

# The Curcumin Analog Da0324 Inhibits the Proliferation of Gastric Cancer Cells *Via* HOTAIRM1/miR-29b-1-5p/PHLPP1 Axis

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## Research article

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# Abstract

## Background

Our previous study has shown that Da0324, a curcumin analog, has significantly improved stability and antitumor activity. However, the molecular mechanisms of action of Da0324 remains poorly understood. Long non-coding RNA (lncRNA) has been certified to play a key role in tumor progression. Here, we aim to investigate the molecular mechanisms underlying the anti-cancer activities of Da0324 through regulation of lncRNA HOTAIRM1.

## Methods

Gastric cancer cell lines were treated with Da0324 and/or transfected with lentiviral vector expressing HOTAIRM1 shRNA, and/or miR-29b-1-5p mimics and/or small interference RNA (siRNA) against PHLPP1, or HOTAIRM1 siRNA or lentiviral vector expressing HOTAIRM1, as required. The expression of HOTAIRM1, miR-29b-1-5p and PHLPP1 in GC cells were determined by Real-Time PCR. Cell growth was examined by CCK-8 assay and colony formation assay in vitro. The targeted relationship between HOTAIRM1 and miR-29b-1-5p was verified by luciferase reporter gene assay. Western blot was utilized to investigate PHLPP1 protein expression levels.

## Results

Da0324 increased the expression of HOTAIRM1 in GC cells. HOTAIRM1 expression was significantly down-regulated in GC tissues, and the low expression of HOTAIRM1 was associated with the shorter survival rate of GC patients based on TCGA database. Knockdown of HOTAIRM1 promoted GC cell proliferation whereas overexpression of HOTAIRM1 inhibited GC cell proliferation as detected by CCK-8 and colony formation assays. Moreover, knockdown of HOTAIRM1 reversed the Da0324-mediated growth inhibition of GC cells. Furthermore, HOTAIRM1 acted as a sponge for miR-29b-1-5p and PHLPP1 is regulated by the HOTAIRM1/miR-29b-1-5p axis in GC cells. Overexpression of miR-29b-1-5p or knockdown of PHLPP1 reversed the ability of Da0324 to inhibit the growth of GC cells.

## Conclusions

Our data suggest that Da0324 exerts antitumor activity by regulating HOTAIRM1/miR-29b-1-5p/PHLPP1 axis in GC cell, and provide new insights into the anti-cancer mechanism of Da0324.

## Background

Gastric cancer (GC) is one of the most common cancers in the world (Bray et al. 2018). It is the second most common cancer in China and the third most frequent cause contributing to cancer-related

deaths(Bray et al. 2018; Chen et al. 2016; Siegel et al. 2019). Because of the lack of specific symptoms, most patients with GC are diagnosed in the advanced stages at the time of diagnosis, resulting in poor prognosis(Saka et al. 2011; Van Cutsem and Ducreux 2016; Van Cutsem et al. 2016). Although advancements of surgical treatment and chemotherapy have improved the outcome of GC in recent decades, the prognosis is still unsatisfactory(Holohan et al. 2013; Liu et al. 2014; Wagner et al. 2006). Hence, more efforts are needed to search for new alternative therapies and improve the effectiveness of comprehensive treatment strategies in GC.

Because the severe side effects of chemotherapy affect the effect of cancer treatment(Lai et al. 2014; Zhao et al. 2010), more and more researches have focused on the anti-cancer properties of natural compounds, which can lead to the discovery of drugs that are more effective in cancer treatment and have lower side effects(Nobili et al. 2009). As an active component extracted from in the spice turmeric, curcumin is widely used as a colorant and spice in food(Farzaei et al. 2018). Curcumin has anti-oxidant, anti-inflammatory, anti-apoptotic and anti-cancer properties, so a growing number of researchers pay attention to it(Benzer et al. 2018; Divya and Pillai 2006). Due to the shortcomings of using curcumin as an anticancer agent, such as low water solubility, which limits oral bioavailability, scientists have adopted a lot of strategies to overcome the existing deficiencies(Shetty et al. 2014). One strategy to address poor aqueous solubility is to use nanoparticle technology to improve bioavailability and cellular uptake(Allam et al. 2015; Chen et al. 2018; Vecchione et al. 2016). In our preceding studies, the strategy to overcome the defects was to synthesize the curcumin analog Da0324, which exhibited excellent target selectivity by inhibiting the activation of NF- $\kappa$ B in GC cells and had low toxicity to normal gastric mucosal epithelial cells(Jin et al. 2016). Da0324 also activated P53 by down-regulating LINC01021 to exert anti-tumor activity against GC(Xu et al. 2020). Nevertheless, there are still knowledge gaps in understanding of the molecular mechanisms involved in Da0324 cytotoxicity to GC. Therefore, it is very important to investigate the molecular mechanism of Da0324 inhibiting gastric cancer.

So far, more and more evidences show that non-coding RNAs (ncRNAs) as important regulators are involved in various physiological and pathological cellular processes(Chen et al. 2016; Kondo et al. 2017). Long non-coding RNA (lncRNA) is a ribonucleotide chain, a group of ncRNAs with a length of more than 200 nucleotides(Beermann et al. 2016). There is ample evidence to support that lncRNA plays an important role in regulating cancer cell proliferation, apoptosis and metastasis(Chen et al. 2018; Wang et al. 2018). One of the functional mechanisms of lncRNAs is to act as competitive endogenous RNAs (ceRNAs), which compete for microRNAs (miRNAs) binding to increase the expression of miRNAs-targeted mRNAs(Song et al. 2019). LncRNA HOTAIRM1 is reported to play an important role in the progression of cancer(Li et al. 2020; Lu et al. 2019). Li et al. reported that lncRNA HOTAIRM1 acted as a ceRNA of miR-107, to regulate proliferation and invasion in papillary thyroid cancer(Li et al. 2020). A recent study also indicated that HOTAIRM1 was found to be downregulated in GC, which regulated the development of GC through miR-17-5p, while the upregulation of HOTAIRM1 was shown to inhibit the proliferation and migration of GC(Lu et al. 2019).

Here, we aimed to investigate the molecular mechanisms underlying the anti-cancer activities of Da0324 through regulation of HOTAIRM1. Collectively, our results revealed that Da0324 treatment upregulated HOTAIRM1, which increased PHLPP1 expression by sponging miR-29b-1-5p in GC cells.

## Materials And Methods

### Cell culture

A normal human gastric mucosa epithelial cell line (GES-1), a human embryonic kidney 293T cell line (HEK-293T), and human gastric cancer cell lines (AGS, and KATO III) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). BGC823 and SGC7901 gastric cancer cell lines were obtained from the China Center for Type Culture Collection (Wuhan, China). AGS cells were grown in F-12K medium (Thermo Fisher Scientific, Waltham, MA, USA), HEK-293T cells were cultured in Dulbecco modified Eagle medium (DMEM) (Thermo Fisher Scientific) and the other cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific). All media were supplemented with 10% fetal calf serum (Sigma, Germany), 100 U/ml penicillin and 10 mg/L streptomycin (Thermo Fisher Scientific). All cells were cultured at 37°C under a humidified atmosphere with 5% CO<sub>2</sub>.

### High-throughput sequencing of lncRNAs

The high-throughput sequencing assay was carried out as previously described. (Xu et al. 2020)

### Cell transfection

The lentiviral vector expressing short hairpin RNA (shRNA) specific to HOTAIRM1 (sh-HOTAIRM1 target sequence: 5'-AGAAACTCCGTGTTACTCA-3') was purchased from Genechem Co. (Shanghai, China). The nonspecific shRNAs were utilized in control group (sh-NC). The full-length sequence of HOTAIRM1 was inserted into pLVX-Puro (Clontech, Mountain View, CA, USA), with empty vectors as the control. Lentivirus were produced as described previously. Besides, small interference RNA (siRNA) against HOTAIRM1 (si-HOTAIRM1-1 sequence: 5'-AGAAACTCCGTGTTACTCA-3'; si-HOTAIRM1-2 sequence: 5'-GCCAGAAACCAGCCATAGT-3')<sup>(Chen et al. 2017)</sup>, siRNA against PHLPP1 (5'-GGAAGACGCUCUUCUGAA-3'), miR-29b-1-5p mimics, and the corresponding negative control (NC) were designed and synthesized by RiboBio Biotech (Guangzhou, China). The transfections were carried out using the Lipofectamine 3000 Reagent (Thermo Fisher Scientific) following the instructions of manufacturer.

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from cultured cells was extracted using TRIzol Reagent (Thermo Fisher Scientific). For lncRNA expression analysis, the cDNA was synthesized by using the InRcute lncRNA First-Strand cDNA Synthesis Kit (Tiangen, China). For miRNA, reverse transcription was performed by using miRNA First Strand cDNA Synthesis (Stem-loop Method) (Sangon Biotech, China). qRT-PCR was performed using SYBR Premix Ex Taq (Takara, Japan) on CFX96 Real-time PCR system (Bio-Rad, Berkeley, CA, USA). GAPDH and U6 were used as internal controls, respectively. The relative gene expression level was calculated using the 2<sup>-ΔΔCt</sup>

method. The primers used in this study were obtained from Sangon Biotech (Shanghai, China) and listed as follows, HOTAIRM1-F: 5'-CCCACCGTTCAATGAAAG-3', HOTAIRM1-R: 5'-GTTTCA AACACCCACATTTC-3'; miR-29b-1-5p-F: 5'-CGGCTGGTTTCATATGGTGG-3', miR-29b-1-5p-R 5'-AGTGCAGGGTCCGAGGTATT-3'; GAPDH-F: 5'-GTCAAG GCTGAGAACGGGAA-3', GAPDH-R: 5'-AAATGAGCCCCAGCCTTCTC-3'; U6-F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', U6-R: 5'-CGCTTCACGAATTTGCGTGTTCAT-3'.

## Subcellular fractionation

The nucleus and cytoplasm were separated using the PARIS™ Kit (Thermo Fisher Scientific). According to the manufacturer's instructions, cells were suspended in cell separation buffer. Then, RNA was extracted and qRT-PCR analysis was conducted to detect the location of HOTAIRM1. GAPDH and U6 served as control transcripts for cytoplasmic and nuclear RNA, respectively.

## Cell counting kit-8 (CCK-8) assay

CCK-8 assay (Dojindo, Japan) was used to evaluate cell viability. Cells (5000/well) were seeded in a 96-well plates, 10 μL of CCK-8 solution was added into each well at indicated time point and incubated for 2 h. The absorbance at 450 nm was measured using microplate reader.

## Plate colony-formation assay

The transfected or Da0324-treated cells were cultivated in six-well plates at a density of 800 cells/well and incubated at 37 °C for 2 weeks. After that, the cells were fixed with 4% paraformaldehyde, stained with crystal violet, and counted under a microscope.

## Dual-luciferase activity assay

The binding site of miR-29b-1-5p to HOTAIRM1 was predicted using IncRNAMap (<http://IncRNAMap.mbc.nctu.edu.tw/>) or TargetScan 7.2 (<http://www.targetscan.org/>), respectively. Luciferase plasmid containing wild-type (pmirGLO-HOTAIRM1-WT) putative miR-29b-1-5p binding sites in the HOTAIRM1 sequence was generated. To detect binding between HOTAIRM1 and miR-29b-1-5p, pmirGLO-HOTAIRM1-WT reporter plasmid was co-transfected with miR-NC or miR-29b-1-5p mimics into HEK-293T cells using the Lipofectamine 3000 reagent (Thermo Fisher Scientific). After transfection for 48 h, the luciferase activity was evaluated through the luciferase reporter assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla luciferase activity was used as control.

## Western blotting

In brief, cells were lysed in RIPA buffer supplemented with phenylmethylsulfonyl fluoride, phosphatase inhibitor and protease inhibitor to obtain total protein. Subsequently, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and shifted to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by 5% nonfat milk for 2 h and then probed with primary antibody against PHLPP1 (Affinity, China) and GAPDH (Cell Signaling Technology) at 1:1000 dilution incubated at 4°C overnight. GAPDH was used as an internal control. The membranes were incubated with appropriate secondary antibody (1:5000, Santa Cruz Biotechnology) for 1 h at room

temperature. Finally, the results were analyzed using the ECL reagent (Thermo Fisher Scientific) and visualized using an imaging system (Bio-Rad).

## Statistical analysis

Data were analyzed using GraphPad Prism 7.0 (GraphPad Prism, Inc., La Jolla, CA, USA), and the results are presented as the mean  $\pm$  standard deviation (SD). Student's t-test was used to compare differences between two groups. The differences among multi-group were analyzed with One-way analysis of variance. A value of  $P < 0.05$  was considered significant.

## Results

### Da0324 inhibits the growth of GC cells and up-regulates the expression of HOTAIRM1

Figure 1A shows that Da0324 treatment significantly inhibits GC cells growth. We have previously reported that the expression levels of various lncRNAs were detected in Da0324-treated SGC7901 cells by high-throughput sequencing (Xu et al. 2020). Interestingly, Da0324 treatment resulted in up-regulation of HOTAIRM1 expression in GC cell lines (Fig. 1B). The results of qRT-PCR analyses verified that HOTAIRM1 levels increased in response to Da0324 treatment (Fig. 1C). Using lncRNA expression data from TCGA, we found that HOTAIRM1 was significantly down-regulated in GC relative to normal samples (Fig. 1D), and GC patients with low expression of HOTAIRM1 had poorer overall survival than patients with high expression of HOTAIRM1 (Fig. 1E). We also analyzed the expression level of HOTAIRM1 in the normal human gastric epithelial GES-1 cell line and GC cell lines by qRT-PCR. As revealed in Fig. 1F, the expression of HOTAIRM1 in KATO III and AGS cells was lower than that of GES-1 cells. Furthermore, in order to better understand the function of HOTAIRM1, we analyzed its subcellular localization in the nucleus and cytoplasm. Our results revealed that HOTAIRM1 was mainly localized in the cytoplasm of KATO III and SGC7901 cells (Fig. 1G). This indicates that HOTAIRM1 may play ceRNA function in GC.

### HOTAIRM1 knockdown facilitates GC cell proliferation

Because the expression of HOTAIRM1 was relatively high in BGC823 and SGC7901 cells, two specific siRNAs targeting HOTAIRM1 (si-HOTAIRM1-1 and si-HOTAIRM1-2) were designed and transfected into BGC823 and SGC7901 cells to investigate the effects of HOTAIRM1 knockdown on GC cell growth. As shown in Fig. 2A, si-HOTAIRM1-1 and si-HOTAIRM1-2 effectively knocked down the expression of HOTAIRM1 in GC cells. Next, CCK-8 assays demonstrated that knockdown of HOTAIRM1 increased cell viability in BGC823 and SGC7901 cells (Fig. 2B). Since si-HOTAIRM1-1 possessed the higher knockdown efficiency, we constructed the lentiviral vector expressing HOTAIRM1 shRNA according to the sequence of si-HOTAIRM1-1. The knockdown efficiency of shHOTAIRM1 was verified in BGC823 and SGC7901 cells by qRT-PCR (Fig. 2C). The CCK-8 assays demonstrated that the viability of BGC823 and SGC7901 cells was

increased after silencing HOTAIRM1 (Fig. 2D and 2E). Similarly, knockdown of HOTAIRM1 promoted the cell colony formation in BGC823 and SGC7901 cells (Fig. 2F and 2G).

## **Overexpression of HOTAIRM1 inhibits gastric cancer cell growth**

To investigate the functional role of HOTAIRM1 in GC cells, we performed gain-of-function experiments. qRT-PCR results showed that HOTAIRM1 was overexpressed in BGC823 and KATO III cells transfected with pLVX-HOTAIRM1 (Fig. 3A). The results of the CCK-8 assay showed that overexpression of HOTAIRM1 suppressed the growth of BGC823 and KATO III cells (Fig. 3B and 3C). The plate colony formation assay also suggested that overexpression of HOTAIRM1 inhibited the colony formation of BGC823 and KATO III cells (Fig. 3D and 3E). These results demonstrated that HOTAIRM1 could inhibit the proliferation of GC cells.

## **Knockdown of HOTAIRM1 alleviate Da0324-induced cytotoxicity in GC cells**

We used HOTAIRM1-silenced BGC823 and SGC7901 cells to evaluate the biological function of HOTAIRM1 in Da0324-induced cytotoxicity *in vitro*. As shown in Fig. 4A and 4B, the results of CCK-8 assays showed that Da0324 treatment markedly reduced GC cell viability and the knockdown of HOTAIRM1 partly reversed the growth inhibition induced by Da0324. In addition, a similar pattern was observed in the plate colony formation assays (Fig. 4C).

## **HOTAIRM1 acts as a sponge for miR-29b-1-5p in GC cells**

To research the effect of HOTAIRM1 on the expression of miRNAs, we used the lncRNAMap online database to predict the miRNAs that interacted with HOTAIRM1. The bioinformatics analysis indicated that miR-29b-1-5p shared complementary bonds in the HOTAIRM1 sequence (Figs. 5A). To further validate the interaction between HOTAIRM1 and miR-29b-1-5p, luciferase reporter constructs were generated. The results of luciferase reporter gene assay showed that miR-29b-1-5p mimic decreased the luciferase activity of the HOTAIRM1 luciferase reporter vector compared with negative control (Fig. 5B). Moreover, miR-29b-1-5p expression was significantly decreased in SGC7901 cells with Da0324 treatment relative to untreated control cells (Fig. 5C), whereas silencing of HOTAIRM1 expression increased miR-29b-1-5p level in SGC7901 cells (Fig. 5D). All these results indicated that Da0324 up-regulated the expression of HOTAIRM1 and HOTAIRM1 could sponge miR-29b-1-5p to suppress its expression.

## **miR-29b-1-5p regulates the Da0324-induced growth inhibition of GC cells**

The role of miR-29b-1-5p in the growth of GC cells was then examined. BGC823 and SGC7901 cells were transfected with miR-29b-1-5p mimics or the corresponding NC and performed the CCK-8 assay to verify

whether miR-29b-1-5p is related to the growth of GC cells. Compared with the NC group, the overexpression of miR-29b-1-5p increased the cell growth (Fig. 5A and 5B). To understand whether miR-29b-1-5p is involved in Da0324 inhibiting the growth of GC cells, BGC823 and SGC7901 cells transfected with miR-29b-1-5p mimics or negative control were treated with Da0324. As shown in Fig. 5C and 5D, overexpression of miR-29b-1-5p reversed the Da0324-induced growth inhibition in BGC823 and SGC7901 cells. Similarly, the results of clone formation assays showed that Da0324 significantly attenuated proliferation of BGC823 and SGC7901 cells; however, these effects could be reversed following transfection with miR-29b-1-5p mimics (Fig. 5E). These results implied that Da0324 inhibited the growth of GC cells may be involved in the regulation of miR-29b-1-5p expression.

## **PHLPP1 is regulated by the HOTAIRM1/miR-29b-1-5p axis in GC cells**

It has been reported that PHLPP1 is a target of miR-29b-1-5p(Datta et al. 2018). Therefore, we investigated the role of PHLPP1 in Da0324 induced growth inhibition of GC cells. Western blot analysis showed that overexpression of miR-29b-1-5p significantly inhibited the protein expression of PHLPP1 in SGC7901 (Fig. 7A). Also, HOTAIRM1 silencing significantly suppressed the level of PHLPP1 protein (Fig. 7B), while administration of Da0324 increased the protein expression of PHLPP1 (Fig. 7C). These findings indicated that Da0324 up-regulated PHLPP1 expression by promotion of HOTAIRM1 and repression of miR-29b-1-5p. Moreover, PHLPP1 expression was significantly down-regulated in GC relative to normal samples based TCGA data (Fig. 7D). Knockdown of PHLPP1 significantly reversed the effect of Da0324 on GC cell growth (Fig. 7E and F). These results suggested that Da0324 inhibited the growth of GC cells *via* the HOTAIRM1/miR-29b-1-5p/PHLPP1 axis (Fig. 7G).

## **Discussion**

There has been an increasing interest in using natural compounds such as curcumin to treat cancer. More and more research on curcumin have demonstrated that it exerts anti-tumor effects by regulating various biological molecules such as cytokines, adhesion molecules, growth factors, and their receptors(Bachmeier et al. 2018). Experimental studies of curcumin have found that it can inhibit the tumor initiation, proliferation, metastasis and invasion of GC cells(Aggarwal et al. 2003). For example, curcumin inhibits proliferation and induces the autophagy and apoptosis in GC cells by activating the P53 signaling pathway and inhibiting the PI3K signaling pathway(Fu et al. 2018). The poor bioavailability and pharmacological kinetics of curcumin hinder its therapeutic potential. In our previous studies, to improve the above shortcomings, we have developed novel analogs of curcumin Da0324, which shows significantly improved stability and anti-cancer activity on GC cells(Jin et al. 2016; Xu et al. 2020). In this study, we further searched for the possible mechanisms, which may provide new ideas to support its fascinating anti-cancer effects.

As potential anticarcinogens, curcumin and its analogues have been explored for potential in regulating lncRNAs(Mishra et al. 2019). For example, curcumin treats glioma by regulating the negative feedback

loop of H19 / miR-675 / VDR(Pan et al. 2020). Furthermore, Yoshida et al. observed that curcumin reduced the resistance of pancreatic cancer cells to gemcitabine by inhibiting the expression of lncRNA PVT1(Yoshida et al. 2017). Here, we identified all statistically altered lncRNAs in GC cells induced by Da0324 with high-throughput sequencing. Notably, we found that HOTAIRM1 was up-regulated in GC cells by Da0324 treatment. HOTAIRM1 is specifically expressed in the myeloid lineage, and was initially identified as a myeloid-specific regulator of the HOXA gene family(Wang and Dostie 2017). It was reported to be involved in various cancers such as GC(Lu et al. 2019), colon cancer(Wan et al. 2016), glioblastoma multiforme(Li et al. 2018) and lung cancer(Tian et al. 2018). These previous findings indicate that HOTAIRM1 may play an important role in suppressing cancer. Here, we found that HOTAIRM1 transcription was activated by Da0324 in GC cells, which indicated that the anti-tumor effects of Da0324 may be caused by the up-regulation of HOTAIRM1. Our study showed that HOTAIRM1 silencing promoted GC cell proliferation whereas overexpression of HOTAIRM1 inhibited GC cell proliferation as detected by CCK-8 and colony formation assays. These findings show that HOTAIRM1 upregulation may be able to inhibit the development of GC, which is similar to the findings of Lu et al(Lu et al. 2019). In addition, functional assays indicated that knockdown of HOTAIRM1 could attenuate the inhibitory effect of Da0324 on GC cell proliferation *in vitro*, suggesting that Da0324 exerts its anti-cancer effects at least partly *via* upregulation of HOTAIRM1.

lncRNAs affect gene regulation through a variety of mechanisms. One of the mechanisms is that they function as ceRNAs and regulate gene expression by sponging corresponding miRNAs(Wang et al. 2017; Wu et al. 2017). In our study, subcellular localization experiments were performed to prove that the expression of HOTAIRM1 was significantly higher in the cytoplasm fraction of GC cells, which supported the potential of HOTAIRM1 in functioning as a ceRNA. In addition, bioinformatics analysis and luciferase reporter assay proved that miR-29b-1-5p was the downstream target of HOTAIRM1. Kim et al. demonstrated that miR-29b-1-5p, acting as a GC development promoter, regulated GC cell migration by targeting CREBZF(Kim et al. 2020). Additionally, it has been reported that the lower expression of miR-29b-1-5p reduced the IC50 of human breast cancer parental cell line MCF-7 selected at 500 nM Adriamycin, and the higher expression of miR-29b-1-5p weakened the effects of liposomal curcumin to Adriamycin-resistance(Zhou et al. 2017). We found that Da0324 treatment down-regulated the expression of miR-29b-1-5p, while knockdown of HOTAIRM1 up-regulated miR-29b-1-5p expression. Also, the manipulation of the expression level of miR-29b-1-5p with a mimic promoted GC cells proliferation. In addition, overexpression of miR-29b-1-5p reversed the Da0324-induced growth inhibition in GC cells. These results indicate that Da0324 suppresses GC cells proliferation may be through up-regulation of HOTAIRM1 expression, which binds to miR-29b-1-5p, resulting in down-regulation of miR-29b-1-5p.

PHLPP1 has been reported as a direct target of miR-29b-1-5p(Datta et al. 2018). Knockdown of miR-29b-1-5p inhibited the migration of AGS cells, knockdown of PHLPP1 augmented migration ratios in Helicobacter pylori-treated AGS cells(Datta et al. 2018). Several research studies have demonstrated that decreasing or lost PHLPP1 expression has been detected in many cancers, such as prostate cancer, colon cancer, pancreatic cancer and glioblastoma(Chen et al. 2011; Liu et al. 2009; Molina et al. 2012; Nitsche et al. 2012). The low expression of PHLPP1 has been related to GC(Wang et al. 2013). Furthermore, Lan

et al. reported that the Neddylation inhibitor MLN4924 triggered autophagy by causing the accumulation of PHLPP1 to inactivate AKT and mTORC1 in GC cells(Lan et al. 2016). In this study, we found that overexpression of miR-29b-1-5p or knockdown of HOTAIRM1 decreased the expression of PHLPP1, while Da0324 treatment increased the expression of PHLPP1 in GC cells. Knockdown of PHLPP1 significantly reversed the effect of Da0324 on GC cell growth inhibition, indicated that the efficiency of Da0324 partly depended on increasing the level of PHLPP1. Overall, these results suggest that the mechanism of anti-cell proliferation induced by Da0324 is achieved by regulating the HOTAIRM1/miR-29b-1-5p/PHLPP1 axis.

## Conclusions

Altogether, our study clarified a new molecular mechanism of anti-cancer activities of Da0324 in GC cells. HOTAIRM1 transcription was activated with Da0324 treatment in GC cells. Subsequently, HOTAIRM1 inhibited GC cell growth through promoting PHLPP1 expression by sponging miR-29b-1-5p. These data reveal new evidence for understanding the biological function and downstream regulatory network of Da0324.

## Abbreviations

CCK-8: Cell counting kit-8; ceRNAs: Competitive endogenous RNAs; DMEM: Dulbecco modified Eagle medium; GC: Gastric cancer; lncRNA: Long non-coding RNA; miRNAs: microRNAs; NC: Negative control; ncRNAs: Non-coding RNAs; PVDF: Polyvinylidene fluoride; qRT-PCR: Quantitative real-time PCR; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD: Standard deviation; shRNA: Short hairpin RNA; siRNA: Small interference RNA.

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

JR, JL, XF and CM conceived and designed the study. XF, CM, CH and WN carried out experiments and analyzed the data. WY, DB, and FQ participated in the experiments, statistical analyses, and data interpretation. XF, CM, JL, and JR drafted and revised the manuscript. All authors read and approved the final manuscript.

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### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare no competing interests.

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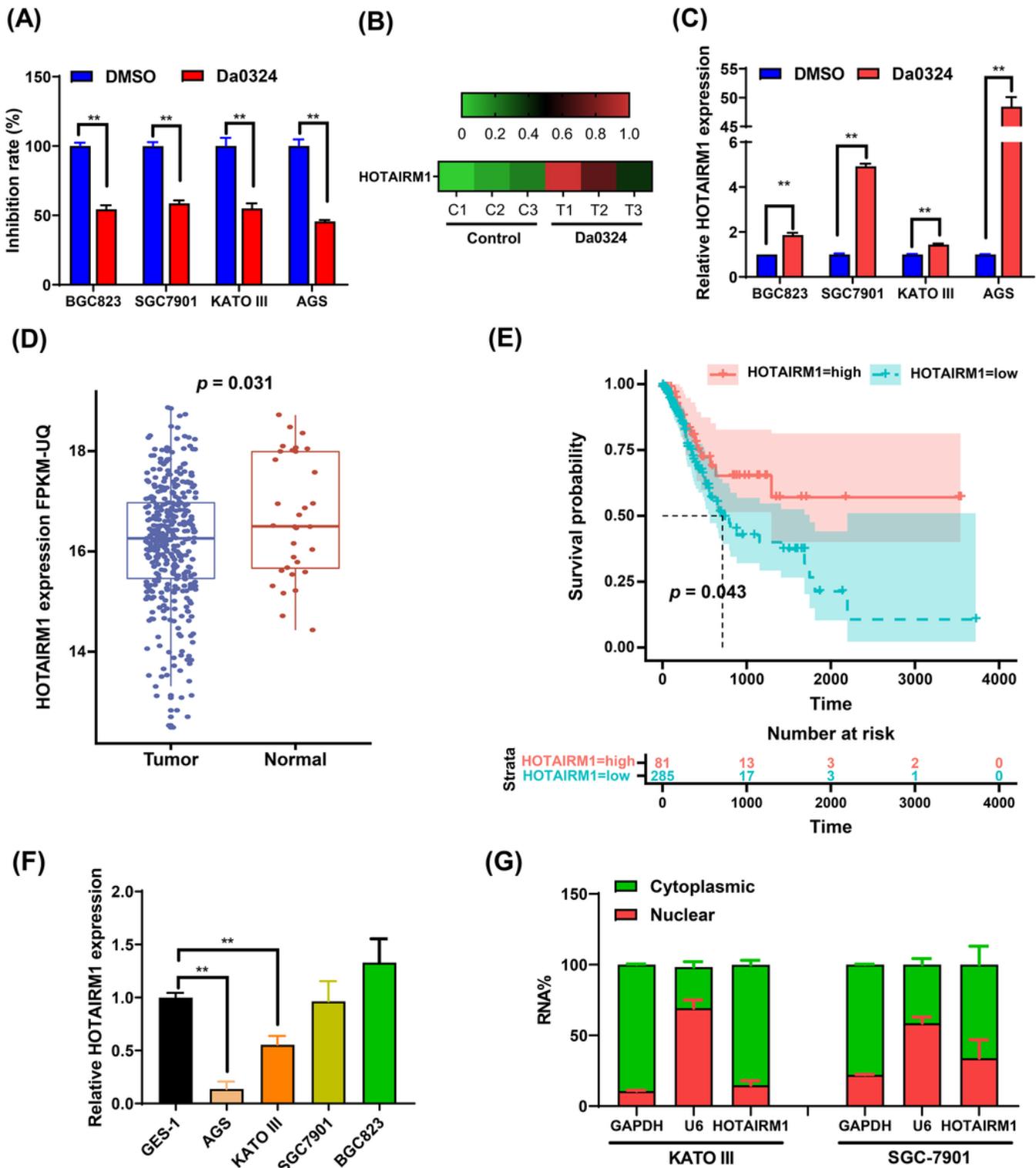
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## Figures



**Figure 1**

Da0324 inhibits the growth of gastric cancer cells and up-regulates the expression of HOTAIRM1. (A) Effects of Da0324 on gastric cancer cell viability. After exposure of GC cell lines (BGC823, SGC7901, KATO III and AGS) to 4 μM Da0324 for 48 h, the cytostatic effect was evaluated by CCK-8 assays. (B) Heatmap of HOTAIRM1 in SGC7901 cells treated with 4 μM Da0324 for 48 h. (C) qRT-PCR analysis of HOTAIRM1 expression in GC cells treated with 4 μM Da0324 for 48 h. (D) The expression of HOTAIRM1 in

normal and gastric cancer tissues based on TCGA data. (E) Survival analysis of gastric cancer patients with high and low expression levels of HOTAIRM1. (F) qRT-PCR analysis of HOTAIRM1 expression in the normal gastric epithelium cell line (GES-1) and GC cell lines (BGC823, SGC7901, KATO III and AGS). (G) Relative HOTAIRM1 expression level in cytoplasm and nuclear of the KATO III and SGC7901 cells was determined by qRT-PCR. GAPDH and U6 were used as cytosolic and nuclear loading controls, respectively. All data represent the mean  $\pm$  SD. Each experiment was performed in triplicate. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

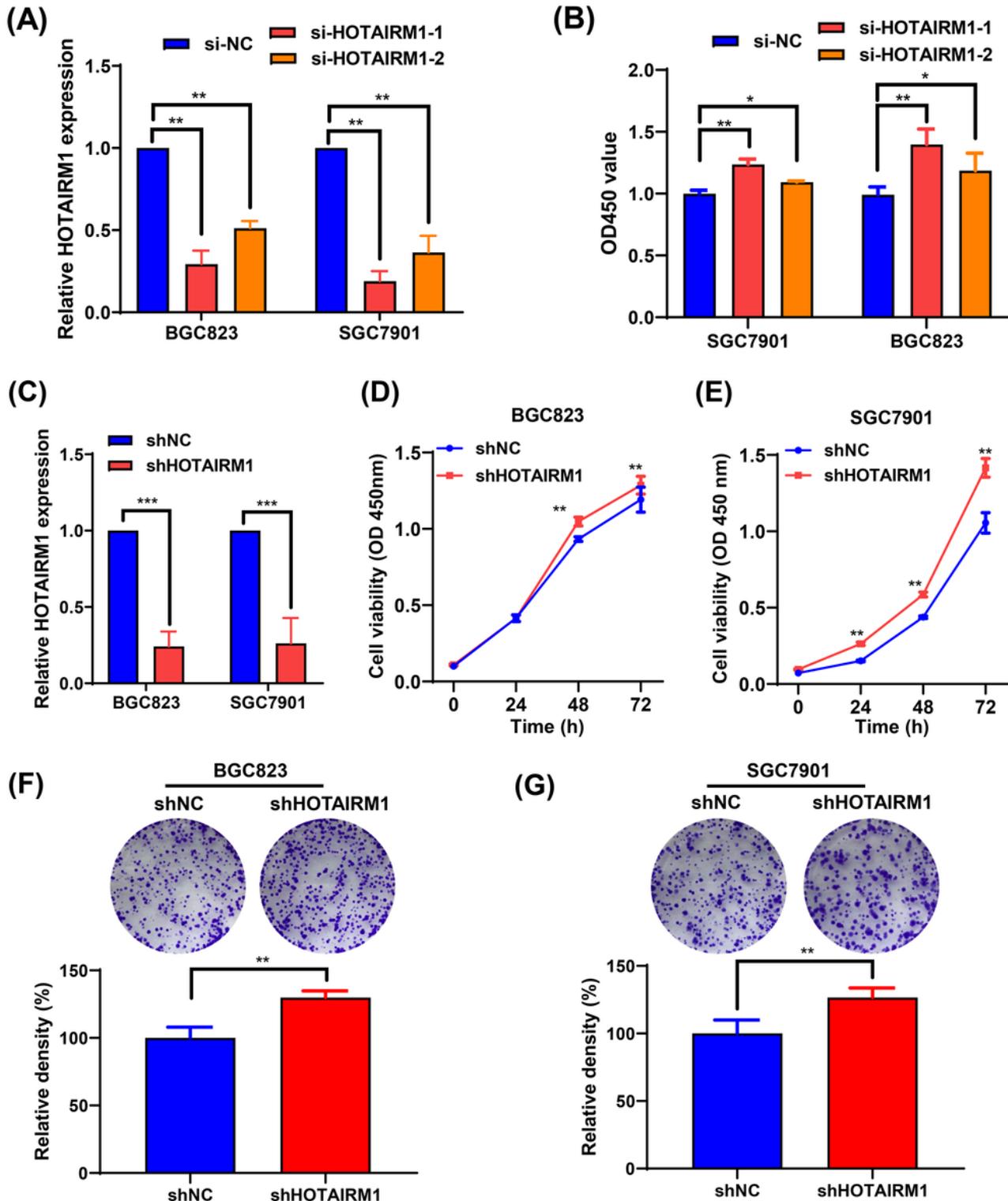
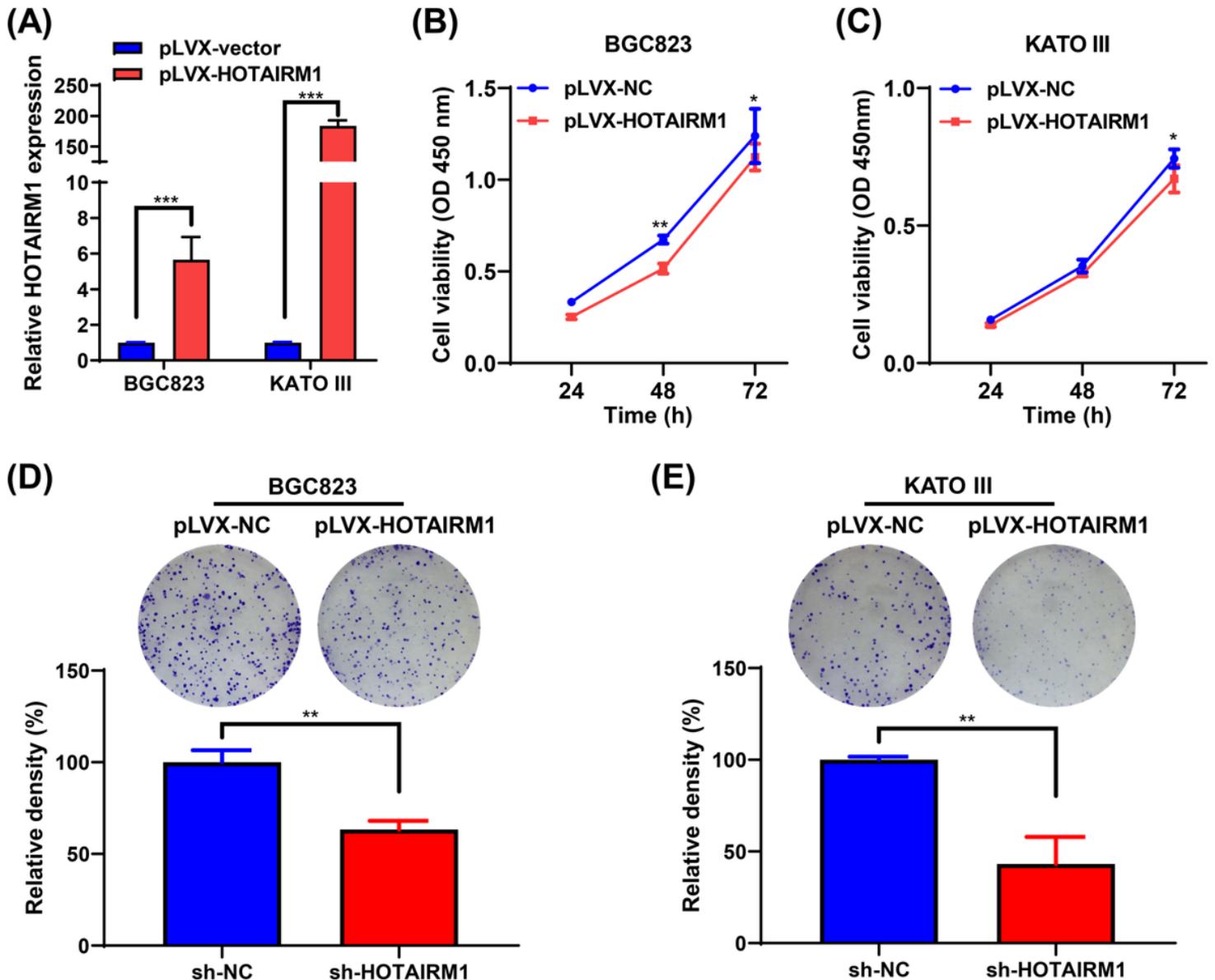


Figure 2

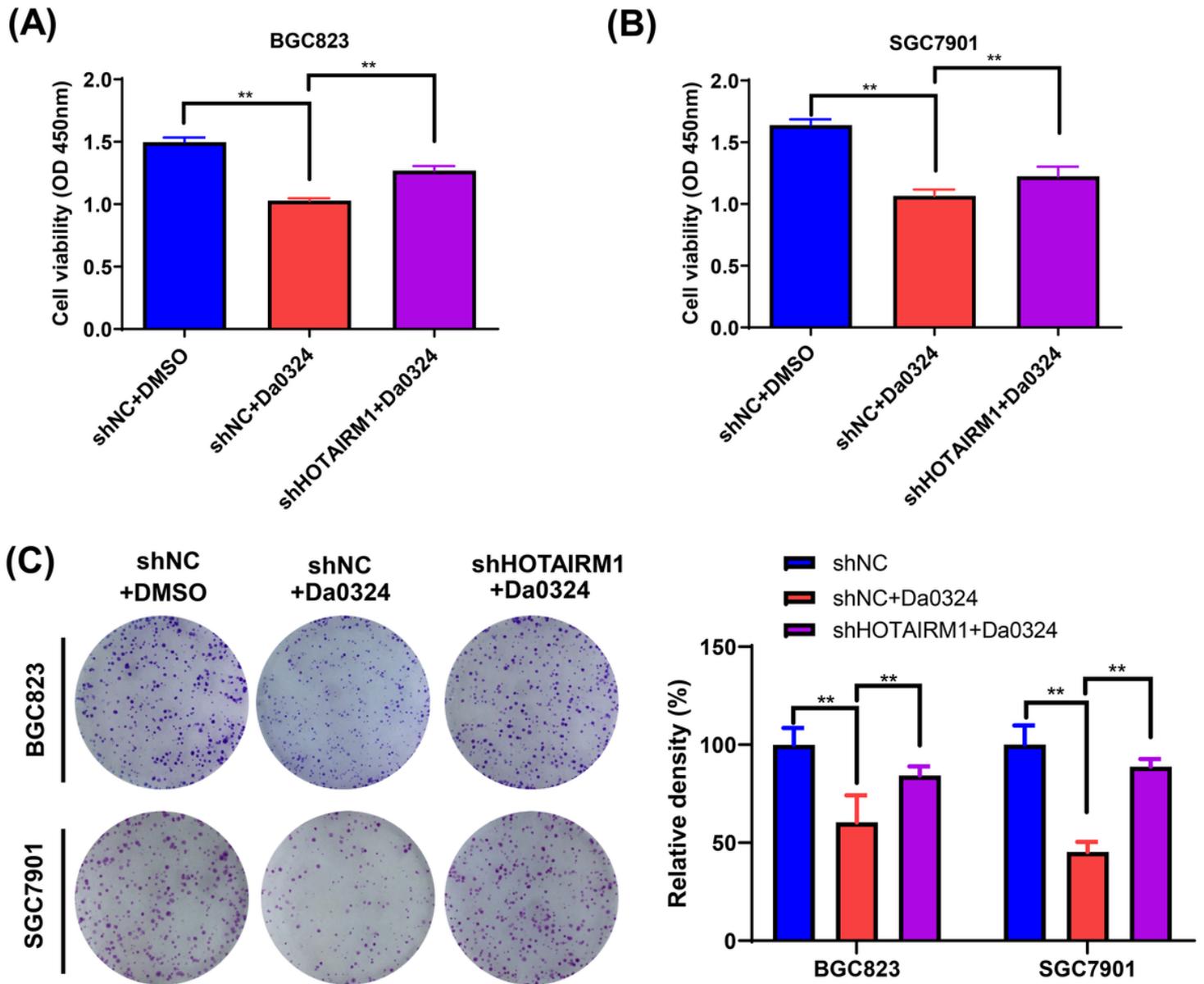
Knockdown of HOTAIRM1 facilitates GC cell proliferation. (A) The expression of HOTAIRM1 was determined in BGC823 and SGC7901 cells transfected with HOTAIRM1 siRNAs using qRT-PCR assays. (B) The relative cell growth was determined in SGC7901 and BGC823 cells transfected with HOTAIRM1 siRNAs by CCK-8 assays. (C) qRT-PCR analysis of HOTAIRM1 expression in BGC823 and SGC7901 cells transfected with shRNA targeting HOTAIRM1 (shHOTAIRM1). (D-E) Cell proliferation assay in BGC823 (D) and SGC7901 (E) cells transduced with lenti-shHOTAIRM1. (F-G) Colony formation in BGC823 (F) and SGC7901 (G) cells transduced with lenti-shHOTAIRM1 was analyzed with colony formation assays. Representative images of clonogenic assay (upper panel panel) and quantitative analysis (lower panel). All data represent the mean  $\pm$  SD. Each experiment was performed in triplicate. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 3**

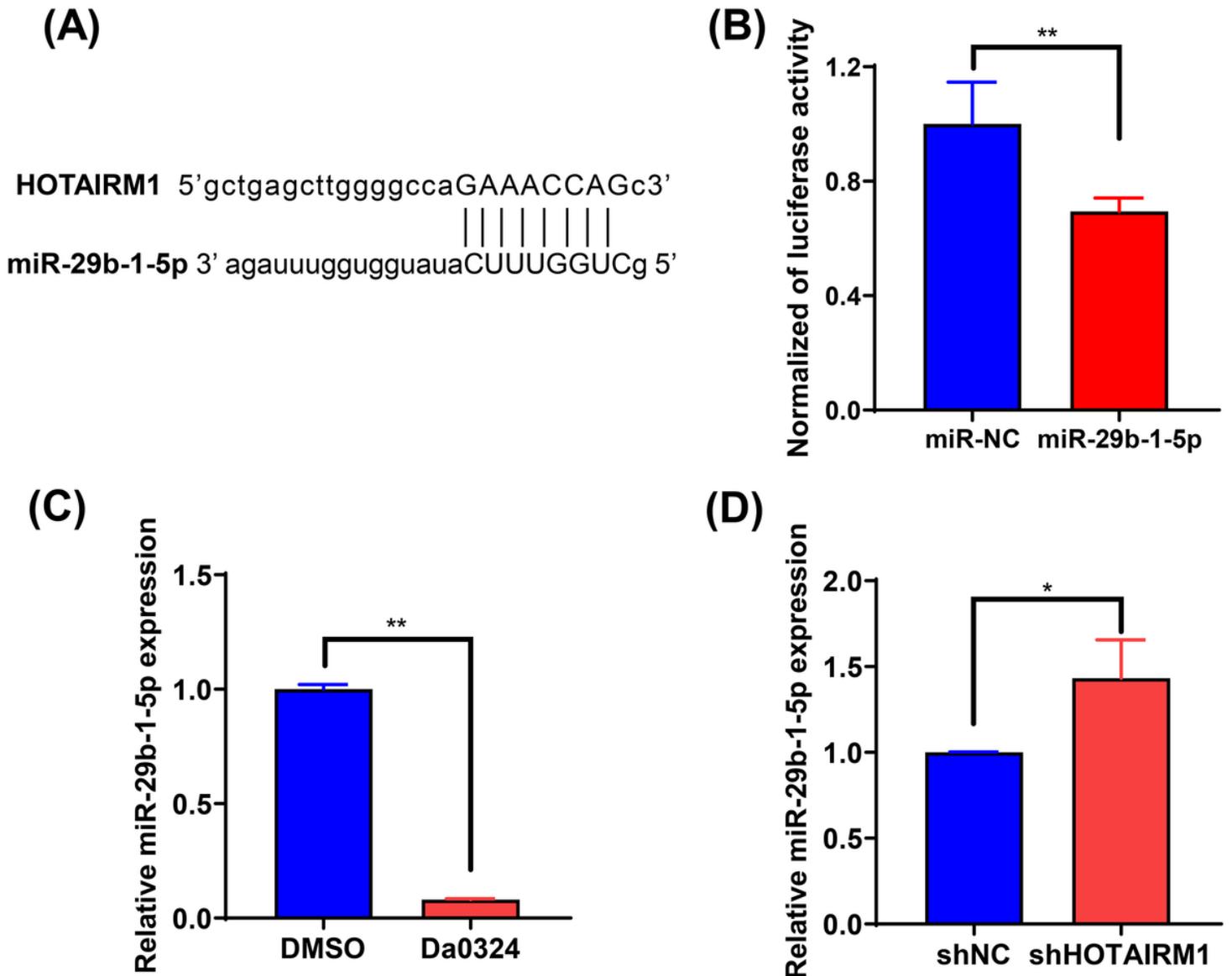
Overexpression of HOTAIRM1 inhibits the proliferation of gastric cancer cells. (A) qRT-PCR analysis of HOTAIRM1 expression in BGC823 and KATO III cells transduced with lenti-HOTAIRM1. (B-C) CCK-8 assays were conducted to determine cell viability of BGC823 (B) and KATO III (C) cells after transduced with lenti-

HOTAIRM1 or control vector. (D-E) Colony-formation assays were performed to identify the effects of HOTAIRM1 on the proliferation of BGC823 (D) and KATO III (E) cells. All experiments were performed in triplicate, and the results are presented as the mean  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



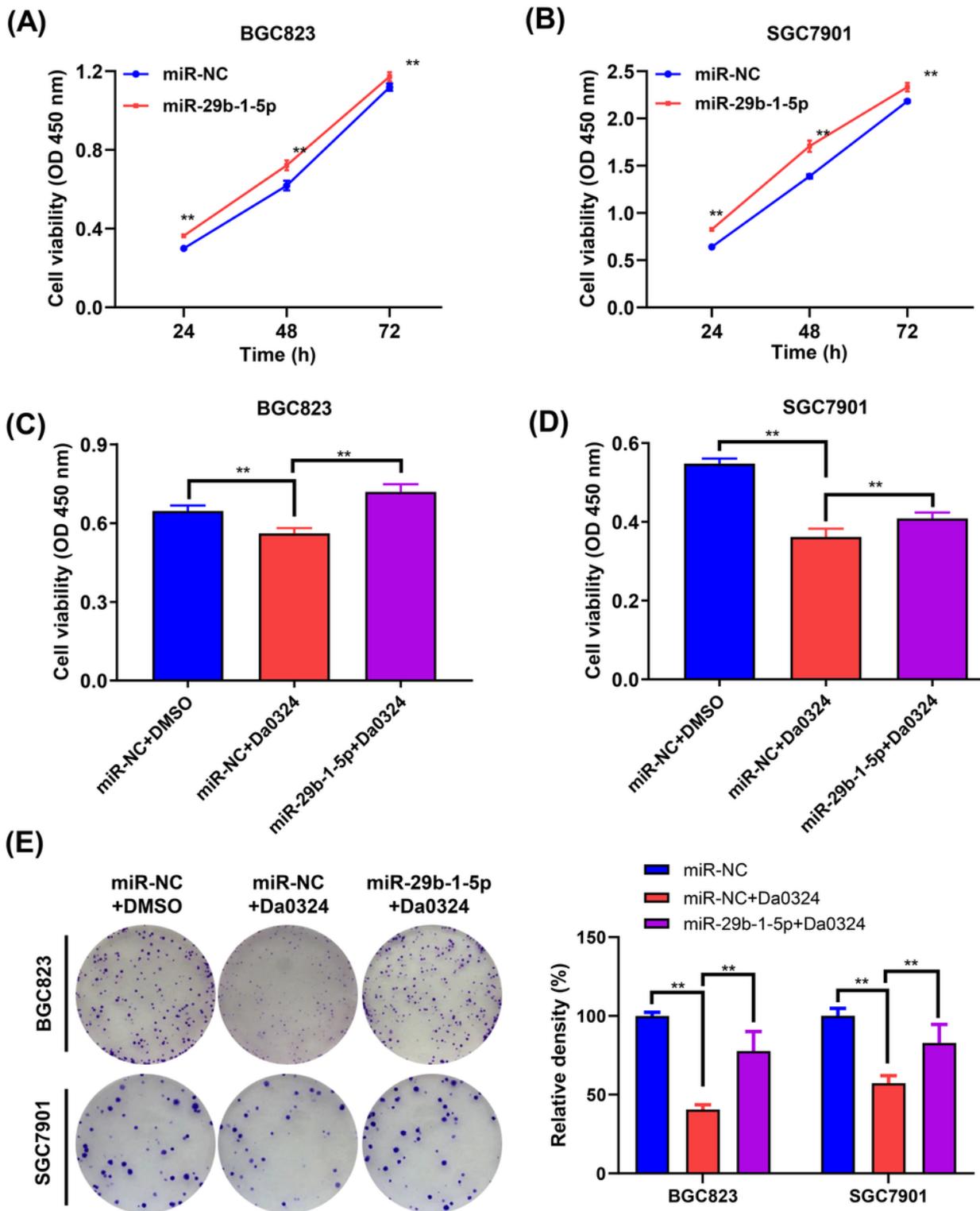
**Figure 4**

Knockdown of HOTAIRM1 alleviates Da0324-induced cytotoxicity in gastric cancer cells. (A-B) BGC823 (A) and SGC7901 (B) cells transduced with shNC or shHOTAIRM1 were exposed to 4  $\mu$ M Da0324 for 48 h, and the cell viability was examined by the CCK-8 assay. (C) BGC823 and SGC7901 cells transduced with shNC or shHOTAIRM1 were exposed to Da0324 for 48 h and colony formation was assessed. All experiments were performed in triplicate, and the results are presented as the mean  $\pm$  SD. \*\*,  $P < 0.01$ .



**Figure 5**

HOTAIRM1 acts as a sponge for miR-29b-1-5p in gastric cancer. (A) IncRNAMap was used to predict the binding site of miR-29b-1-5p in the HOTAIRM1 sequence. (B) Dual-luciferase assay was performed to verify the binding of miR-29b-1-5p to HOTAIRM1. (C) Relative expression level of miR-29b-1-5p was determined in SGC7901 cells treated with 4  $\mu$ M Da0324 for 48 h. (D) qRT-PCR analysis of miR-29b-1-5p expression in SGC7901 cells transduced with lenti-shHOTAIRM1. All experiments were performed in triplicate, and the results are presented as the mean  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 6**

miR-29b-1-5p regulates the Da0324-induced cytotoxicity in gastric cancer cells. (A-B) Cell proliferation was examined by CCK-8 assays in BGC823 (A) and SGC7901 (B) cells transfected with miR-NC control or miR-29b-1-5p mimics. (C-D) Relative viability of BGC823 (C) and SGC7901 (D) cells pre-transfected with miR-29b-1-5p mimics was determined by CCK-8 assays after 24 h Da0324 (4  $\mu$ M) treatment. (E) BGC823 and SGC7901 cells transfected with miR-29b-1-5p mimics were treated with Da0324 (4  $\mu$ M) for 48 h, and

then the clonogenic assay was used to detect the colony-formation abilities of BGC823 and SGC7901 cells. Data are showed as mean  $\pm$  SD (n = 3). \*\*, P < 0.01.

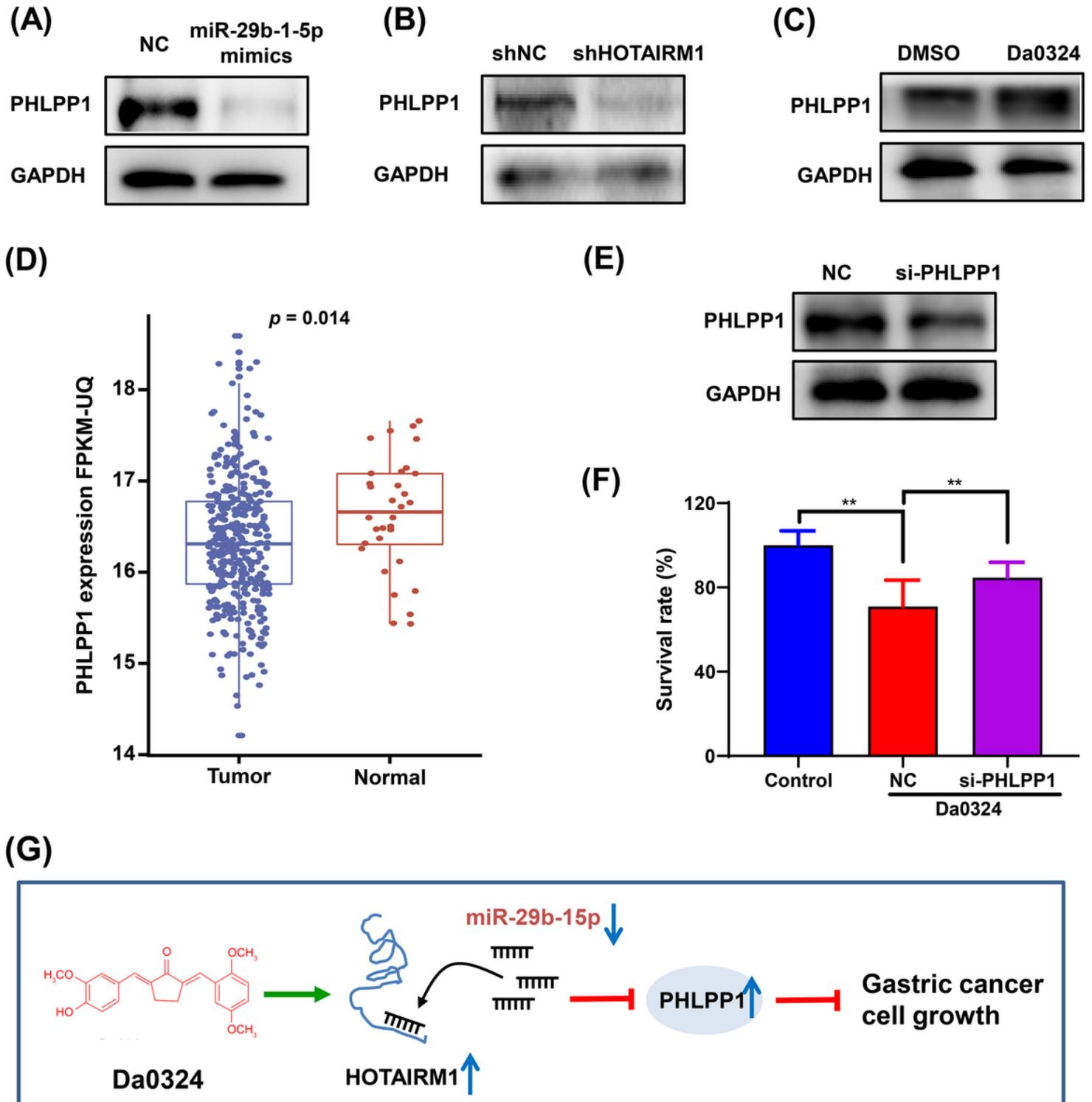


Figure 7

PHLPP1 is regulated by the HOTAIRM1/miR-29b-1-5p axis in gastric cancer cells. (A) Western blot analysis of PHLPP1 protein level in SGC7901 cells transfected with miR-29b-1-5p mimics. (B) Western blot analysis of PHLPP1 protein level in SGC7901 cells transfected with shHOTAIRM1. (C) SGC7901 cells

were treated with Da0324 (4  $\mu$ M) for 48 h, PHLPP1 protein expression was determined by Western blotting. (D) The PHLPP1 expression in normal and gastric cancer samples based on TCGA data. (E) The knockdown efficiency of si-PHLPP1 was verified in SGC7901 cells by Western blotting. (F) Cell proliferation of SGC7901 cells transfected with si-PHLPP1 or negative control and treated with Da0324, according to the CCK-8 assay. Data are showed as mean  $\pm$  SD (n = 3). \*\*, P < 0.01. (G) Mechanistic model of Da0324-induced growth inhibition of gastric cancer cells.