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Propofol suppresses proliferation, migration and invasion of gastric cancer cells *via* regulating miR-29/MMP-2 axis

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Abstract. – OBJECTIVE: Propofol (2,6-diisopropylphenol) is a commonly used intravenous anesthetic agent. Previous studies suggested that propofol might act as anti-tumor drug in various cancers, including gastric cancer. However, the underlying mechanism is still largely unknown.

MATERIALS AND METHODS: 1, 5, 10 and 20 μg/ml of propofol were used to treat gastric cancer cell MKN45 for 24, 48 or 72 hours. MTT assay was used to detect the proliferation. Transwell assay was employed to measure the invasion migration with or without matrigel. The expression of miR-29a, 29b and 29c was assessed by quantitative real time polymerase chain reaction (qRT-PCR). Luciferase assay was introduced to confirm the relationship between miR-29 family member and MMP-2. Western blot was adopted to measure the expression of MMP-2 protein.

RESULTS: The proliferation, migration and invasion of gastric cancer cell MKN45 were gradually decreased after propofol treatment in time- and dose- dependent manners. MiR-29a, b and c were downregulated in MKN45 cells compared with normal gastric mucosa epithelial cell GES-1 and upregulated by propofol. Inhibition of miR-29a, b or c promoted cell proliferation, migration and invasion of MKN45 cells under propofol treatment. MMP-2 was a target and regulated by miR-29 family. Propofol MMP-2 silencing reversed the stimulative effect of miR-29 inhibitor.

CONCLUSIONS: Propofol inhibited cell proliferation, migration and invasion by upregulating miR-29a, miR-29b and miR-29c and downregulating MMP-2.

Key Words:
Propofol, miR-29, MMP-2, Gastric cancer.

Introduction

Gastric cancer is one of the most commonly diagnosed gastrointestinal malignancies and the second leading cause of cancer-related death with high mortality worldwide. Like other cancers, the occurrence of gastric cancer is chalked up to multifactors such as unhealthy dietary habit, improper lifestyle, alcohol abuse, inflammation and family history[1-3]. Despite the advances made in early diagnosis and treatment, the prognosis of gastric cancer patient is still unsatisfactory[4]. It has been reported that the 5-year survival rate of gastric cancer is approximately 60% for patients with primary carcinoma but only 2% for the metastatic-stage patients[5]. Thus, it is urgent and helpful to search novel and effective therapeutic strategy for gastric cancer treatment.

Propofol (2,6-diisopropylphenol) is a commonly and extensively used intravenous anesthetic in clinic[6]. Besides, propofol also exerts various non-anesthetic functions, such as antiemetic, anxiolytic, neuroprotective and immunomodulatory activity[7]. Emerging evidence[8] has suggested that propofol has anti-tumor effects. Liu et al[9] reported that propofol could inhibit cell proliferation and epithelial-mesenchymal transition in lung cancer cells *in vitro*. Du et al[10] revealed that propofol inhibited the proliferation, migration and invasion as well as induced apoptosis in endometrial cancer cells. Huang et al[11] suggested that propofol inhibited the proliferation and invasion of ovarian cancer cells. Bai et al[12] proved that propofol suppressed the migration and invasion of breast cancer. In term of gastric cancer, propofol also acts as an anti-tumor medicine. Wang et al[13] concluded that propofol could inhibit the expression of microRNA-221 and suppressed cell proliferation and invasion in gastric cancer cells. However, the detailed molecular mechanism of propofol in gastric cancer is needed to be further explored. MicroRNAs (miRNAs) are a group of short, sin-

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gle-strand, endogenous and non-protein-coding RNAs. Mechanically, miRNAs mediate the degradation or translational suppression of target messenger RNAs (mRNAs) *via* directly binding to the 3'-untranslated regions (3'-UTR) [14]. It has been reported that miRNAs are related with many biological processes such as cell growth, differentiation, development and apoptosis. Moreover, dysregulated expression of miRNAs is involved in many diseases including cardiovascular disease [15], immune disease [16], neurodegenerative disease [17] and even cancers [18]. Meanwhile, miRNAs are a providing therapy target in cancer treatment [19]. MiR-29 family in human contains hsa-miR-29a, hsa-miR-29b and hsa-miR-29c, which contribute to the progression of various cancers. For example, miR-29a acts as a tumor suppressor in pancreatic cancer [20]; miR-29b can regulate the migration of human breast cancer cells [21]; miR-29c can inhibit cell proliferation and induce apoptosis in hepatocellular carcinoma [22]. However, it is not clear the relationship between miR-29 family and gastric cancer.

In the present study, we firstly analyzed the effects of propofol on cell proliferation, migration and invasion in gastric cancer cell line MK Subsequently, we suggested that miR-29 fa was downregulated in gastric cancer, which co be elevated by propofol treatment. Th we co firmed miR-29 regulated the expr MMP-2 *via* directly binding and sileno MMP which could attenuate the promoti mi tor on cell proliferation, m vation nvasion of MKN45 cells. This fi provide e mechanism of propofol in ric cer treat t.

Materials and Methods

Cell Culture and Treatment

Human normal gastric mucosa epithelial cell line GES- gastric cancer cell line MKN45 and 2 cells were purchased from CoBioer Biosci Cp., LTD (Nanjing, China). All the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin solution (Gibco, Thermo Fisher, Waltham, MA, USA). The medium was changed every two days. The cells were treated with propofol (Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 0, 1, 5, 10 and 20 μg/ml for 0, 24, 48 or 72 hours.

Transfection

miR-29a, miR-29b and miR-29c inhibitor, siRNA-MMP-2 and their corresponding negative control were purchased from GenePharma Co. (Shanghai, China) and transfected into MKN45 cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 hours, the cells were treated with 10 μg/ml propofol for another 48 hours.

MTT Assay

Cell proliferation was termi using TT assay. For the untransf d cells, 1x were seeded into each of 9 ell pla for the transfected cells the ls re tran fected firstly in 6-well plat d aft hour e cells were trypsinized made int cell suspension, then 1x1 cel ere seed into each well of 96-well plate. A that, the cells were treated wi thout 10 μ l propofol for another 48 h s. Next, 10 μl MTT was added into each well a incubated r 4 h at 37°C. After discarding t edium, μl dimethyl sulfoxide (DMSO) (Sig , St. Louis, MO, USA) was added and read the absorbance at 490 nm.

Transwell Assay

The migration was performed with transwell chamber (Sigma-Aldrich, St. Louis, MO, USA). The untransfected or transfected cells were seeded into 6-well plate and treated with 10 μg/ml propofol for 48 hours. Next, $1x10^5$ cells of each sample were seeded into the upper chamber with serum-free DMEM. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) was added into the lower chamber. After incubation at 37°C for 24 hours, the cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA) and stained with 0.1% crystal violet for 30 min followed by washing with PBS three times. For invasion assay, matrigel (BD Bioscience, Franklin Lakes, NJ, USA) was coated in the transwell.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total small RNA (<200 nt) was isolated with mirVanamiRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA). Then cDNA was reversed transcribed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primers for miR-29a, b and U6 were purchased from GenePharma

Co. (Shanghai, China). U6 small nuclear RNA (snRNA) was used to normalize miR-29a, b and c. Fluorescence was detected with SYBR Green (Promega, Madison, WI, USA) and QuantStudio™ 3 Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The primers used in this research are as follows: miR-29a forward: 5'-TGCGCTAGCACCATCTGAAAT-3' and reverse: 5'-CAGTGCAGGGTCCGAGGAT-3'. miR-29b forward: 5'-ACACT CCAGCTGGGTAGCACCATCTGAAA-3' and reverse: 5'-CTCAACTGGTGTCGTGGA-3', miR-29c forward: 5'-CGCGCGTGACCGATTTCTCCTG-3' and reverse: 5'-ATCCAGTGCAGGGTCCGAGG-3', U6 forward: 5'-GCGCGTCGTGAAGCGTTC-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3'. β-actin forward: 5'-GGACCTGACTGACTACCTC-3' and reverse: 5'-CCTGCTTGCTG-3', respectively. The relative expression of miR-29a, b and c was calculated by the $2^{-\Delta\Delta}$Ct method.

Luciferase Assay

Wild-type (WT) MMP-2 3' untranslated region (3'UTR) containing the putative miR-29 binding sites or a mutant sequence was inserted into downstream of the firefly luciferase gene in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, Madison, WI, USA). The reconstructed plasmids were designated as MMP-2 3'UTR WT and MMP-2 3'UTR MUT. 293T cells were transfected with miR-29a, miR-29b and miR-29c mimic or negative control (miR-NC) and MMP-2 3'UTR WT (or MUT) were collected at 48 h after the transfection. Then, the luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). Luciferase activity values were normalized to that of the Renilla luciferase internal control.

Western Blot

Proteins were extracted with RIPA lysis buffer following the protocol. Equal amount of each protein sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA, USA). The membranes were blocked with 5% dried non-fat milk in TBS, and then they were incubated with specific primary antibodies including anti-MMP-2 (ab40994, Abcam, Cambridge, MA, USA, 1:100) and anti-β-actin (ab8227, Abcam, Cambridge, MA, USA, 1:1000) antibody at 4°C overnight. The polyvinylidene difluoride (PVDF) membrane was washed three times for 10 min each with Tris-buffered saline with Tween 20 (TBST), following incubation with horseradish peroxidase (HRP)-conjugated IgG secondary antibody (1:5000 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature for 1 hr. After the protein signals were washed with TBST three times, they were detected using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

The data were displayed as mean ± SD (standard deviation) from three biological replicated experiments or as indicated. One-way analysis of results was analyzed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Student's *t*-test was used to analyze the group comparisons. The *p*-values less than 0.05 were regarded as statistically significant.

Results

Propofol Inhibited Cell Proliferation, Migration and Invasion in MKN45 Cells

Previous studies suggested that propofol can suppress cell proliferation and invasion in various cancers [23]. Then we checked the effects of propofol in gastric cancer. Firstly, we treated gastric cancer cell MKN45 with propofol at the concentration of 0, 1, 5, 10 and 20 μg/ml for 48 hours and used MTT assay to assess the proliferation. As shown in Figure 1A, it significantly decreased under 5, 10 and 20 μg/ml propofol treatment compared with the control, which was treated with 0 μg/ml. Next, we used 10 μg/ml propofol to treat MKN45 cells for 24, 48 and 72 hours, and MTT results indicated that cell proliferation gradually decreased while the treating time was prolonged (Figure 1B). Subsequently, we introduced transwell assay to measure the effects of propofol in cell migration. Similarly, we treated MKN45 cells with different concentration of propofol and the number of migrated cells dramatically reduced when the concentration increased (Figure 1C). At the same time, cell migration was ablated seriously treated with 10 μg/ml propofol for 48 and 72 hours (Figure 1D). Meanwhile, the invasion of MKN45 was weakened by propofol treatment (Figure 1E-1F). These results indicated that the proliferation, migration and invasion of MKN45

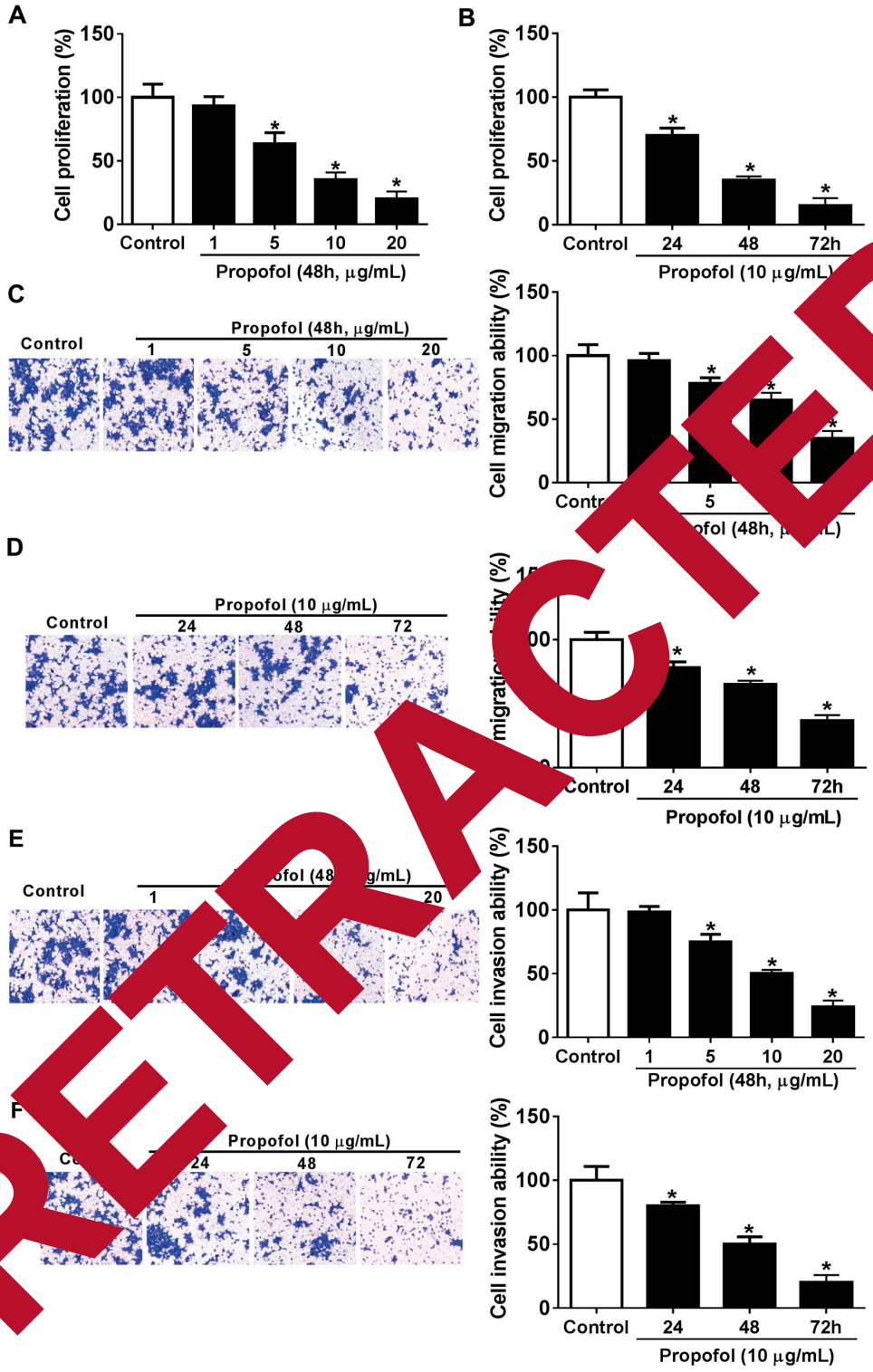

Figure 1. Propofol inhibited cell proliferation, migration and invasion in MKN45 cells. **A,** MTT assay analysis of MKN45 after treatment with 0, 1, 5, 10 and 20 μg/ml propofol for 48 hours. **B,** The proliferation of MKN45 after treatment with 10 μg/ml propofol for 0, 24, 48 and 72 hours. **C,** Transwell assay of MKN45 after treatment with 0, 1, 5, 10 and 20 μg/ml propofol for 48 hours. **D,** The migration of MKN45 after treatment with 10 μg/ml propofol for 0, 24, 48 and 72 hours. **E,** The invasion ability of MKN45 after treatment with 0, 1, 5, 10 and 20 μg/ml propofol for 48 hours. **F,** The migration of MKN45 after treatment with 10 μg/ml propofol for 0, 24, 48 and 72 hours. * mean p value < 0.05.

cells were inhibited by propofol in dose- and time- dependent manners.

MiR-29 Family was Upregulated After Propofol Treatment

It was suggested that miR-29c can be induced by chemotherapy and suppressed the metastasis in gastric cancer [24]. Thus, we firstly surveyed the expression of all miR-29 family members including miR-29a, b and c in gastric cancer cells. Compared with that in human normal gastric mucosa epithelial cell line GES-1, miR-29a, b and c expressed lower in MKN45 cells (Figure 2A). Considering that propofol inhibited the metastasis in gastric cancer, to test whether miR-29 family was involved in this process, we assessed their expression in MKN45 cells after propofol treatment. The expression of miR-29a, b and c was increased about 2 to 4 folds by propofol in gastric cancer (Figure 2B). These results suggested that miR-29 family was downregulated in gastric cancer and could be increased by propofol.

Effects of miR-29 Inhibitors on Proliferation, Migration and Invasion in Propofol Treated Gastric Cells

Next, to better understand the relation[ship] between propofol and miR-29 family, we sy[n]thetized miR-29 inhibitors (in-miR-29a, in-miR-29b, in-miR-29c) and the [mu]tant control (in-miR-NC); then, we trans[fected] the inhibitors into MKN45 cells, re[spec]tively. The results showed that the knock[down] efficiencies were around 60% (Figure 3A). After the transfection for 24 hours, we treated the cells with 10 μg/ml propofol for another 48 hours. Next, the proliferation of transfected MKN45 was promoted after propofol treatment (Figure 3B). Simultaneously, the migration (Figure 3C) and invasion (Figure 3D) ability was elevated in the transfected and treated cells. These data hinted that miR-29 family knockdown could hoist the proliferation, migration and invasion in MKN45 cells after propofol treatment.

MMP-2 was a Target of miR-29

MicroRNA may regulate the gene expression by directly binding to the 3'UTR, inhibiting the translation or mediating the degradation, we predicted the mRNA targets of miR-29 family using TargetScan. Then the bioinformatics analysis showed that MMP-2 was a potential target. So, we constructed the luciferase reporter vectors containing the complementary sequences in the 3'UTR of MMP-2 or the mutant form (Figure 4A) and co-transfected the reporter vectors into 293T cells with different miR-29 inhibitors. The luciferase activity decreased when co-transfected the reporter vector containing with the 3'UTR of MMP-2 and miR-29a, b and c, but not with the mutant (Figure 4B). In addition, we measured MMP-2 expression after propofol treatment and propofol inhibited MMP-2 protein expression in MKN45 cells (Figure 4C). To further validate the relationship between miR-29a and MMP-2, we quantified endogenous MMP-2 protein expression in MKN45 cells transfected with miR-29a, b and c mimics. The Western blot results demonstrated that MMP-2 was significantly reduced after transfection (Fig-

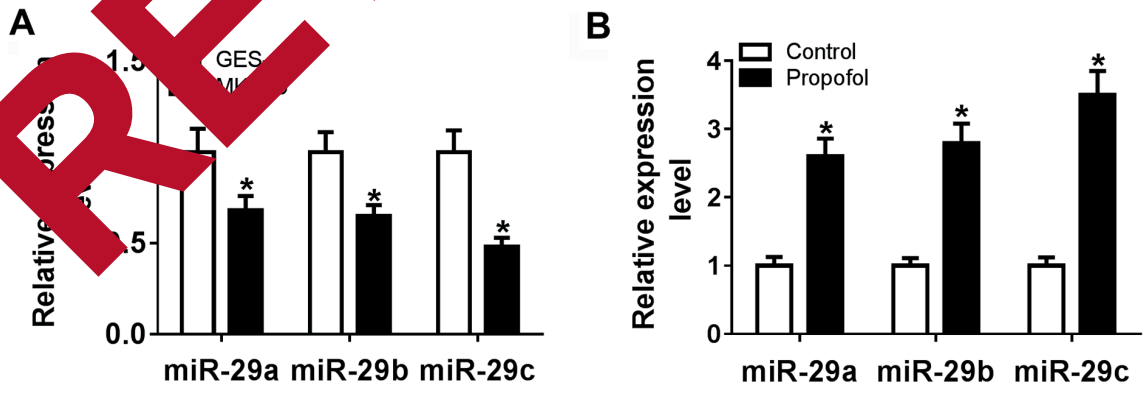

Figure 2. MiR-29 family was upregulated after propofol treatment. **A,** Quantitative real-time PCR analysis of miR-29a, b and c in human normal gastric mucosa epithelial cell line GES-1 and gastric cancer cell line MKN45. **B,** The expression of miR-29a, miR-29b and miR-29c in gastric cancer cell line MKN45 after 0 or 10 μg/ml propofol treatment for 48 hours. * mean p-value < 0.05.

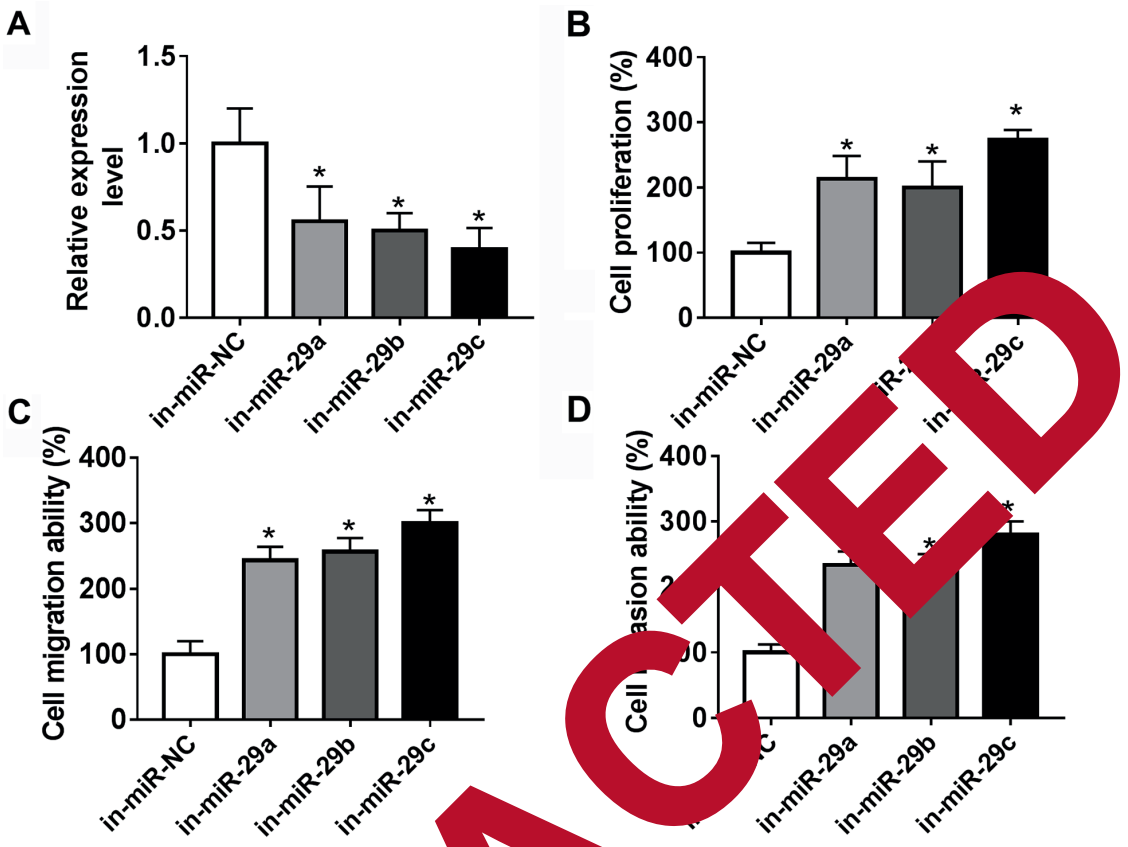

Figure 3. MiR-29 inhibitor promoted cell proliferation, [illegible] invasion. **A,** The expression of miR-29 family member in MKN45 cells after transfection with corresponding mi[illegible]A inhibitor. **B,** Proliferation of MKN45 cells transfected with miR-29a, miR-29b and miR-29c inhibitor[illegible]ive con[illegible]followed with 10 μg/ml propofol treatment for 48 hours. **C,** Migration ability of MKN45 cells transfe[illegible]with [illegible]R-29a, m[illegible]9b and miR-29c inhibitor and negative control followed with 10 μg/ml propofol treatment for 48 h[illegible]. **D,** Inv[illegible]n ability [illegible]MKN45 cells transfected with miR-29a, miR-29b and miR-29c inhibitor and negative control fol[illegible]we[illegible]h 1[illegible]fol treatment for 48 hours. * mean p value < 0.05.

ure 4D). These [illegible]ults cued[illegible]t miR-29 family regulated MM[illegible]2 expression [illegible]irect binding.

MMP-2 Si[illegible]in[illegible]ever[illegible]ed the Effects of miR-29 Inhi[illegible]

To [illegible]r ex[illegible]e role of miR-29 and M[illegible]P-2 in [illegible]opofol[illegible]ated gastric cells, we intro-[illegible]d s[illegible]fore RNA (si-MMP-2) to knock-do[illegible]e MMP-2 expression in MKN45 cells (Figu[illegible]A). Next, we co-transfected si-MMP-2 into MK[illegible]5 cells with the inhibitor of miR-29a, b or c for 24 hours followed with 10 μg/ml propofol for another 48 hours. The cell proliferation was attenuated in co-transfected cells compared with the cell transfected with miR-29 inhibitor and siRNA negative control (Figure 5B, 5C and 5D). Moreover, the migrated cells were decreased in co-transfected cells (Figure 5E, 5F and 5G) as well as the invaded cells (Figure 5H, 5I and 5J). These data indicated the MMP-2 silencing attenuated the effects of miR-29 inhibitor in cell proliferation, migration and invasion in gastric cells.

Discussion

Propofol has been suggested to act as tumor suppressor in various cancers. In pancreatic cancer, propofol inhibits cell migration as well as the expression of VEGF, and HIF-1α, and makes inactive ERK and AKT[26]. In lung cancer[27], propofol decreases the cell viability *via* regulating miR-486, FOXO1, FOXO3, BMI and caspase 3. Propofol can inhibit hepatocellular cell growth by activating the macrophage to secrete microvesicles that could deliver miR-142-3p to the tumor cells[28]. In gastric cancer, propofol also performs anti-tumor effects such as inhibiting proliferation, pro-

moting apoptosis[29]. In the present work, we found that propofol could inhibit the proliferation, migration and invasion ability of gastric cancer cell MKN45 that was consistent with previous studies. To further explore the mechanism of propofol in gastric cancer, we focused on miR-29 family.

MiR-29 family includes three mature members, miR-29a, 29b and 29c, which are encoded by two genes cluster and share the conservative sequences. MiR-29 family has been suggested to be involved in many biological processes including innate and adaptive immune response[30], cellular senescence[31] and neuronal maturation[32]. Recently, many studies hinted that miR-29 may serve as tumor suppressor in several cancers and downregulation of miR-29 family is correlated with the progression of hepatocellular cancer, colon cancer, cervical cancer and etc. We found that miR-29 family members were downregulated in MKN45 cells and propofol treatment elevated the expression of miR-29a, 29b and 29c. So, we hypothesized that propofol displayed the anti-tumor effect *via* upregulating miR-29. Next, we predicted that MMP-2 is a poten[illegible] mRNA target of miR-29 *via* bioinformati[illegible]rase assay. Besides, MMP-2 expre[illegible] was reg[illegible]ed by propofol and miR-29 in M[illegible] cells.

MMP-2 (Matrix Met[illegible]pepti[illegible]2) b[illegible]gs to the matrix metall[illegible]oteinase [illegible]which is capable of cleav[illegible] com[illegible]nts o[illegible] extra-

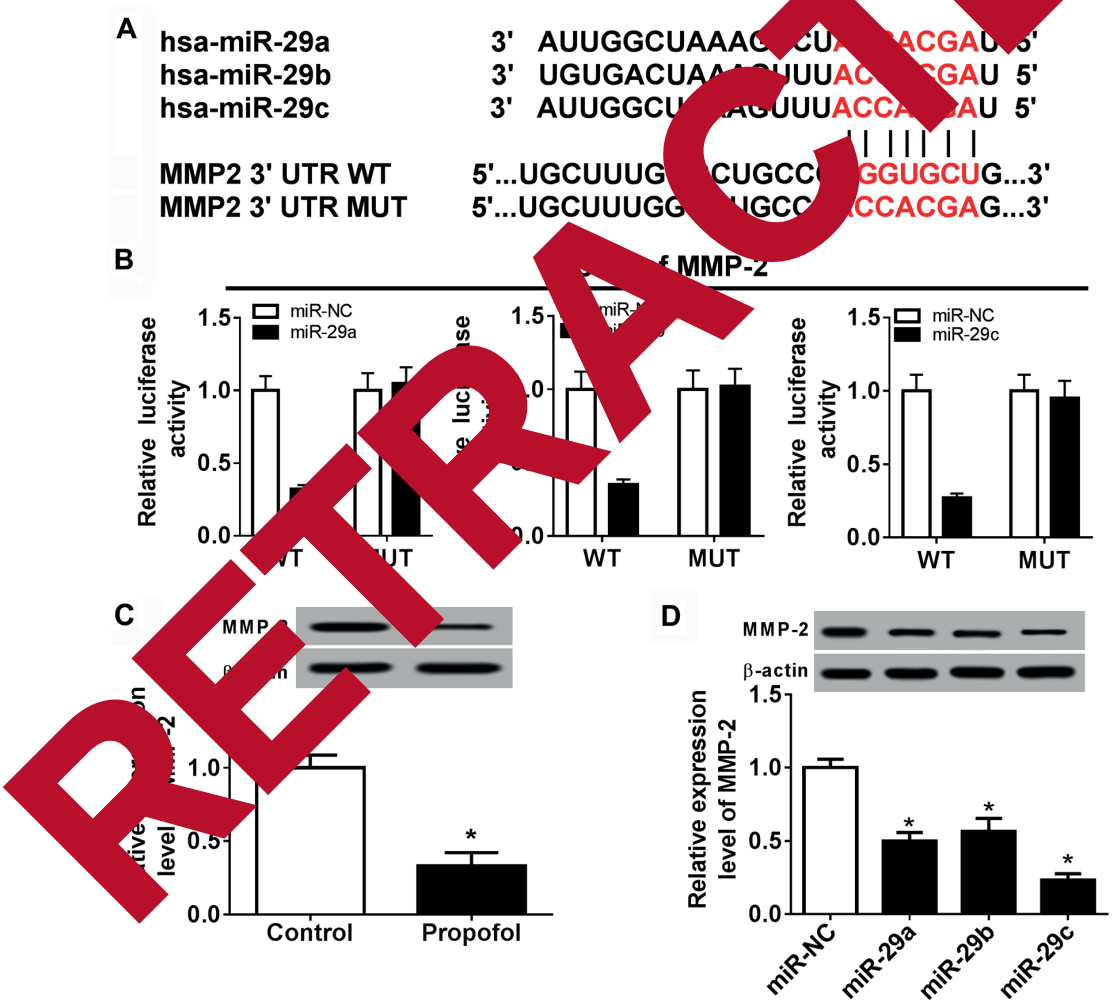

Figure 4. MMP-2 was a target of miR-29. **A,** The predicted binding sites between miR-29 family member and MMP-2. **B,** Luciferase assay of miR-29a, miR-29b and miR-29c and MMP-2 3'UTR WT or MUT in HEK293T cells. **C,** Western blot analysis of MMP-2 in MKN45 cells after 10 μg/ml propofol treatment for 48 hours. **D,** The protein expression of MMP-2 in MKN45 after transfected miR-29a, miR-29b and miR-29c mimic followed by 10 μg/ml propofol treatment for 48 hours. * mean p-value < 0.05.

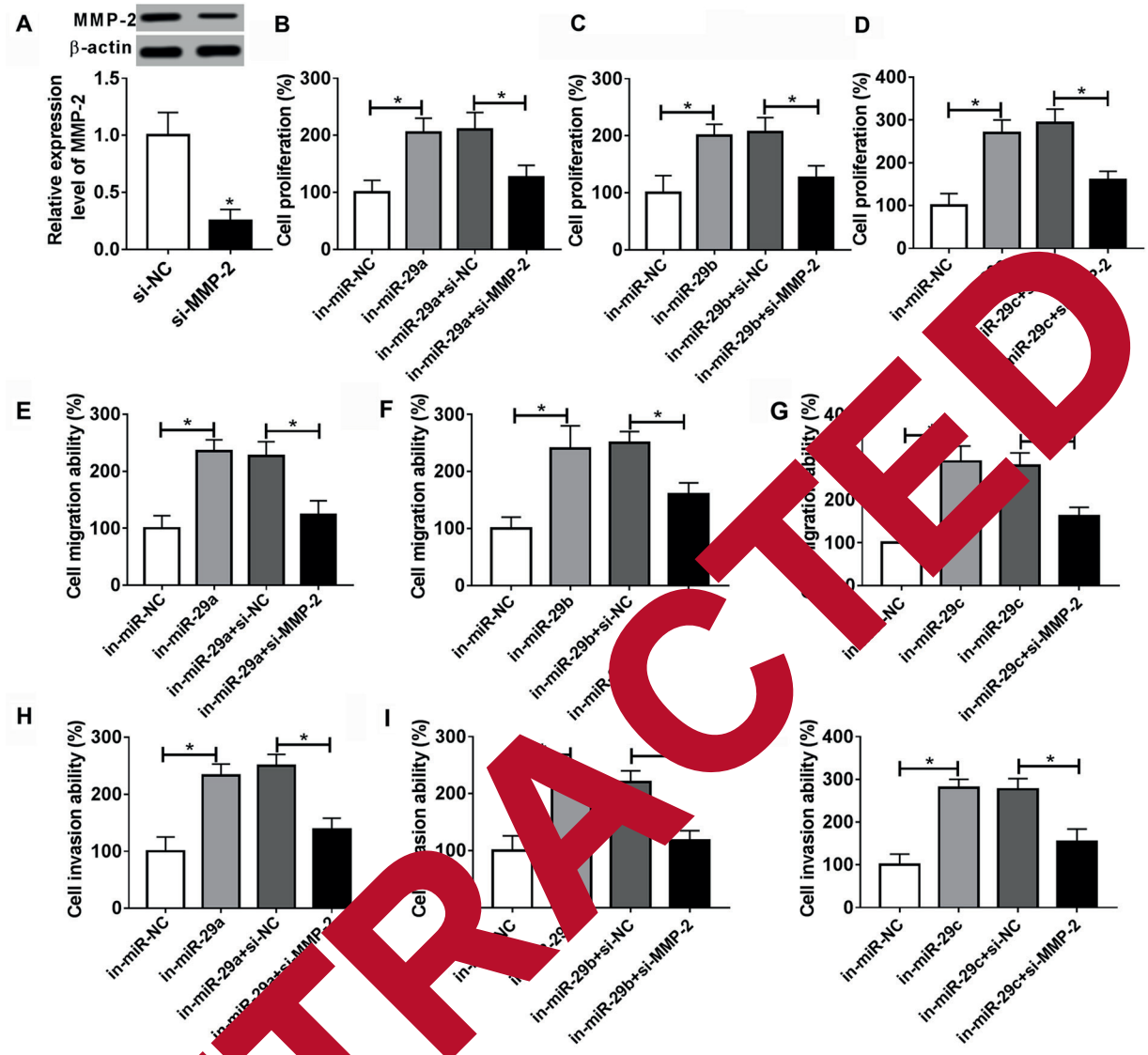

Figure 5. MMP-2 silencing reverses the effects of miR-29 inhibitor. **A,** MMP-2 protein level in MKN45 cells after transfection with small interfere RNA of MMP-2 (si-MMP-2). **B-D,** Cell proliferation of MKN45 cells after co-transfection with miR-29a **B**, miR-29b **C**, miR-29c **D,** inhibitor and si-MMP-2 followed with 10 μg/ml propofol treatment for 48 hours. **E-G,** Cell migration of MKN45 cells after co-transfection with miR-29a **E**, miR-29b **F**, miR-29c **G,** inhibitor and si-MMP-2 followed with 10 μg/ml propofol treatment for 48 hours. **H-J,** Cell invasion ability of MKN45 cells after co-transfection with miR-29a **H**, miR-29b **I**, miR-29c **J** inhibitor and si-MMP-2 followed with 10 μg/ml propofol treatment for 48 hours. * mean p-value < 0.05.

cellular matrix and molecules involved in signal transduction. It has been reported that MMP-2 is involved in the metastasis of various cancers including breast cancer[33], bladder cancer[34], papillary thyroid cancer[35] and esophageal cancer[36]. In gastric cancer, MMP-2 is related with the proliferation, migration, invasion and prognosis[37,38]. We claimed that MMP-2 was upregulated in gastric cancers and inhibited by propofol treatment. In addition, MMP-2 silencing attenuated the stimulative effects of miR-29 inhibitor in cell proliferation, migration and invasion.

Conclusions

Taken together, our work showed that propofol exerts anti-tumor effects by inhibiting cell

proliferation, migration and invasion in gastric cancer. Moreover, propofol upregulated the expression of miR-29a, b and c in MKN45 cells while miR-29 inhibitor reversed the inhibitory effects of propofol. In addition, silencing MMP-2, which is a downstream target of miR-29, could attenuate the effects of miR-29 inhibitor. However, there are still many mysteries which need to be explored, for example how propofol regulates the expression of miR-29, whether there are any other downstream targets of propofol and miR-29, whether there will be a better outcome if combining propofol with other anti-tumor drugs, and these will be our future work proposal. In brief, propofol exerts the anti-tumor effects *via* regulating miR-29/MMP-2 axis, which provides a promising therapy strategy for the treatment of gastric cancer.

Conflict of interest

The authors declare no conflicts of interest.

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