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# LMP1–miR-146a–CXCR4 axis regulates cell proliferation, apoptosis and metastasis

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ABSTRACT

Epstein–Barr virus (EBV), the first human tumor virus to have been discovered, sustains an asymptomatic lifelong infection in ~95% of the world's population. Reportedly, EBV infection induces the expression of specific cellular microRNAs (miRNAs), such as miR-155, miR-146a, miR-21, which can contribute to the persistence of latently infected cells. In this study, we investigated whether C-X-C chemokine receptor type 4 (CXCR4) is a cellular target of human miR-146a. We also investigated the role of miR-146a and CXCR4 in EBV-associated cells. The results indicate that miR-146a is more abundantly expressed in EBV-positive than in EBV-negative cells. MiR-146a down-regulated CXCR4 expression in a dose- and time-dependent manner. Phenotypic experiments detected miR-146a mimics that could suppress cell proliferation and cell migration and promote cell apoptosis by targeting CXCR4. In addition, miR-146a mimics suppressed cell survival by decreasing the population of G0/G1 phase cells. Latent membrane protein (LMP)1, an importance oncoprotein, can stimulate miR-146a and inhibit the CXCR4 expression. Our findings indicate that LMP1–miR-146a–CXCR4 axis functions as a regulator in the EBV-associated cells.

## 1. Introduction

Epstein–Barr virus (EBV) was the oncogenic herpesvirus to be discovered. It is estimated that ˜95% of the world's population has an asymptomatic lifelong EBV infection (Khan and Hashim, 2014; Young et al., 2016). EBV sequences can be detected in a number of malignant diseases such as endemic Burkitt lymphoma (BL), lymphoepithelioma-like nasopharyngeal carcinoma (NPC), Hodgkin lymphomas (HL), non-Hodgkin lymphomas (NHL) in immunocompromised patients, sporadic BL, some T and NK cell lymphomas, and gastric adenocarcinomas (Delecluse et al., 2007). Depending on EBV-determined nuclear antigen (EBNA) and latent membrane protein (LMP) expression, EBV exhibits four different gene expression patterns during latency (Tempera et al., 2011). Latency 0 is observed in healthy carriers, with no viral proteins or low levels of EBNA-1 and LMP2A. Latency I shows viral protein expression limited to EBNA-1 and LMP2A, such as in BL. Latency II is characterized by EBNA-1, LMP1, LMP2A, and LMP2B expression and is commonly found in NPC, HL and NK/T-cell lymphomas. latency III, often seen in immunedeficiency-associated lymphoma, shows expression of all six EBNAs and three LMPs.

Although EBNA-1 and LMP2A are expressed and LMP1 is absent in EBV-associated gastric cancer (EBVaGC), several studies have reported that EBVaGC tissues express LMP1 messenger RNA (mRNA) (Luo et al., 2005; Sugiura et al., 1996). At the same time, GT38 and GT39, EBV-positive gastric epithelial cells, are identified with LMP1 expression (Sairenji, 1999). Therefore, EBVaGC might have a latency pattern between I and II.

MicroRNAs (MiRNAs) are highly conserved, small non-coding RNAs that form complementary duplexes with their targets mRNAs, leading to the down-regulation of genes (Macfarlane and Murphy, 2010). Numerous studies have shown that miRNAs play an important role in oncogenesis and tumor metastasis, implying that miRNAs might function as diagnostics, either alone or in combination with other known biomarkers (McManus, 2003; Mohr and Mott, 2015). To date, > 2600 human miRNAs (cellular miRNAs) have been archived in miRBase (http://www.mirbase.org). So far, the association between miRNAs and EBV has not been intensively investigated. Studies have reported that EBV infection induces the expression of specific cellular miRNAs, such as miR-155, miR-146a, miR-21, which can contribute to the persistence of latently infected cells (Skalsky, 2017). In some studies, miR-146a has shown up-regulation in EBVaGC compared with EBV-negative gastric cancer (EBVnGC) cells (Kim et al., 2016). However, few studies have taken into account the underlying mechanisms of miR-146a function in EBV-associated cells.

Chemokines are a large family of small, structurally related cytokines.

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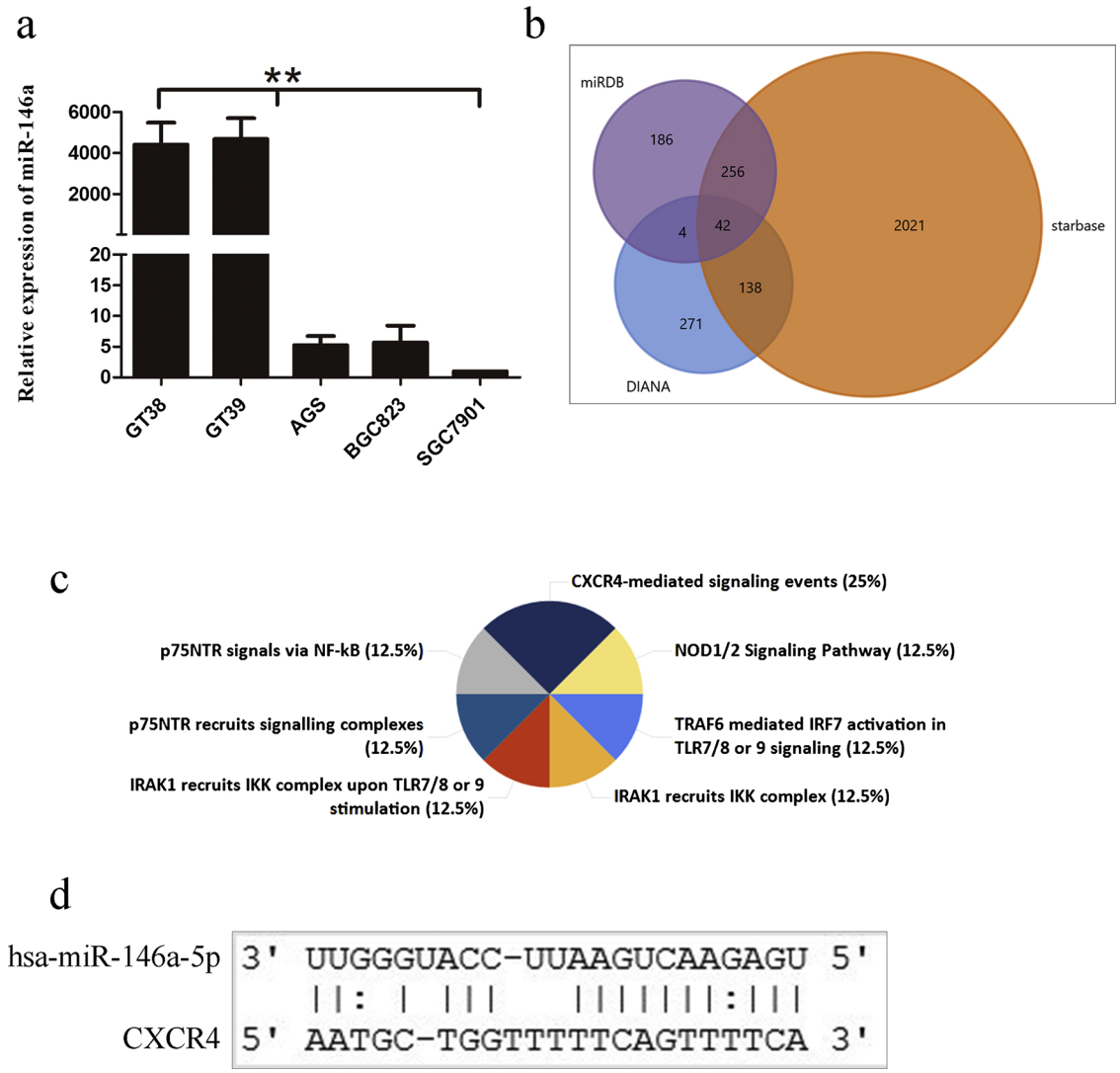


**Fig. 1.** The expression and roles of miR-146a in EBV-positive and EBV-negative cells.
(a) Relative expression of miR-146a was calculated using the comparative Cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and U6 as the internal standard. Data was represented as mean ± standard error of mean (SEM) and came from at least three replicates. (b) Venn diagram of predicted miR-146a targets by three programs (miRDB, starbase and DIANA). The overlapping part is the co-predicted genes, including 42 genes in total. (c) Enrichment analysis of predicted miR-146a targets was assessed by KEGG. Only the most enriched seven cell signaling pathways of predicted miR-146a targets were shown. (d) The potential binding sequence between hsa-miR-146a-5p and CXCR4 was predicted by informatics tools.

So far, nearly 50 chemokines and 20 chemokine receptors have been identified in humans (Viola and Luster, 2008).C-X-C chemokine receptor type 4 (CXCR4), one of the most well-studied chemokine receptors, functions as a co-receptor for human immunodeficiency virus entry (Farzan et al., 2002). Several studies have indicated CXCR4 overexpression in > 23 different types of human cancers (e.g., kidney, lung, breast, melanomas), which contributes to the tumor growth, angiogenesis, metastasis, and therapeutic resistance (Chatterjee et al., 2014).However, there are few studies on the expression and specific involvement of CXCR4 in EBV-associated carcinoma cells. In this study, we investigated whether CXCR4 is a cellular target of human miR-146a. We also investigated the role of miR-146a and CXCR4 in EBV-associated cells.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

GT38 and GT39, EBV-positive (with LMP1 expression) gastric epithelial cell lines, were kind gifts by Sarienji T (Tottori University). In addition, three EBV-negative gastric epithelial cell lines, that is, AGS, BGC823, and SGC7901, were purchased from the Cell Culture Center (China). All cell lines were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries Company, Israel) and 2% penicillin-streptomycin at 37 °C with 5% $CO_2$.

### 2.2. RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, USA) and reverse transcribed using First Strand cDNA synthesis kit (Takara, Japan). These products were detected using a Faststart Essential DNA Green Master kit (Roche, Switzerland) with the manufacturer's instructions. All reactions were done in triplicate and the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) for GAPDH or U6(supplied in the kit) were used as internal standard. The primers were as follows: for CXCR4, 5′-GCGTCTCAGTGCCCTTTTGT-3′ and 5′-TGAAGTAGTGGGCTAAGGGC-3′, for LMP1,5′- CCTTGGTCTACTCCT ACTGATGATCA -3′and 5′- CAGCACAATTCCAAGGTACAATG-3′, for GAPDH, 5′- CAAATTCCATGGCACCGTCA-3′ and 5′-UUGAAACGCACU GUCUGUGTT-3′, for microRNA-146a, 5′-CGCTGAGAACTGAATTCCAT GGGTT-3′.

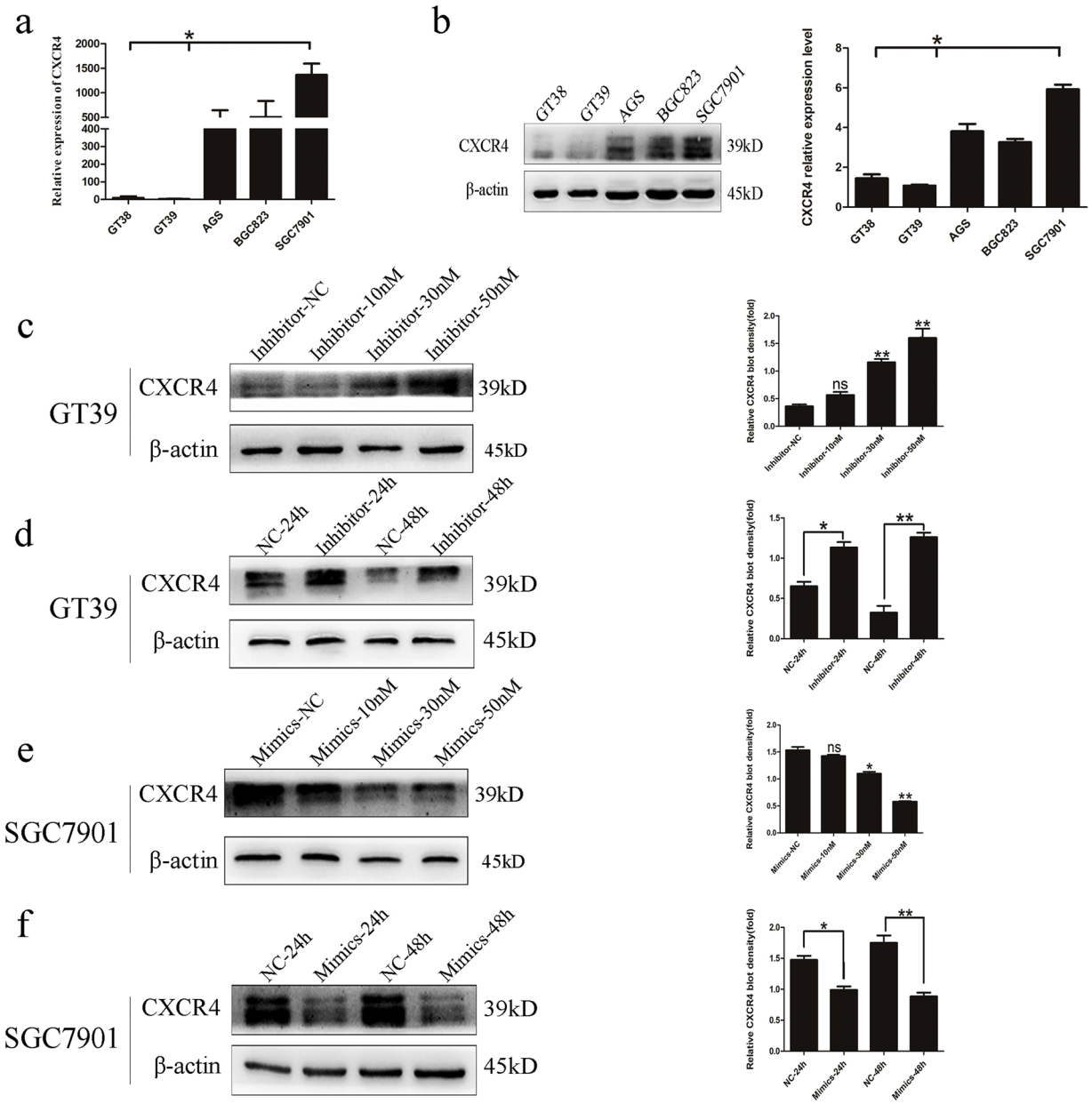


**Fig. 2.** The expression of CXCR4.
(a) Relative expression of CXCR4 was calculated using the comparative cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) and the expression of GAPDH as the internal standard. (b) The protein level of CXCR4 was analyzed by Western blot. (c)Relative protein expression of CXCR4 was detected in GT39 cell after 48 h treatment of miR-146a inhibitor (with the three concentrations of 10 nM, 30 nM, 50 nM). (**$p < 0.01$, ns: no significant). (d) Relative protein expression of CXCR4 was detected in GT39 cells after the treatment of miR-146a inhibitor at 24 h and 48 h. (**$p < 0.01$, *$p < 0.05$). (e)The protein level of CXCR4 was determined with the treatment of NC and mimics(10 nM, 30 nM, 50 nM)in SGC7901 cell (**$p < 0.01$, *$p < 0.05$, ns: no significant, respectively) (f) The protein level of CXCR4 was detected with the treatment of 24 h and 48 h in SGC7901(**$p < 0.01$,*$p < 0.05$). Data are representative of three independent experiments.

### 2.3. Western blot analysis

All cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with strong RIPA buffer containing a mixture of phenylmethanesulfonylfluoride (PMSF), a protease inhibitor, and phosphatase inhibitors (ratio 100:1:1) to obtain proteins. The proteins obtained were measured using a bicinchoninic acid assay kit (CWBIO, China), separated by 10% SDS–PAGE, and then blotted onto polyvinylidene fluoride membrane (Millipore, USA), The blotted proteins were blocked with 5% non-fat milk for 2 h at room temperature and incubated with a primary antibody overnight at 4 °C, followed by a second antibody for the chemiluminescence detection. Finally, proteins of interest were visualized using the Quantum-ST5 (Vilber Lourmat, France) enhanced chemiluminescence detection system with chemiluminescence substrates (Millipore). The specific antibodies were as follows: CXCR4 monoclonal antibody (Abcam, USA,1:100), anti-beta-actin antibody (Novus, USA, 1:2000), LMP1 monoclonal antibody (Abcam, USA, 1:500), anti-ERK1/2 antibody (CST, USA, 1:1000), anti-p-ERK1/2 antibody (CST, USA, 1:1000), anti-AKT antibody (CST, USA, 1:1000), anti-p-AKT antibody (CST, USA, 1:1000).

### 2.4. Transfection with microRNA-146a inhibitor and mimics

We purchased miRNA-146a-5p mimics, inhibitor, and scramble controls from Genepharma (China). The cells were seeded in six-well plates at a density of $1 \times 10^6$ cells/well and transfected with

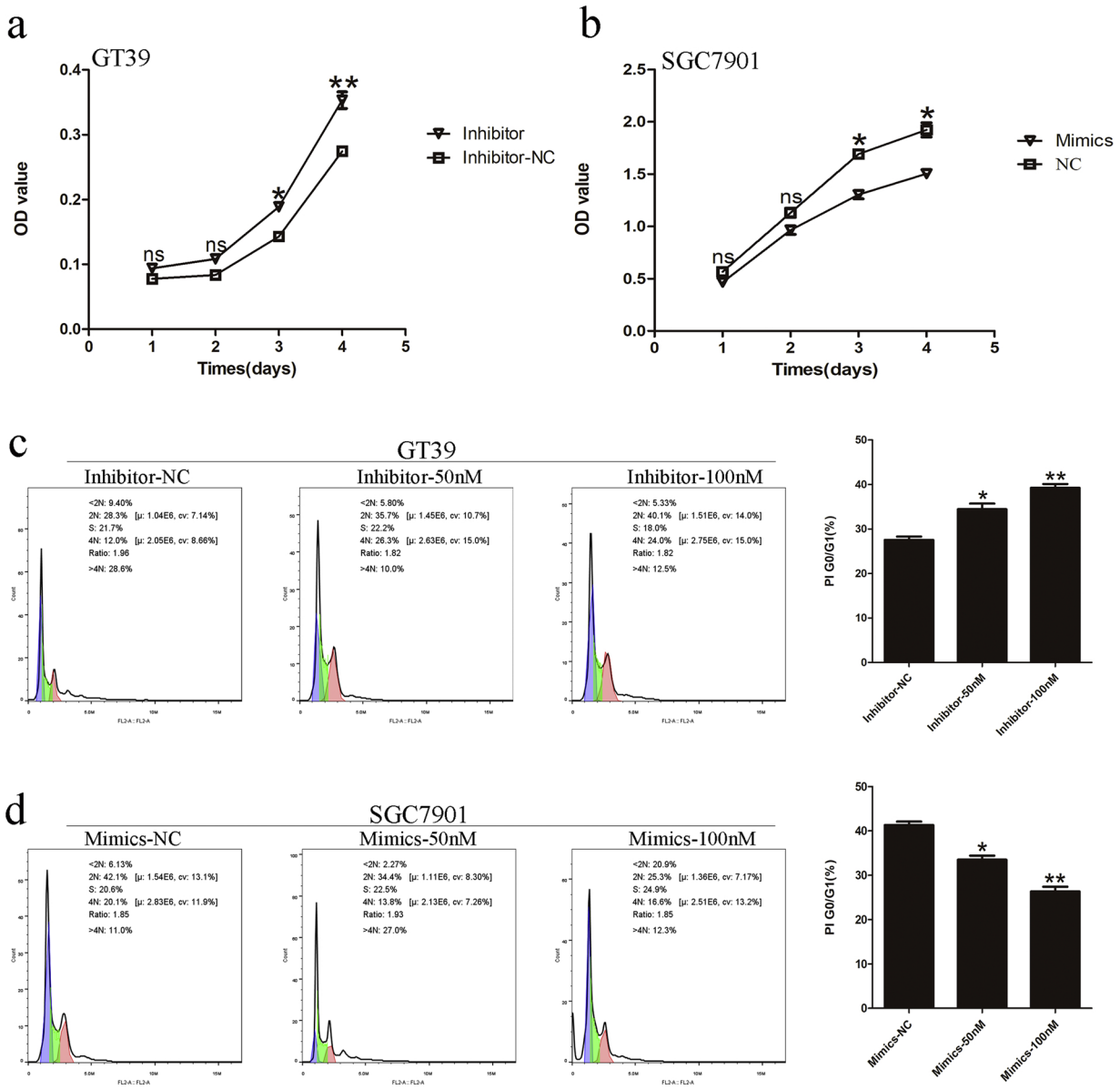


**Fig. 3.** Effects of miR-146a on cell proliferation and cell cycle.
(a)The proliferative capacity of miR-146a inhibitor by CCK8 assay in GT39 cell with 50 nM concentration at 24 h, 48 h, 72 h and 96 h. (**$p < 0.01$, *$p < 0.05$, ns: no significant) (b) The effect of miR-146a mimics on cell proliferation by CCK8 assay in SGC7901 cell. (*$p < 0.05$, ns: no significant) (c) Transfected with miR-146a inhibitor (50 nM, 100 nM) and inhibitor NC, cell cycles were analyzed by PI staining assays at 48 h in GT39 cell. The 2N in cell cycle represents for G0/G1 phase of DNA replication. (**$p < 0.01$, *$p < 0.05$) (d)The cell cycle analysis of miR-146a mimics was detected by PI staining assays using flow cytometry (**$p < 0.01$, *$p < 0.05$). Data are representative of three independent experiments.

Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The sequences were as follows: for microRNA-146a-5p mimics, 5′- UGAGAACUGAAUUCCAUGGGUU-3′, 5′-CCCAUGGAAUUCAGUUCUCAUU-3′; for microRNA-146a-5p inhibitor, 5′-AACCCAUGGAAUUCAGUUCUCA-3′, for mimics negative control, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′-ACG UGACACGUUCGGAGAATT-3′; for inhibitor negative control, 5′-CAGU ACUUUUGUGUAGUACAA-3′.

### 2.5. Transfection with siRNAs of LMP1 and CXCR4

Specific siRNAs against CXCR4 were designed and synthesized by HANBIO (China). The sequences are as follows: hs-CXCR4-si-1: 5′-AGAUAUACACUUCAGAUAAdTd-3′, 5′-UUAUCUGAAGUGUAUA UCUdTd-3′; hs-CXCR4-si-2: 5′-GAAGCAUGACGGACAAGUAdTd-3′, 5′-UACUUGUCCGUCAUGCUUCdTd-3′. The siRNA target sequence to LMP1 mRNA was 5′-AAGAGCCUUCUCUGUCCACU-3′. The siRNAs were transfected using Lipofectamine 2000 Reagent (Invitrogen, Thermo Fisher Scientic, Germany) and assayed at mRNA and protein levels after transfection. All experiments were performed in triplicate.

### 2.6. Cell proliferation assay

Cell proliferation was detected using cell-counting kit 8 (CCK8; Boster Biological Technology, China). For CCK8 assay, $5 \times 10^3$ cells/100 μL were seed in a 96-well plate for 24 h. Then, 10 μL of CCK8 solution was added to culture medium after incubation for 24, 48, 72, and 96 h, and the cells were incubated for an additional 2 h at 37 °C. Finally, the absorbance was measured at 450 nm using a microplate reader.

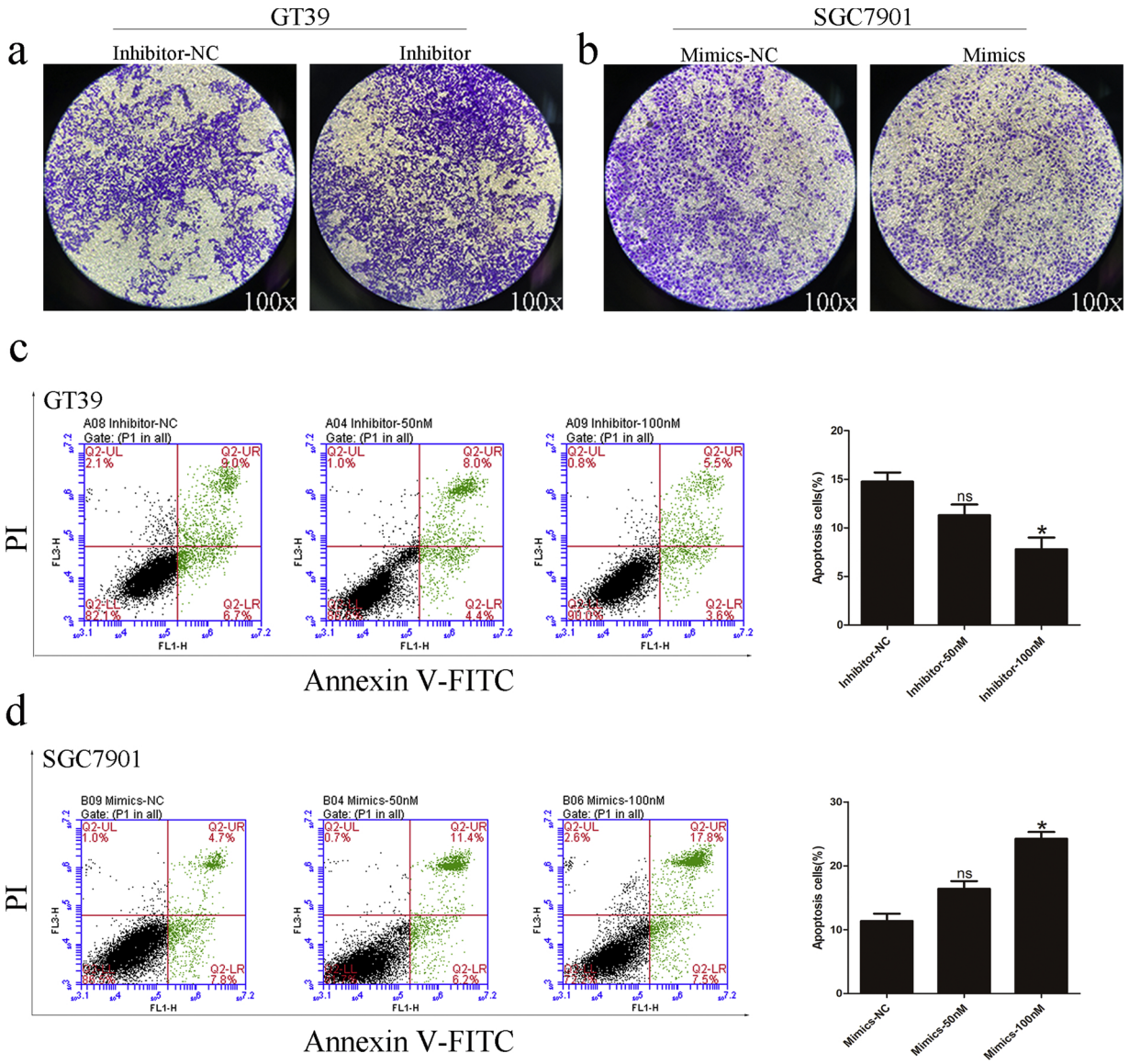


**Fig. 4.** Effects of miR-146a on cell migration and cell apoptosis.
(a, b) The role of miR-146a inhibitor (50 nM) and mimics (50 nM) on cell migration were assessed by transwell migration assay at 48 h under the microscope (100x). (c, d) cells were treated with miR-146a inhibitor and mimics at 48 h and then double staining of annexin V and propidium iodide (PI) was detected by flow cytometry. Total apoptosis was analyzed compared to the control group (*$p < 0.05$, ns: no significant). Data are representative of three independent experiments.

### 2.7. Propidium iodide staining analysis

Cells were collected 48 h after indicated treatments, washed with PBS, and fixed in 66% ethanol overnight at 4 °C. Using a propidium iodide (PI) flow cytometry kit (abcam, USA), the cells were incubated with PI and RNase staining solutions for 30 min at 37 °C in the dark. Finally, according to the standard protocol, > 10,000 cells were detected by fluorescence-activated cell sorting (FACS) using a FACSCalibur apparatus (BD Biosciences, USA) according to the standard protocol.

### 2.8. Cell apoptosis detection

Cells were seeded in six-well plates at a density of $1 \times 10^6$ cells/well, cultured for 48 h, washed twice with PBS, and then detected using the Annexin V-FITC Apoptosis detection kit (BD Biosciences, USA) according to the manufacturer's instructions. After incubating for 10–15 min at room temperature in the dark, the specimens were analyzed by FACS using a FACSCalibur apparatus (BD Biosciences, USA).

### 2.9. Migration assay

For transwell migration assay, $1 \times 10^5$ cells were plated in the top chamber with a non-coated membrane with 8 μm pores (Corning, USA). The cells were plated in culture medium without serum, and the medium was supplemented with 20% FBS as a chemoattractant in the lower chamber. The cells were incubated for 48 h, fixed with methanol, and stained with hematoxylin. Stained cells were counted at 10× magnification under a microscope. All experiments were performed in triplicate.

### 2.10. Overexpression of LMP1

The plasmids of LMP1 (pcDNA3.1-LMP1) was purchased from Invitrogen Inc. (Hanbio, China). The plasmid was transfected using Lipofectamine 2000 Reagent (Invitrogen, USA) and fluorescent activity was assayed under a fluorescence microscope. The transfected SGC7901 cell line was called SGC7901-LMP1 (SGC7901-NC was the corresponding control with a pcDNA3.1 expression vector).

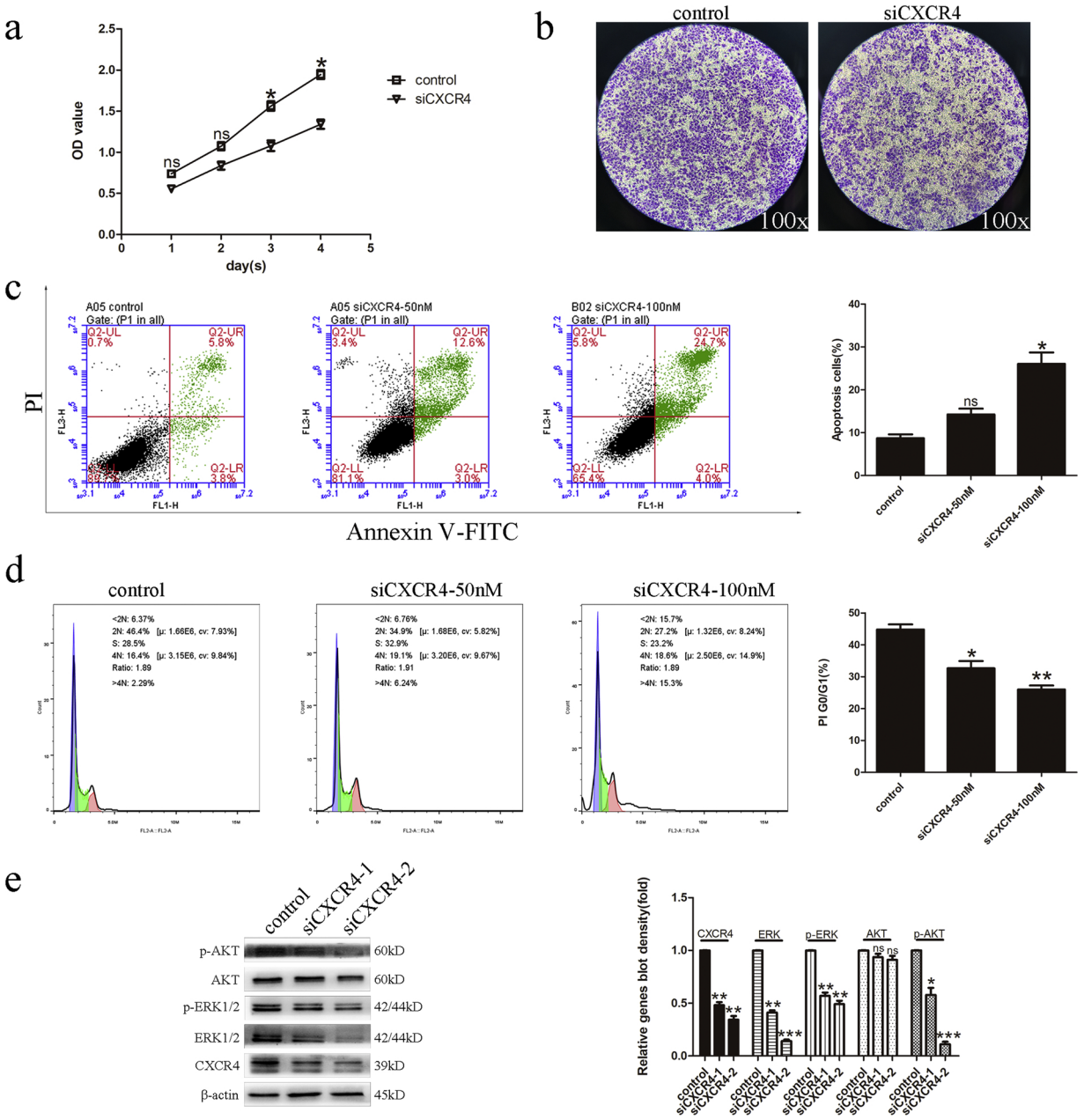


**Fig. 5.** Effects of CXCR4 on cell proliferation, migration and apotosis.
(a)The proliferative capacity of CXCR4 was evaluated by CCK8 assay in SGC7901 cell (*$p$ < 0.05, ns: no significant). (b) The role of CXCR4 on cell migration were assessed by transwell migration assay at 48 h under the microscope (100x). (c) Treated with siCXCR4 at 48 h, cells were detected using double staining of annexin V and propidium iodide (PI) by flow cytometry (*$p$ < 0.05, ns: no significant). (d) The cell cycle analysis of siCXCR4 was detected by PI staining assays using flow cytometry (**$p$ < 0.01, *$p$ < 0.05). (e)The effects on PI3K/AKT and ERK signal pathways were analyzed by Western blot after interference of CXCR4 (***$p$ < 0.001, **$p$ < 0.01, *$p$ < 0.05, ns: no significant). Data are representative of three independent experiments.

### *2.11. Statistical analysis*

Data were analyzed using the Student's t-test, two-way repeated-measure analysis of variance (ANOVA). Analyses were performed using GraphPad Prism software (GraphPad Software, USA). All data was expressed as means ± standard error of the mean (SEM).

## 3. Results

### *3.1. Relative expression of miR-146a in EBV-positive and EBV-negative cancer cells*

The abnormal expression of miR-146a has been reported in different types of cancer. In this study, we measured miR-146a expression in EBV-positive (GT38 and GT39) and EBV-negative (AGS, BGC823 and SGC7901) cells via the quantitative reverse transcription polymerase chain (qRT-PCR). MiR-146a expression was much higher in EBV-positive cells than in EBV-negative cells, as shown in Fig. 1a. We predicted the potential target genes by using the microRNA target prediction databases miRDB, DIANA, and starbase. Co-targeting genes (Fig. 1b) were subjected to enrichment analysis of cell signaling pathways using KEGG (http://www.genome.jp/kegg/). CXCR4-mediated signaling was significantly enriched by the predicted targets of miR-146a (Fig. 1c). Bioinformatics analysis showed that the binding region of miR-146a was located in the 3′-UTR of CXCR4 (Fig. 1d). These results suggest that CXCR4 might be a cellular target of human miR-146a in EBV-associated

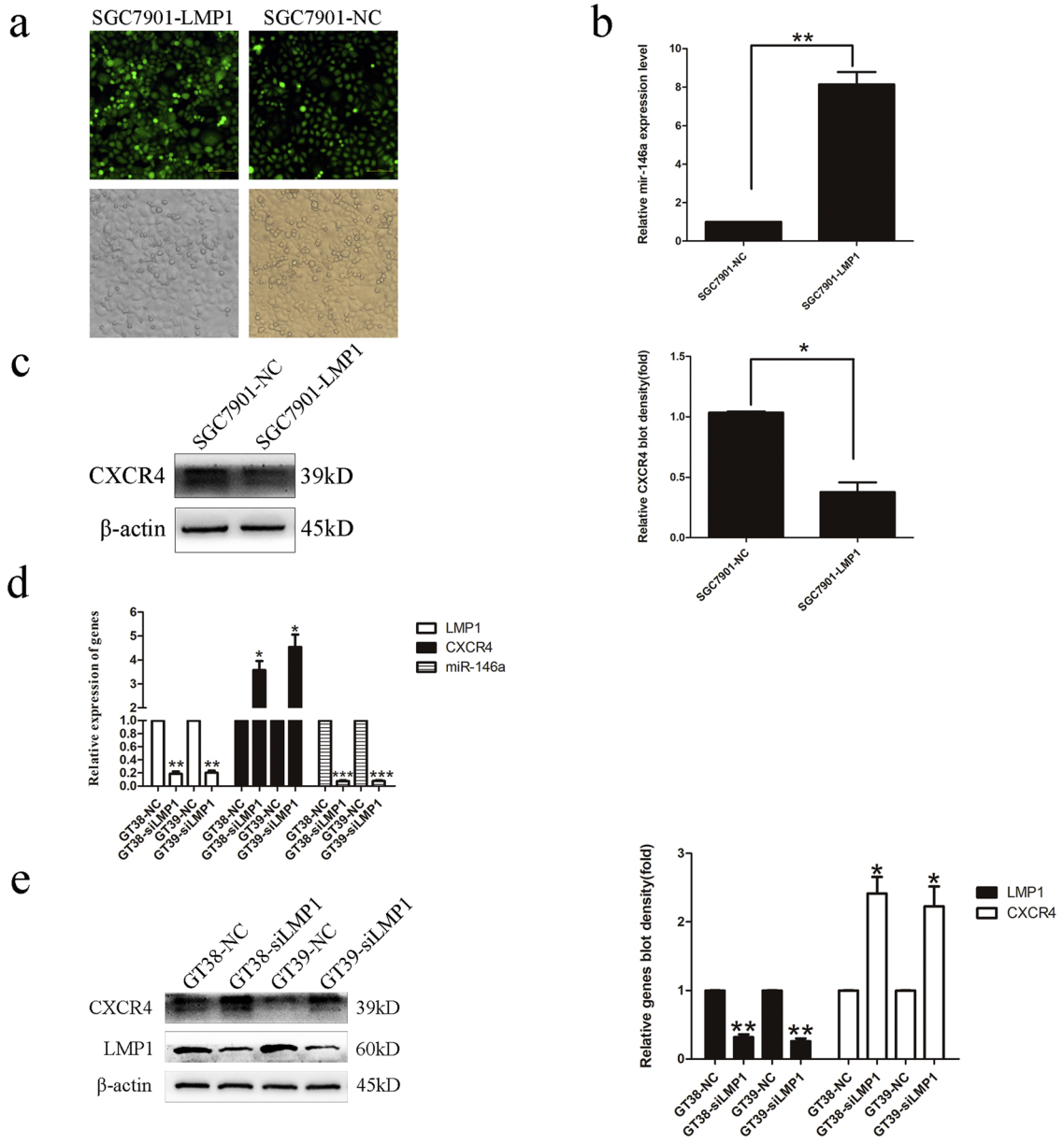


**Fig. 6.** Effects of EBV LMP1 on miR-146a–CXCR4 expression.
(a) Transfection efficiency was detected by fluorescence microscopy in SGC7901-LMP1 and SGC7901-NC cells. (b)The expression of miR-146a was detected by transfected with LMP1 plasmid using the comparative Cycle threshold (Ct) value ($2^{-\Delta\Delta Ct}$) (**$p < 0.01$). (c) The protein level of CXCR4 in SGC7901-LMP1 cell was detected by Western blot (*$p < 0.05$). (d) The levels of miR-146a and CXCR4 were assessed by inferencing of LMP1 in GT38 and GT39 cells (***$p < 0.005$, **$p < 0.01$, *$p < 0.05$). (e) The protein of CXCR4 was analyzed by Western blot with the treatment of siLMP1 (**$p < 0.01$, *$p < 0.05$). Data are representative of three independent experiments.

cells.

### 3.2. Relative expression of CXCR4 in EBV-associated cell lines

qRT-PCR and Western blot analysis showed significantly higher CXCR4 expression in EBV-negative cells than in EBV-positive cells (Fig. 2a and b). As expected, CXCR4 was up-regulated in EBV-negative cells, which is reverse-correlated with miR-146a expression.

### 3.3. MiR-146a regulates the expression of CXCR4 in a dose- and time-dependent manner

In this study, we investigated GT39 cells with high miR-146a levels and SGC7901 cells with low miR-146a levels, repression of miR-146a in GT39 cells by miR-146a inhibitor, and overexpression of miR-146a in SGC7901 cells by miR-146a mimics. As shown in Fig. 2c, 10, 30, and 50 nM miR-146a inhibitors were transfected in GT39 cells, and we found a gradual increase in CXCR4 levels compared with negative controls (NC), with no reduction in the 10 nM miR-146a inhibitor. Simultaneously, GT39 cells transfected with the 50 nM miR-146a inhibitor for 24 and 48 h showed an obvious increase in CXCR4 levels in a time-dependent manner (Fig. 2d). SGC7901 with miR-146a mimics expressed an adverse tendency (Fig. 2e and f) compared with GT39 cells transfected with miR-146a inhibitors. These results suggest that miR-146a functions as a negative modulation factor for CXCR4 in EBV-associated cells.

### 3.4. Effects of miR-146a on cell growth, migration and apoptosis

To study the potential role of miR-146a in EBV-associated cell

progression, miR-146a inhibitors were transfected into GT39 cells. As shown in Fig. 3a, transfection of miR-146a inhibitors facilitated cell proliferation, whereas restoration of miR-146a suppressed cell proliferation (Fig. 3b). We next detected the effects of miR-146a on cell cycle propagation using PI staining analysis. Compared with the controls, GT39 cells showed an increased accumulation of G0/G1 phase cells 48 h after treatment with miR-146a inhibitors ($p < 0.05$ and $p < 0.01$) (Fig. 3c). Similarly, SGC7901 cells showed an obvious decrease in G0/G1 phase cells by miR-146a mimics (Fig. 3d). In addition, miR-146a disruption clearly promoted the migration of GT39 cells compared with GT39-NC cells (Fig. 4a), whereas miR-146a mimics could suppressed the migration of SGC7901 cells (Fig. 4b). Moreover, cell apoptosis notably decreased after suppression of miR-146a and increased after miR-146a overexpression in SGC7901 cells (Fig. 4c and d). These results suggest that miR-146a suppresses cell proliferation, decreases the population of G0/G1 phase cells, inhibits cell migration, and increases cell apoptosis.

### 3.5. Effects of CXCR4 on cell growth, migration and apoptosis in vitro

SGC7901 cells were treated with CXCR4 siRNA to explore the effects of CXCR4 on cell phenotypic functions. The transfection efficiency was assayed using qRT-PCR and Western blot analysis. The CCK8 assay showed that CXCR4 siRNA decreased the OD of SGC7901 cells compared with SGC7901-NC cells (Fig. 5a). And migration assay was performed to test the effects of CXCR4 siRNA on the migration ability of SGC7901 cells (Fig. 5b). We found that the number of migrated cells decreased in the group treated with CXCR4 siRNA. In addition, CXCR4 siRNA treatment increased the apoptosis rate in SGC7901 cells (Fig. 5c). We also assessed the effects of CXCR4 on the cell cycle using PI staining analysis with CXCR4 siRNA transfection. We found that the population of G0/G1 phase cells decreased compared with the controls (Fig. 5d). These results demonstrate that CXCR4 siRNA promotes SGC7901 cell apoptosis while inhibiting the proliferation, migration, and accumulation of G0/G1 phase cells. In addition, we detected the expression of ERK1/2 and PI3K/AKT-related proteins using Western blot analysis after CXCR4 treatment (Fig. 5e). The results suggest that CXCR4 siRNA significantly decreases AKT and ERK phosphorylations in SGC7901 cells compared with the controls.

### 3.6. Effects of LMP1 on miR-146a and CXCR4 expression

Fig. 6a shows the transfection efficiency of LMP1 and NC plasmids in SGC7901 cells, and Fig. 6b shows the increased expression of miR-146a after transfection of LMP1 in SGC7901 cells (SGC7901-LMP1) compared with SGC7901-NC cells. CXCR4 expression in SGC7901-LMP1 cells was lower than in SGC7901-NC cells (Fig. 6c; $p < 0.05$), which suggests that LMP1 might enhance miR-146a expression to down-regulate CXCR4 expression. Simultaneously, treated with LMP1 siRNA, GT38 and GT39 cells show an obvious decrease of miR-146a expression, followed by up-regulation of CXCR4 at the mRNA and protein levels (Fig. 6d and e).

## 4. Discussion

MiRNAs, found in many different cancers, are associated with the development of tumors, including oncogenesis and tumor suppression (Sassen et al., 2008). In this study, miR-146a was up-regulated in EBV-positive compared with EBV-negative cells. Previous studies have shown that miR-146a mainly participates in adaptive immunity and innate immune responses (Nahid et al., 2009; Taganov et al., 2006). In addition, miR-146a has been proven to inhibit tumor growth and metastasis in breast cancer (Hurst et al., 2009). MiR-146a overexpression inhibits the invasive capacity of pancreatic cancer cells by down-regulation of EGFR and NF-κB regulatory kinase interleukin 1 receptor–-associated kinase 1(IRAK-1) (Li et al., 2010). However, the role of miR-146a in EBV-associated cells has not yet been researched.

We predicted potential target genes by three bioinformatics tools: miRDB, DIANA and starbase. We found 42 genes, the co-predicting target genes, by enrichment analysis of cell signaling pathways. CXCR4-mediated signaling was significantly enriched. We investigated whether CXCR4 is the most potential target gene regulating the biological functions of cancer cells. Studies have reported that miR-146a aggravates lipopolysaccharide-induced inflammatory injury by targeting CXCR4 in the articular chondrocytes, and the targeting effects of miR-146a on the 3′-UTR of CXCR4 have been assessed by luciferase activity analysis (Sun et al., 2017).

In this study, we found CXCR4 expression is down-regulated in EBV-positive cells with high miR-146a expression, whereas CXCR4 expression is up-regulated in EBV-negative cells with low miR-146a expression. Moreover, in GT39 cells transfected with miR-146a inhibitors, CXCR4 expression showed a gradual increase in a dose- and time-dependent manner, whereas SGC7901 cells with miR-146a mimics showed the exactly opposite results. In summary, miR-146a influences the biological functions of EBV-associated cells by targeting CXCR4.

CXCR4, a seven-transmembrane G-protein coupled receptor, transduces its signal to various pathways on binding with CXCL12 (Katsumoto and Kume, 2011). CXCR4 can activate the Ras/Raf/MEK/ERK pathway, which modulates cell cycle progression via phosphorylation of the adaptor protein Shc (Chang et al., 2003; Loetscher et al., 1994). In parallel, CXCR4-oriented migration is mediated by PI3Ks (Ward, 2006). Moreover, CXCR4 plays a critical role in promoting cancer cell survival by activating the NF-κB signaling pathway (Vlahakis et al., 2002). In this study, CXCR4 activated the PI3K/AKT and ERK1/2 signals, which might be part of the mechanism through which CXCR4 regulates cellular function. On the basis of existing research, we detected the role of miR-146a by targeting CXCR4. MiR-146a mimics suppressed cell survival by decreasing the population of G0/G1 phase cells, which was detected by PI staining analysis. MiR-146a mimics also repressed cell migration and induced cell apoptosis, which had the same cellular effects as interference with CXCR4. In addition, miR-146a inhibitors, indicating moderately increased CXCR4 expression, could be advantageous for cell proliferation and migration and disadvantageous to cell apoptosis. EBV-positive cells had specific biological features different from EBV-negative cells, regulated by miR-146a–CXCR4 axis, suggesting that miR-146a–CXCR4 might be a potential treatment target.

By transfecting the LMP1 plasmid and LMP1-siRNA, we verified that LMP1 could increase miR-146a expression. The result is consistent with several studies. Studies have shown substantial expression of miR-146a in latency III cell lines (which express LMP1) but not in latency I cell lines (Cameron et al., 2008). In this study, LMP1 enhanced miR-146a expression in EBV-positive cells (GT38 and GT39) that expressed LMP1 (Tajima et al., 1998). Studies have also indicated the LMP1 induces miR-146a expression via the NF-κB signaling pathway (Motsch et al., 2007). Our previous data proved that LMP1 promoted NF-κB pathway activation in EBV-associated cells. Therefore, LMP1 induces miR-146a expression by NF-κB signaling in EBV-associated cells, thereby influencing cell phenotypes by regulating CXCR4 expression.

This study also demonstrated that miR-146a could decrease cell proliferation, migration and increase cell apoptosis by down-regulating CXCR4 expression, implying that miR-146a acts as a suppressor in EBV-associated cells. LMP1 might regulate EBV-associated cells growth, migration and apoptosis, thereby evading the immune system. Studies have shown that LMP1 mimics CD40 signaling as part of its “normal” biological functions (Uchida et al., 1999) and expresses viral miRNAs or regulates host miRNA expression (Polakovicova et al., 2018) to evade the immune system. Studies have also shown that CXCR4 antagonists could augment antitumor immune responses and lead to tumor-free survival (Komorowski et al., 2016). Therefore, the LMP1–miR-146a–CXCR4 axis might be another potential mechanism of LMP1-mediated immune system evasion. In addition, latency cells treated

with lytic-inducing agents resulted in the arrest of G0/G1 phase cell growth (Rodriguez et al., 2001). This study found that miR-146a decreased the arrest of G0/G1 phase cell growth, which may lead to maintain of latently infected cells. Previous studies have shown that LMP1 signals through TRAFs or TRADD and activates NF-KB, JNK, and p38 signaling pathways to promote the survival and growth of EBV-infected cells (Soni et al., 2007). Therefore, a balance might exist between the ability of LMP1 to promote cell growth and its role in escaping anti-tumor immune responses.

In summary, the LMP1–miR-146a–CXCR4 axis plays an important role in EBV-positive cells, providing a therapeutic alternative for successful treatment. However, considering the difference between cells and tissues, experimental results at the cell level cannot be analogized to histological levels; therefore, further investigations on the possible mechanisms of EBV infection and the role of CXCR4 in EBV-associated carcinoma tissues are needed.

## Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Medical Ethics Committee at the Medical College of Qingdao University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

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