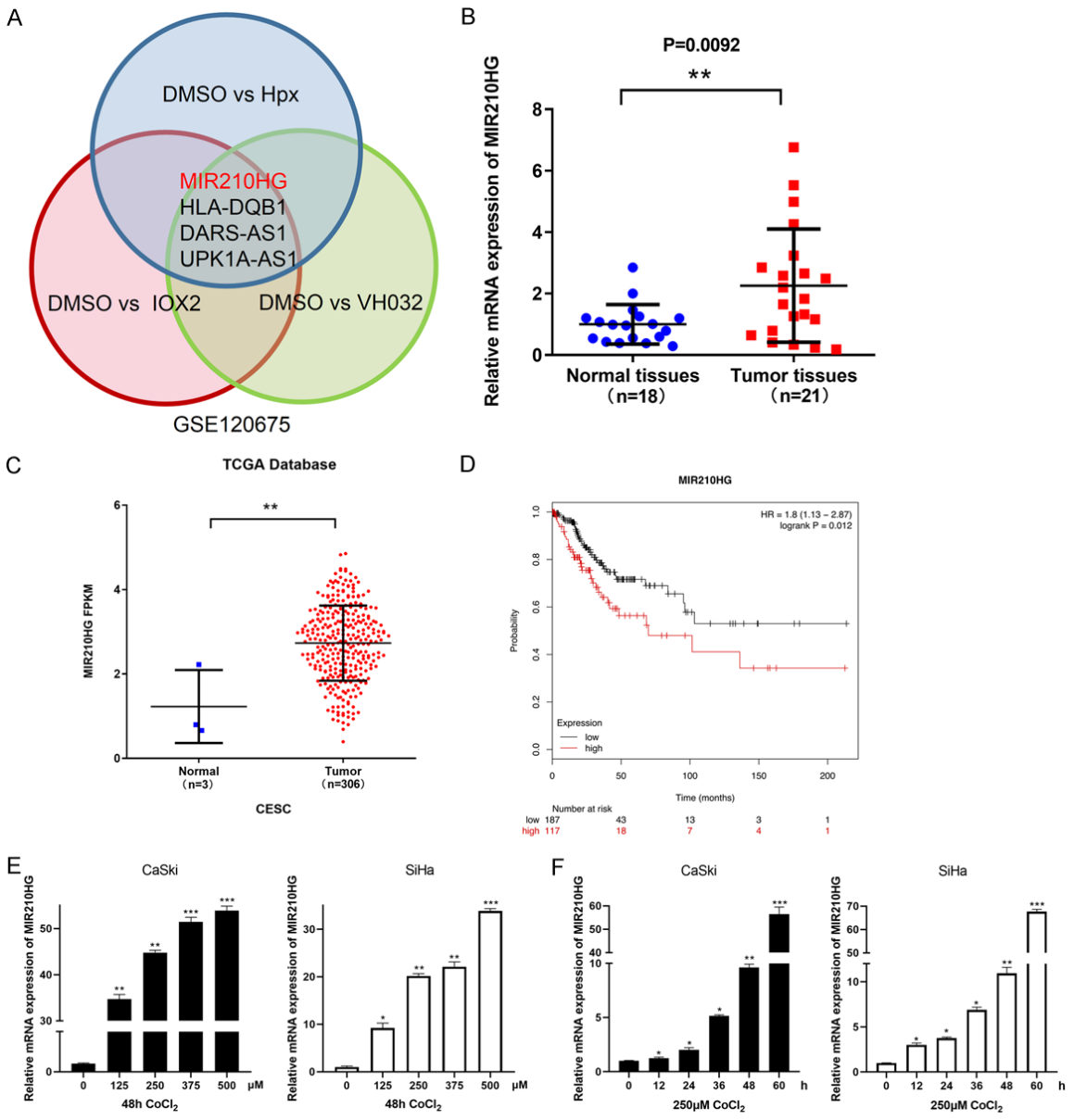

Figure 1. MIR210HG was a hypoxia-inducible and aberrantly upregulated lncRNA in CC. A. The Venn diagrams of the GEO dataset (GSE120675) revealed that four genes were identified that overlapped under different treatments of hypoxic pathway inhibition. B. qRT-PCR analysis showed MIR210HG was upregulated in CSCC tissues compared with normal cervical epithelial tissues. C. The expression level of MIR210HG in the TCGA database. D. Overall survival (OS) of high- and low-expression MIR210HG patients determined using the Kaplan-Meier Plotter website (http://kmplot.com/analysis/). E, F. MIR210HG were measured by qRT-PCR in samples derived from cells subjected to dose- and time- dependent hypoxia treatment. The asterisk (*) indicates $P < 0.05$.

significant. All the experiments were repeated at least three times.

Results

MIR210HG was a hypoxia-inducible and aberrantly upregulated lncRNA in CSCC

To uncover the function of noncoding transcripts under physiological conditions such as hypoxia, we explored the gene expression profiles of cervical cancer cell in response to hypoxic pathway inhibition from Gene Expression Omnibus (GEO) database (GSE120675). The results provided the expected hypoxic coding signature and four overlapping lncRNAs (**Figure 1A**). Among them, MIR210HG was one of notably overexpressed lncRNAs in HPV-positive CSCC compared to HPV-negative nor-

mal controls from our previous studies (unpublished data). qRT-PCR analysis found that MIR210HG was up-regulated in 21 CSCC tissues as compared to 18 normal tissues (**Figure 1B**). In the TCGA database, MIR210HG was highly expressed in the cervical cancer tissues (**Figure 1C**). The Kaplan-Meier survival analysis revealed that patients with high MIR210HG expression levels had a shorter overall survival time (**Figure 1D**). Cobalt (II) chloride ($CoCl_2$), as a prolyl hydroxylase (PHD) inhibitor, is a commonly used hypoxia mimetic agent in establishing experimental hypoxia. Interestingly, MIR210HG was significantly upregulated exposed to hypoxia mimetic $CoCl_2$ in a dose- and duration-dependent manner by qRT-PCR analysis (**Figure 1E** and **1F**). In summary, we identified MIR210HG as a hypoxia inducible lncRNA related with CSCC.

Downregulation of MIR210HG inhibits the proliferation, migration and invasion of CSCC cells

To explore the biological function of MIR210HG in CSCC cells, we performed CCK-8 and transwell assays to evaluate the effect of MIR210HG on the proliferation, migration and invasion of CSCC cells. Next, we transfected si-MIR210HG into CaSki and SiHa cells, finding that MIR210HG expression was significantly silenced after the transfection. The knockdown efficiency of MIR210HG in CSCC cells was checked by qRT-PCR assay (**Figure 2A**). Subsequently, CCK-8 assay was implemented in CSCC cells to detect cell proliferation. As a result, MIR210HG knockdown significantly suppressed the proliferation of CSCC cells (**Figure 2B** and **2C**) under normoxia or treatment of $CoCl_2$. We also performed transwell assays under normoxic and hypoxic conditions. Consistently, downregulation of MIR210HG significantly inhibited the migration and invasion capacities of CSCC cells under hypoxic conditions (**Figure 2D**).

Upregulation of MIR210HG promotes the proliferation, migration and invasion of CSCC cells

On the other hand, we also upregulated the expression of MIR210HG in CSCC cells. MIR210HG overexpression pcDNA3.1 vector were used to establish gain-of-function cell models in CaSki and SiHa cells. As shown in **Figure 3A**, MIR210HG was significantly increased after the transfection. The proliferative ability was then analyzed by CCK-8 assay and the results showed that the proliferation ability of CSCC cells was significantly enhanced under normoxia or treatment of $CoCl_2$ (**Figure 3B** and **3C**). The migration and invasion abilities were detected by transwell assay and the results indicated that upregulation of MIR210HG remarkably enhanced the migration and invasion capacities of CSCC cells under normoxic and hypoxic conditions (**Figure 3D**). Taken together, these results strongly suggested that MIR210HG promotes the proliferation, migration and invasion of CSCC cells.

MIR210HG could be modulated by HPV16 E6/E7 through HIF-1α

Our previous data indicated that MIR210HG was one of remarkably overexpressed lncRNAs in HPV-positive CSCC compared to HPV-negative normal cervical epithelial tissues, we next conducted qRT-PCR analysis to determine whether HPV16 E6 and E7 could affect the expression of MIR210HG. As expected, MIR210HG was downregulated after HPV16 E6 or E7 silencing, while upregulated after HPV16 E6 or E7 overexpression (**Figure 4A** and **4B**). Considering that HPV16 E6 and E7 oncoproteins have been demonstrated to induce the expression of HIF-1α in CC cells [12] and MIR210HG was a hypoxia-inducible lncRNA, we speculated whether E6 and E7 could regulate MIR210HG via HIF-1α. Further studies revealed that HIF-1α protein level was suppressed by E6 or E7 repression (**Figure 4C**), and MIR210HG upregulation by HPV16 E6 or E7 overexpression could be abolished by HIF-1α depletion (**Figure 4D**). Consequently, these findings indicated HPV16 E6 and E7 might enhance the expression of MIR210HG partially by modulating HIF-1α.

MIR210HG forms a positive feedback loop with HIF-1α to promote the expression of PGK1

To elucidate the potential underlying mechanisms of MIR210HG in tumorigenesis, Gene Set Enrichment analysis (GSEA) was performed in cervical cancer cohort from TCGA database. Surprisingly, GSEA analysis revealed that high MIR210HG expression was positively correlated with “GLYCOLYSIS” and “HYPOXIA” pathway (**Figure 5A**), and we confirmed that MIR210HG knockdown dramatically inhibited mRNA expression level of HIF-1α and its downstream

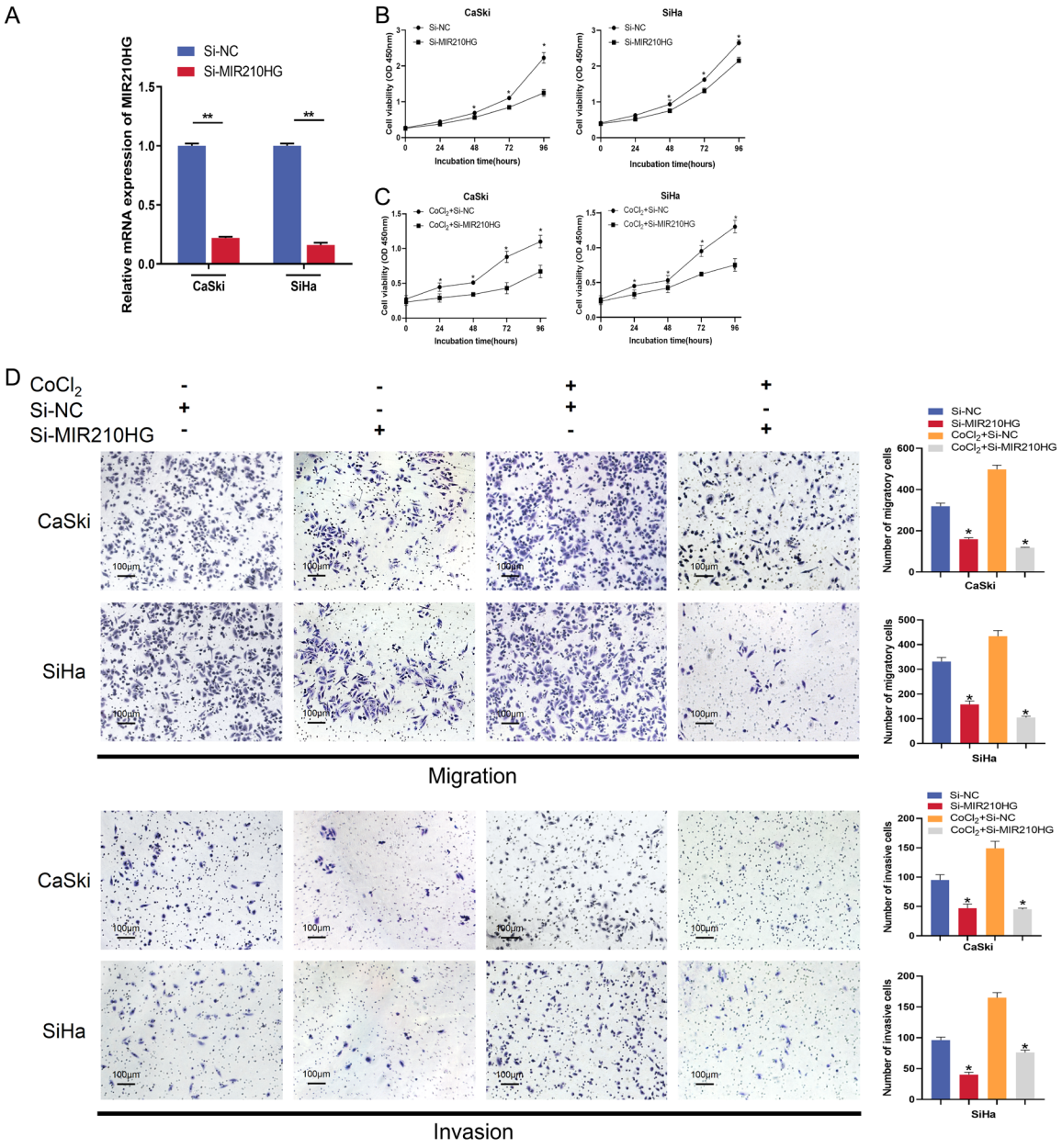

Figure 2. Knockdown of MIR210HG inhibited CSCC cells proliferation, migration and invasion in vitro under treatment of $CoCl_2$. A. qRT-PCR was conducted to examine the efficiency of si-MIR210HG delivering into the CaSki and SiHa cells. B, C. Cell Counting Kit-8 assays showed that suppressing MIR210HG significantly reduced cell proliferation in comparison with the control under normoxia or chemical hypoxia conditions. D. In transwell assay, the cell migration and invasion ability of CaSki and SiHa cells were notably increased after treatment of $CoCl_2$. MIR210HG knockdown could abolish the effect of $CoCl_2$ treatment on cell migration and invasion. The asterisk (*) indicates $P < 0.05$. Scale bar represents 100 μm.

gene PGK1 under normoxia or treatment of $CoCl_2$ (**Figure 5B**). However, HIF-1α reduction could in turn diminish the RNA level of MIR-210HG and PGK1, suggesting a positive feedback loop between MIR210HG and HIF-1α (**Figure 5C**). Subsequently, we observed that depletion of MIR210HG expression could notably decrease HIF-1α and PGK1 protein level, which was in line with the qRT-PCR results (**Figure 5D**). Moreover, the expression of HIF-1α in CaSki and SiHa cells was significantly elevated in accordance with upregulated PGK1 expression during hypoxia condition (**Figure 5E**). qPCR analysis of nuclear and cytoplasmic

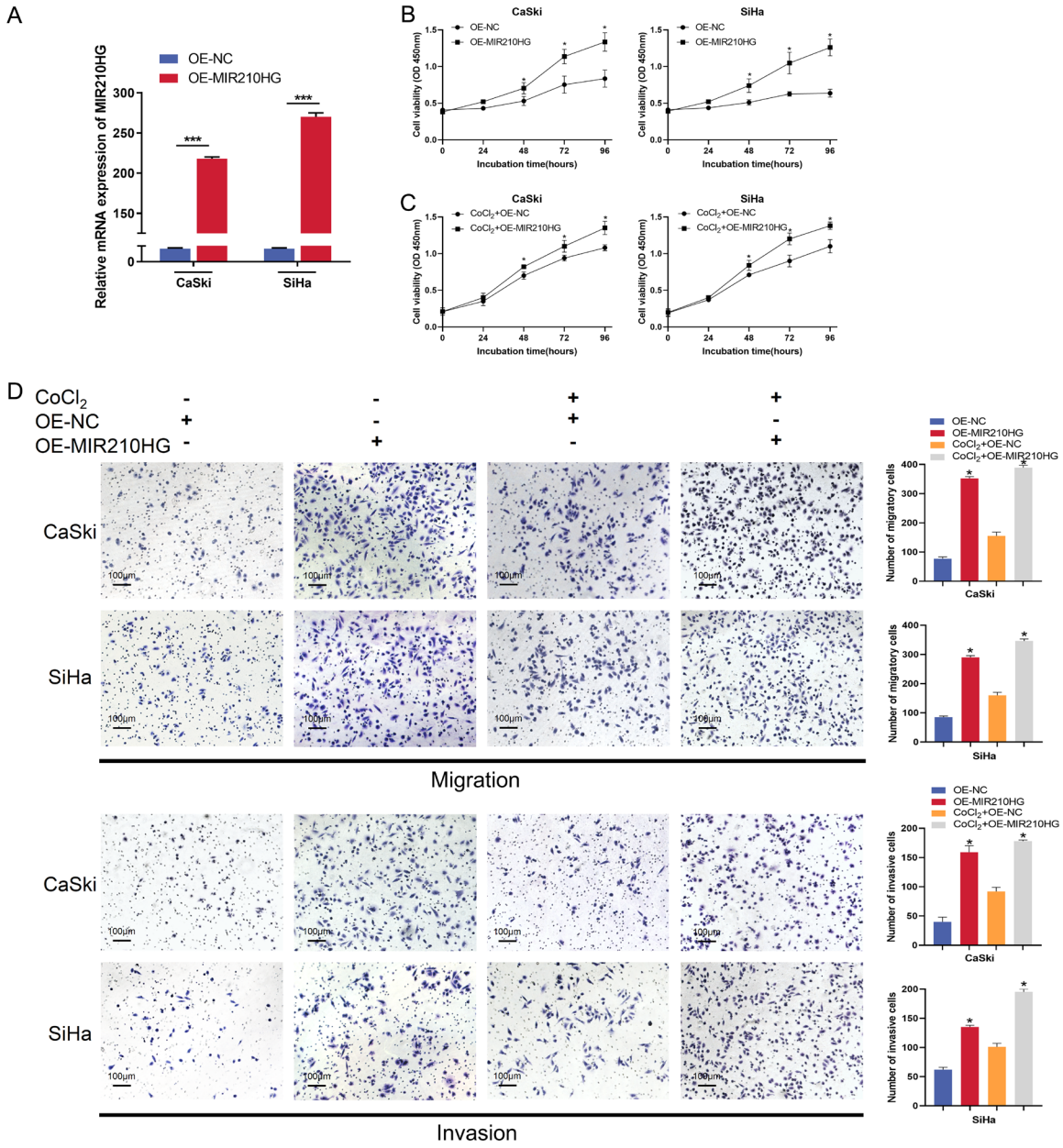

Figure 3. Overexpression of MIR210HG promoted CSCC cells proliferation, migration and invasion in vitro under treatment of $CoCl_2$. A. qRT-PCR was conducted to examine the efficiency of OE-MIR210HG delivering into the CaSki and SiHa cells. B, C. Cell Counting Kit-8 assays showed that MIR210HG overexpression dramatically promoted cervical cancer cell proliferation in comparison with the control under normoxia or chemical hypoxia conditions. D. MIR210HG overexpression could increase the impact of the treatment of $CoCl_2$ on the migration and invasion ability of CaSki and SiHa cells. The asterisk (*) indicates $P < 0.05$. Scale bar represents 100 μm.

lncRNA showed that MIR210HG was mainly detected in the nucleus of CaSki and SiHa cells, indicating that MIR210HG might exert its functions in nucleus (**Figure 5F**). To further validate the ability of HIF-1α to transactivate MIR210HG, the promoter region of MIR210HG were inserted into firefly luciferase reporter plasmid. Luciferase assay showed that HIF-1α knockdown significantly suppressed the luciferase activity in both normoxia and chemical hypoxia conditions. Additionally, the expression of MIR210HG is positively correlated with HIF-1α in CSCC tissues (**Figure 5H**). Collectively, our results strongly suggested that the existence positive feedback loop between MIR-210HG and HIF-1α, and HIF-1α activates the

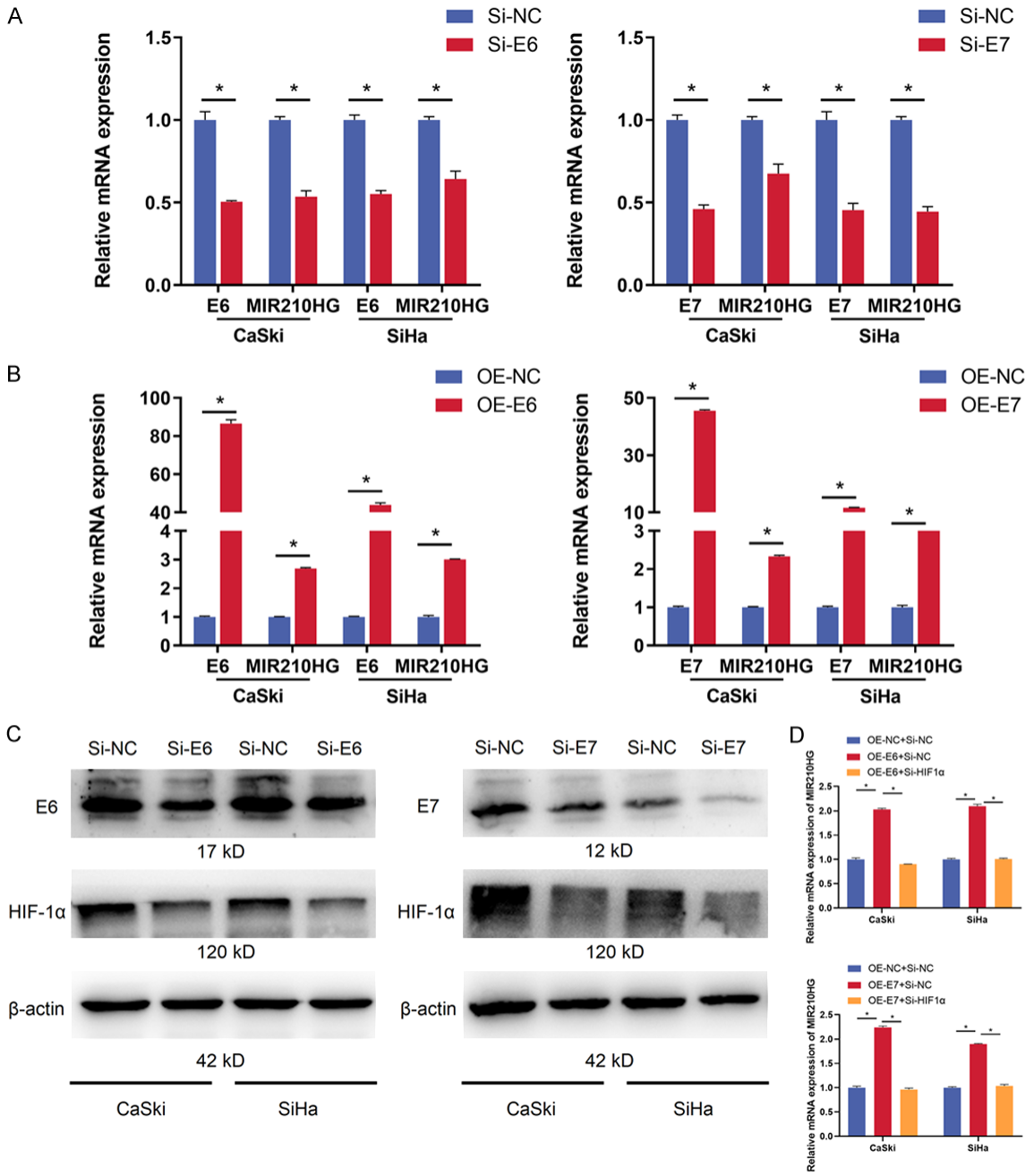

Figure 4. MIR210HG could be modulated by HPV16 E6/E7 through HIF-1α. A, B. The expression levels of MIR210HG were modulated by HPV16 E6 or E7 by qRT-PCR analysis. C. Western blot analysis showed the effect of HPV16 E6 or E7 knockdown on the expression of HIF-1α. D. qRT-PCR analysis revealed that enhanced expression of MIR210HG after overexpression of HPV16 E6 or E7 could be reversed by HIF-1α knockdown. The asterisk (*) indicates $P < 0.05$.

transcription of MIR210HG via binding its promoter.

MIR210HG promotes CSCC cells migration and invasion via PGK1

Considering that MIR210HG forms a positive feedback loop with HIF-1α to affect expression of PGK1, we next examined whether MIR210HG or HIF-1α synergistically regulate the expression of PGK1. The results showed that co-transfection of si-HIF1α and OE-MIR210HG greatly rescued the mRNA and protein expression of PGK1 in CSCC cells caused by only knockdown of HIF-1α under normoxia or chemical hypoxia conditions (**Figure 6A-C**). Then, further experi-

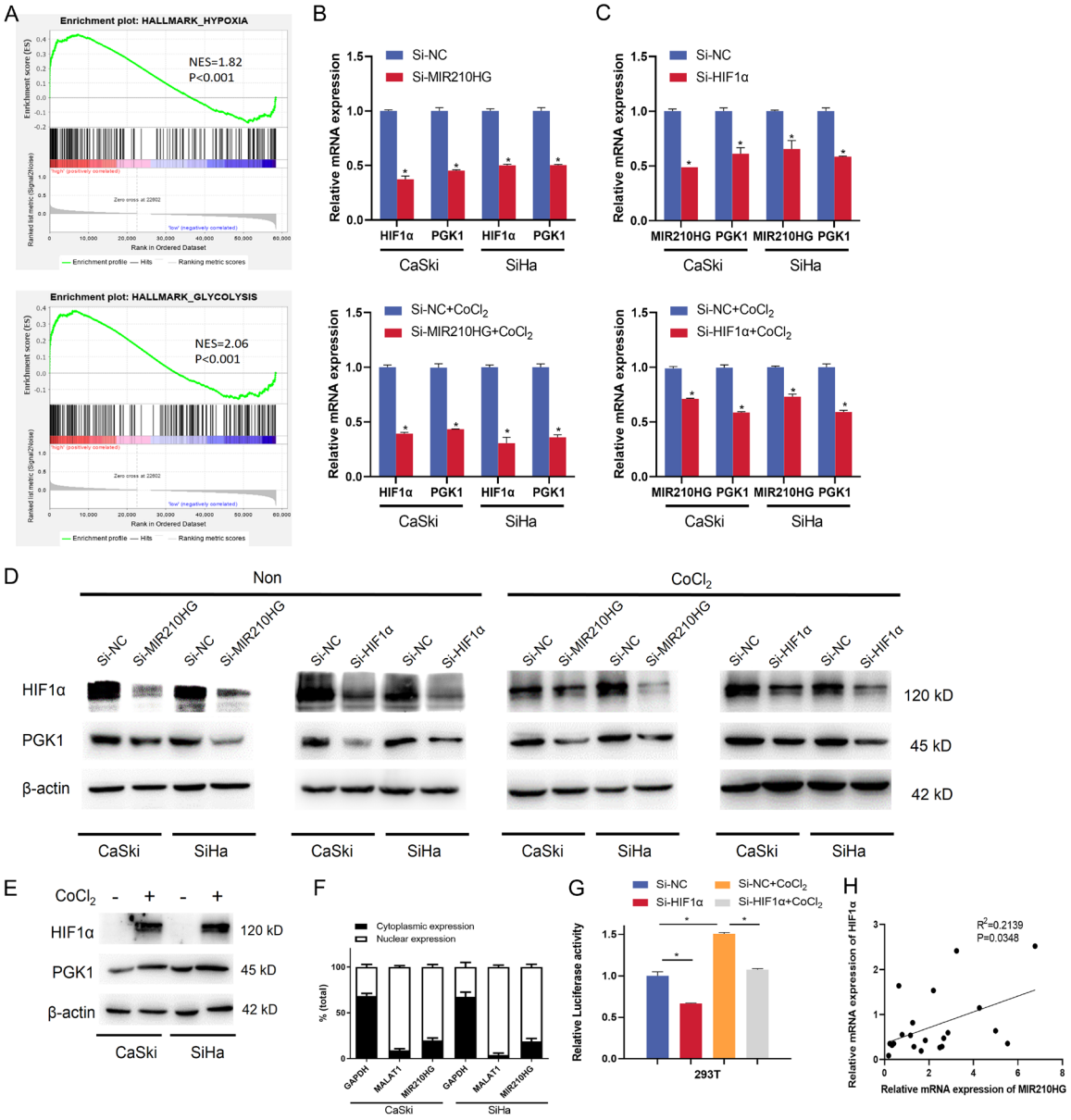

Figure 5. MIR210HG forms a positive feedback loop with HIF-1α to promote the expression of PGK1. A. Gene Set Enrichment Analysis (GSEA) indicated that "GLYCOLYSIS" and "HYPOXIA" pathways were significantly associated with high expression of MIR210HG. B. qRT-PCR was used to verify the expression of HIF-1α and PGK1 after transfected with si-MIR210HG under normoxia or chemical inducer $CoCl_2$. C. qRT-PCR was used to validate the expression of MIR210HG and PGK1 after transfected with Si-HIF1α under normoxia or chemical inducer $CoCl_2$. D. Western blot analysis of PGK1 in CSCC cells after transfected with si-MIR210HG or si-HIF1α under normoxia or chemical inducer $CoCl_2$. E. Western blot analysis of HIF-1α and PGK1 in CSCC cells under normoxia or chemical inducer $CoCl_2$. F. MIR210HG subcellular distributions in CaSki and SiHa cells were determined by qRT-PCR. G. Luciferase reporter assay was used to determine the effect of HIF-1α knockdown on MIR210HG promoter under normoxia or chemical inducer $CoCl_2$. H. Pearson correlation analysis showed a positive relationship between MIR210HG and HIF-1α in cervical cancer tissues. The asterisk (*) indicates $P < 0.05$.

ments were carried out to verify whether regulation work of MIR210HG on cell migration and invasion was related with PGK1. The efficiency of silencing PGK1 was validated by western blot (**Figure 6D**). As expected, the results of transwell assay indicated that suppression of PGK1 apparently abrogated the enhanced effect of MIR210HG upregulation on cell migration and

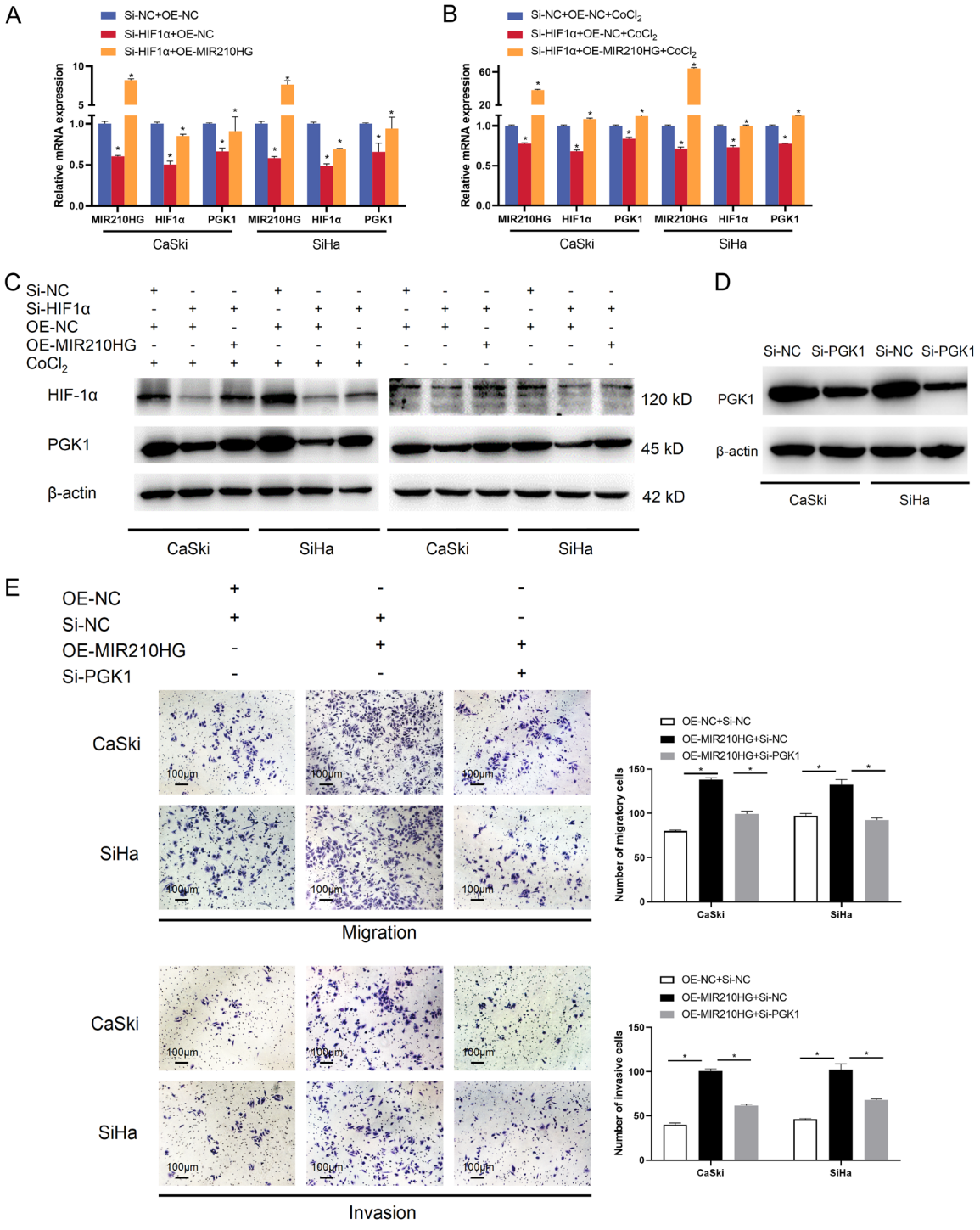

Figure 6. MIR210HG regulated cell migration and invasion via PGK1 in CaSki and SiHa cells. A-C. qRT-PCR and western blot analysis showed that the protein and mRNA expression of PGK1 was decreased after HIF-1α knockdown, and the mRNA and protein expression of PGK1 was reversed after MIR210HG overexpression under normoxia or $CoCl_2$ treatment. D. The efficiency of silencing PGK1 was confirmed by western blot. E. PGK1 knockdown could abolish the increased cell migration and invasion capacity caused by overexpression of MIR210HG. The asterisk (*) indicates $P < 0.05$. Scale bar represents 100 μm.

invasion (**Figure 6E**). Taken together, PGK1 was a crucial downstream target of MIR210HG in the regulation of migration and invasion in CSCC cells.

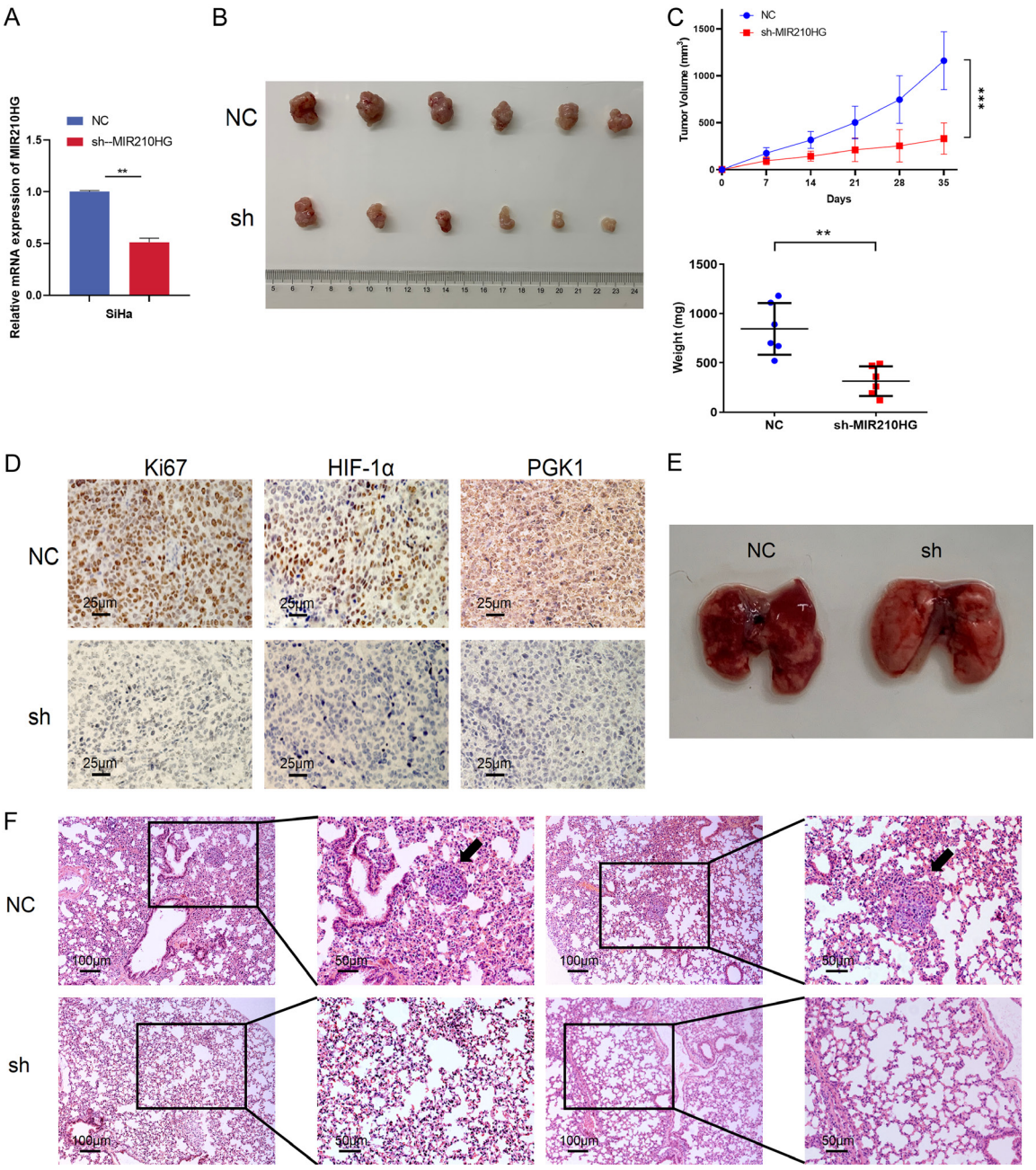

Figure 7. Inhibition of MIR210HG repressed CSCC cells tumorigenesis and pulmonary metastasis in vivo. A. qRT-PCR indicated suppression of the expression of MIR210HG by sh transfection compared to the control group. B, C. Tumor volume and weight of each group were measured at the time of the injection. On day 35, the mice were sacrificed. The tumors were monitored every 7 days and were obtained at Day 35. D. Expression of Ki67, HIF-1α and PGK1 in the xenografts were detected by IHC. E. Effects of MIR210HG knockdown on tumor metastasis in nude mice models. F. H&E analysis of histologic features in NC and sh group was shown in pulmonary metastasis model. The asterisk (*) indicates $P < 0.05$. Scale bar represents 25 or 50 or 100 μm.

Silencing of MIR210HG impairs xenograft tumor growth and pulmonary metastasis in vivo

To further investigate the oncogenic role of MIR210HG, SiHa cells stably transfected with sh-MIR210HG or NC were used to establish xenograft model and pulmonary metastasis model in vivo. The infection efficacy was confirmed in **Figure 7A**. After cell injection for 5 weeks, tumor volume and weight were signifi-

cantly reduced in sh-MIR210HG group in contrast to that in sh-NC group (**Figure 7B** and **7C**). Meanwhile, compared with sh-NC group, sh-MIR210HG transformed cells had the milder capacity for pulmonary metastasis (**Figure 7E** and **7F**). Immunohistochemical analysis further revealed a significant downregulation of Ki-67, HIF-1α and PGK1 in tumor tissues of sh-MIR210HG group compared with that in sh-NC group (**Figure 7D**). These results supported the function of MIR210HG in promoting proliferation and metastatic capacity in vivo.

Discussion

Although the complex biological functions of lncRNAs are still largely unknown, accumulating evidence has shown that lncRNAs contribute to the initiation and development of various cancers by acting as oncogenic or tumor suppressive regulators [13]. MIR210HG has been reported to play oncogenic functions in cervical cancer [14], non-small cell lung cancer (NSCLC) [15], hepatocellular carcinoma [16] and so on. Majority of these researches revealed that MIR210HG could act as a competing endogenous RNA (ceRNA) in tumorigenesis. For example, Wang et al. [14] demonstrated that it promoted CC progression through regulating the miR-503-5p/TRAF4 axis. However, the role and mechanism of MIR210HG in cervical cancer have not fully been elucidated.

Adaptation of cancer cells to a hypoxic tumor microenvironment is of great importance for their malignant growth and distant metastasis [17]. Based on bioinformatics analysis using GEO database, four hypoxia-related lncRNAs (MIR210HG, HLA-DQB1, DARS-AS1 and UPK-1A-AS1) in cervical cancer were screened and DARS-AS1 has been identified as a hypoxia-induced lncRNA [18]. Interestingly, MIR210HG was remarkably upregulated in HPV-positive CSCC tissues, when compared to HPV-negative normal cervical epithelial tissues from our previous microarray data. Further investigation showed that MIR210HG was significantly upregulated in samples derived from cells subjected to dose- and time-dependent hypoxia treatment. And gain-and loss-of-function studies strongly suggested that MIR210HG could play a stimulative role in the tumor migration, invasion and metastasis. Recent studies also indicated the oncogenic function of MIR210HG in human cancers, which was consistent with our findings. For instance, MIR210HG was found to be highly expressed in NSCLC which promoted proliferation and migration of NSCLC cells by inhibiting CACNA2D2 via binding to DNMT1 [19]. Similarly, Bu et al. [20] suggested that MIR210HG is associated with NSCLC cell progression through regulating the miR-874/STAT3 axis. Meanwhile, Li et al. [21] reported that MIR210HG sponge miR-1226-3p to facilitate the invasion and metastasis of breast cancer cells by regulating mucin-1c and EMT pathway. Thus, our results provided important evidence that MIR210HG was highly expressed in CC and may be used as a potential biomarker.

Persistent infections of high-risk human papillomavirus (HR-HPV) is regarded as the most significant risk factor for cervical carcinogenesis. Until now, several lncRNAs including the metastasis associated lung adenocarcinoma transcript 1 (MALAT-1), thymopoietin pseudogene 2 (TMPOP2) and small nucleolar RNA host gene 12 (SNHG12) have been identified to be regulated by oncoproteins E6 or E7 [22-24]. However, the relationship between MIR210HG and HPV viral proteins has not been investigated. To explore whether the expression of MIR-210HG could be induced by HPV 16 E6 or E7, we transfected HPV16 E6- or E7-encoding plasmids and siRNAs targeting E6 or E7 into SiHa and Caski cells and found that both E6 and E7 stimulated MIR210HG expression, indicating the involvement of viral proteins in the regulation of MIR210HG in CC cells. Furthermore, the mechanism by which viral proteins promote the expression of MIR210HG has not been clarified. Subsequently, we performed the analysis of the promoter region of MIR210HG using the JASPAR core database and found the presence of putative hypoxia response elements. Interestingly, previous studies showed that E6 and E7 can induce HIF-1α protein accumulation [12]. Considering that MIR210HG was induced by hypoxia as aforementioned, we further provided the first evidence that hypoxia-induced MIR210HG is upregulated by HPV16 E6/E7 via HIF-1α. Luciferase reporter assays demonstrated that HIF-1α might bind directly to the MIR-210HG promoter region and activate its transcription. Our results were in line with the previous report suggesting that HIF-1α may interact directly with the promoter of MIR210HG in

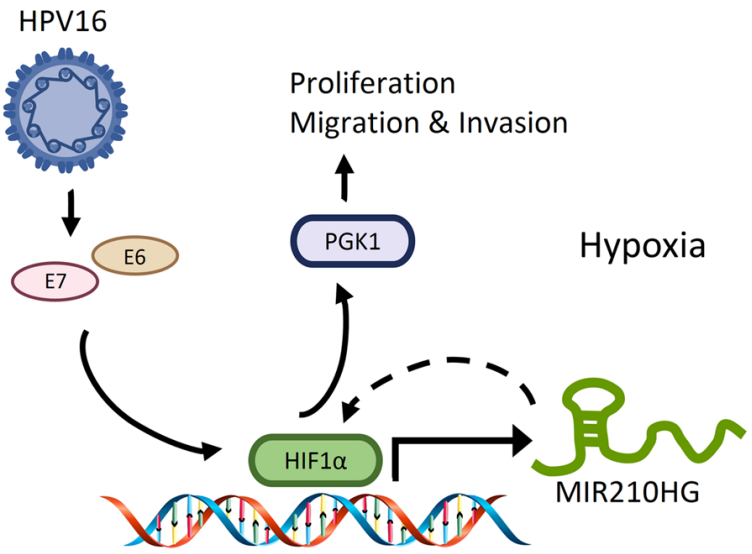

Figure 8. A schematic depicture of the mechanism underlying the role of MIR210HG in CSCC. Our results indicate that MIR210HG could be modulated by HPV16 E6/E7 through HIF-1α. Moreover, hypoxia-induced MIR210HG forms a positive feedback loop with HIF-1α to promote expression of PGK1, simultaneously HIF-1α binds the promoter of MIR210HG and activate it expression at transcriptional level. As a consequence, CSCC cells migration, invasion and glycolytic metabolism capabilities are enhanced.

Hs578T, MDA-MB-231 and HCC1937 cells by chromatin immunoprecipitation assay (ChIP) and qPCR [25]. Even so, further experiment such as ChIP are needed in the future. Additionally, it doesn't exclude other HPV oncoproteins (such as E5, E2) or viral factors participate in the regulation of MIR210HG in CC cells because we only investigated the relationship between HPV16 E6/E7 and MIR210HG.

In addition, silencing MIR210HG or HIF-1α respectively decreased the mutual expression level, implying that a positive feedback loop between MIR210HG and HIF-1α. Given its importance in sensing hypoxic tension, it is not surprising that cells utilize feedback mechanisms to precisely control HIF-1α signals. A recent study showed that HIF-1α binds the promoter region of LINC00511 to active its transcription and LINC00511 indirectly regulates the expression of HIF-1α through sponging miR-153-5p, forming a positive feedback loop of HIF-1α/LINC00511/miR-153-5p in CRC [26]. And lncRNA HITT was demonstrated to form a regulatory loop with HIF-1α to modulate angiogenesis and tumor growth [27]. In the present study, MIR210HG was primarily located in the nuclear portion, indicating that MIR-210HG might modulate the expression of HIF-1α in the nucleus. Interestingly, Wang et al. [28] showed that lncRNA PVT1 promotes nasopharyngeal carcinoma cell proliferation via a KAT2A/H3K9ac/TIF1β/NF90/HIF-1α signaling pathway. Therefore, future studies will clarify the exact mechanisms how MIR210HG engages the transcriptional and epigenetic machinery to regulate the HIF-1α expression. Our findings also showed that either MIR210HG or HIF-1α knockdown could attenuate the expression level of PGK1, a HIF-1α downstream target which acts as the first ATP-producing enzyme in glycolysis. Ectopic overexpression of MIR210HG could reverse the reduced PGK1 expression caused by HIF-1α silencing. Many studies suggested that PGK1 is highly expressed in various cancers, such as breast cancer [29], liver cancer [30], and colon cancer [31]. In particular, PGK1 has also been proved to be transactivated by some transcription factors including HIF-1α [32, 33]. Nevertheless, it remains unknown that the roles of PGK1 in MIR210HG-mediated cancer progression. Our data confirmed that PGK1 depletion notably reversed the migration and invasion capacities in MIR-210HG overexpression CSCC cells.

Conclusion

In conclusion, our findings indicate that MIR-210HG functions as an oncogenic lncRNA in CSCC and its high levels are associated with tumor progression and unfavourable prognosis of patients. The present study provides the first evidence that hypoxia-inducible MIR210HG is induced by HPV16 E6/E7 via transcription factor HIF-1α, and might act as a tumor promoter in CSCC by enhancing the expression of PGK1. Investigation of the molecular mechanism showed that existence of a positive feedback loop between MIR210HG and HIF-1α, while HIF-1α could directly bind to the MIR210HG promoter region and activate its transcription (**Figure 8**). Therefore, MIR210HG has now emerged as a

novel prognostic biomarker and a potential new target for CC treatment.

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Disclosure of conflict of interest

None.

Abbreviations

lncRNA, long non-coding RNA; CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; HIF-1α, hypoxia-inducible factor 1α; PGK1, phosphoglycerate kinase 1; MIR210HG, MIR210 host gene; GEO, Gene Expression Omnibus; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; $CoCl_2$, Cobalt (II) chloride PHD, prolyl hydroxylase; CCK-8, Cell Counting Kit-8; GSEA, Gene Set Enrichment analysis; NSCLC, non-small cell lung cancer; ceRNA, competing endogenous RNA; HR-HPV, high-risk human papillomavirus; MALAT-1, metastasis associated lung adenocarcinoma transcript 1; TMPOP2, thymopoietin pseudogene 2; SNHG12, small nucleolar RNA host gene 12; ChIP, Chromatin immunoprecipitation assay; NCE, normal cervical epithelial; siRNAs, small interfering RNAs.

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Table S1. siRNA used for RNA interference

Gene Symbol	Sense 5'-3'	Antisense 5'-3'
si-E6	GUUACCACAGUUAUGCACATT	UGUGCAUAACUGUGGUAACTT
si-E7	AGGAGGAUGAAAUAGAUGGTT	CCAUCUAUUUCAUCCUCCUTT
si-MIR210HG	CCCACUUGGCCUAUGCAUUTT	AAUGCAUAGGCCAAGUGGGTT
si-HIF1α	AACCAAGTAGCCTGTTATCAA	GATAACAGGCTACTTGGTTAA
si-PGK1	GCAUCAAAUUCUGCUUGGATT	UCCAAGCAGAAUUUGAUGCTT

Table S2. Primers used for qRT-PCR

Gene Symbol	Forward 5'-3'	Reverse 5'-3'
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
MIR210HG	GGGTTCTGGCTTGCTGACA	GCAACTCGGCTTGGTTATTTC
HPV16 E6	TGCACAGAGCTGCAAACAAC	AGCATATGGATTCCCATCTC
HPV16 E7	ATTAAATGACAGCTCAGAGGA	GCTTTGTACGCACAACCGAAGC
HIF1α	CACCACAGGACAGTACAGGAT	CGTGCTGAATAATACCACTCACA
PGK1	ATGTCGCTTTCTAACAAGCTGA	GCGGAGGTTCTCCAGCA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
MALAT1	GACGGAGGTTGAGATGAAGC	ATTCGGGGCTCTGTAGTCCT