Int J Clin Exp Med 2016;9(6):10055-10062
www.ijcem.com /ISSN:1940-5901/IJCEM0022207

Original Article

Identification of IRF1, PPP2R5E and IL-6R, the target genes of miR-23a in gastric cancer cells

Li-Hua Zhu[1,2,3], Hong Zhao[4], Zong-Tao Li[5], Lin-Lin Zhao[6], Jun-Jie Huang[1], Juan Li[1], Zhi-Hong Hou[1], Ya-Nan Xiong[1], Ai-Hua Liu[1], Gai Li[4]

[1]Department of Pathogeny Biology, School of Basic Medical, North China University of Science and Technology, Tangshan 063000, Hebei, China; [2]Hebei Key Laboratory for Chronic Diseases, School of Basic Medical Sciences, North China University of Science and Technology, Tangshan 063000, Hebei, China; [3]Tangshan Key Laboratory for Preclinical and Basic Research on Chronic Diseases, North China University of Science and Technology, Tangshan 063000, Hebei, China; [4]Affiliated Hospital, North China University of Science and Technology, Tangshan 063000, Hebei, China; [5]Tangshan Gongren Hospital, China; [6]Pharmacy Disciplines, Pharmaceutical College, Hebei United University, Tangshan 063000, Hebei, China

Received December 18, 2015; Accepted March 5, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Objective: We applied dual-fluorescent protein vector system to the identify IRF1, PPP2R5E and IL-6R, the target genes of miR-23a. Method: 3'UTR sequence containing IRF1, PPP2R5E and IL-6R gene was inserted into the EGFP-expressing plasmid. Then MGC803 cells were co-transfected with this plasmid and the plasmid expressing RFP and miR-23a. Detection was performed using spectrophotofluorometer. mRNA expressions of IRF1 and PPP2R5E gene in MGC803 cells with inhibited expression or over-expression of miR-23a were detected using real-time quantitative PCR. After it was confirmed that the mRNA expressions of IRF1 and PPP2R5E gene were inhibited by siRNA, MTT method and clone formation assay were carried out to observe the effect on the proliferation of MGC803 cells. Results: miR-23a significantly inhibited the expressions of IRF1, PPP2R5E and IL-6R gene. After silencing of miR-23a, mRNA expressions of IRF1 and PPP2R5E gene in MGC803 cells increased; mRNA expressions of IRF1 and PPP2R5E gene in MGC803 cells that overexpressed miR-2323a decreased. With the silencing of IRF1 and PPP2R5E gene, the MGC803 cells showed enhanced cell activity and clone-forming ability. Conclusion: miR-23a promoted the proliferation of MGC803 cells by regulating the expression of IRF1, PPP2R5E and IL-6R gene.

Keywords: Gastric cancer, miR-23a, target gene

Introduction

Gastric cancer is the most common malignant tumor of the gastrointestinal tract. Gastric adenocarcinoma is derived from the epithelial cells of gastric mucosa with high malignancy, accounting for 95% of all gastric cancers. The incidence of gastric cancer remains high in China, and it varies from one region to another. Usually the incidence is higher in north China and the coastal regions than in south China and the non-coastal regions. In recent years, the incidence of gastric cancer is rising among young adults and in rural areas of China. The pathogenesis of gastric cancer is associated with high-salt high-energy diet and *Helicobacter pylori* (HP) infection [1]. Family history of gastric cancer also plays a role in addition to chronic atrophic gastritis, adenomatous polyps and history of gastric surgery. Although the specific pathogenesis of gastric cancer is unclear, it is generally believed that the activation of oncogenes or the deactivation of tumor suppressor genes will lead to proliferation of tumor cells and hence gastric cancer. The oncogenes linked to the occurrence and progression of gastric cancer include ras, c-myc, bcl-2, neu and cyclin D1, while the tumor suppressor genes include p53, pRb and p16 [2, 3]. miRNA plays a regulatory role in the occurrence and progression of various malignant tumors [4-7]. Mature miRNA inhibits the transcription or translation of genes by binding to the 3'non-translated region of the mRNA of its target genes [8].

According to the report, miR-23a is overexpressed in liver cancer [9]. As shown by our previous

Table 1. PCR primers for the construction of recombinant plasmid

Primer	Sequence (5'-3')	Length
miR-23a F	GCGAGATCTGGCTCCTGCATATGAG	324 bp
miR-23a R	GATGAATTCCAGGCACAGGCTTCGG	
IRF1 3'UTR	CGCGGATCCAGAAAAGCATAACACCAATCC	270 bp
IRF1 3'UTR	CGGAATTCGTGGCAAGATCCACACCGA	
IRF1 3'UTR-MS	CCAAAGCCAGTGATAAGAGTGAAAGTGGG	270 bp
IRF1 3'UTR-MS	CCCACTTTCCTACTCTTATCAC TGGCTTTGG	
PPP2R5E 3'UT	CGCGGATCCAAATTCCATTATCGGGAG	194 bp
PPP2R5E 3'UT	CGGGATTCTCCAGAGGAGGATGTTACAC	
PPP2R5E 3'UT-MS	GATACAATTTTCATAAGAGTACAATCTTAAATTTAGC	194 bp
PPP2R5E 3'UT-MS	GCTAAATTTAAGATTGTACTCTTATGAAAATTGTATC	
IL-6R 3'UTR	CCGAGATCTGGCTTTTACTTAAACCG	959 bp
IL-6R 3'UTR	CAGGAATTCACTTGCTCTGTCACCC	

gene chip assay, miR-23a expression in gastric adenocarcinoma was higher than that in the normal gastric tissues. In contrast, the expression of IRF1, PPP2R5E and IL-6R in gastric adenocarcinoma was lower than that in the normal gastric tissues. The fluorescent protein plasmids containing 3'UTR of IRF1, PPP2R5E and IL-6R were used for the co-transfection of MGC803 cells along with pcDNA3-pri-miR-23a plasmid. Then the mRNA and protein expressions of IRF1 and PPP2R5E genes in the MGC-803 cells were detected after silencing or overexpression of miR-23a. Next the expressions of IRF1 and PPP2R5E, the target genes of miR-23a, were inhibited, and the effect of cell activity and clone-forming ability was observed.

Materials and method

Cell line and cell culture

Human gastric cancer cell line MGC803 was donoted by Peking University Health Science Center. The cells were cultured in RPMI 1640 medium (Gibco) containing 1% penicillin/streptomycin (Gibco) and 10% FBS (Institute of Hematology & Blood Diseases Hospital Chinese Academy of Medical Sciences). The cells were placed in a 5% CO_2 incubator at 37°C with saturated humidity.

Construction of recombinant plasmid

3'UTR of IRF1, PPP2R5E and IL-6R and their mutants was amplified using the primers in **Table 1**. Then the sequence was inserted into downstream of the EGFP gene in pcDNA3/EGFP plasmid (constructed by our laboratory) to construct the fluorescent protein vector [8]. The primary transcript of miR-23a obtained in **Table 1** was inserted into pcDNA3 plasmid to construct the recombinant plasmid of miR-23a.

Cell transfection

Log phase cells were inoculated to a 48-well plate at 2×10^4 cells per well (4 replicates). Transfection was performed using Lipofectamine 2000 24 h later. For each group, EGFP-expressing plasmid was added at 0.4 μg per well. To quantify the fluorescent protein, RFP-expressiong plasmid (pDsRed2N1) was added into each group as internal reference at 0.1 μg per well. The total content of plasmid was 0.5 μg per well, and the volume of ASO was 50 pmol.

Fluorescence detection

After transfection for 48 h, the culture medium was discarded. The cells were washed with PBS once, and cell lysis at 4°C for 30 min was inititaed by adding 250 μl RIPA lysis buffer into each well. Quantification was performed using spectrophotofluorometer. The excitation wavelength was 488 nm and 558 nm and the emission wavelength was 507 nm and 583 nm when detecting green fluorescence and red fluorescence, respectively. GFP/RFP ratio was calculated. Each experiment was repeated 3 times, and the fluorescence values were analyzed.

Detection of mRNA expressions

After transfection for 48 h, the MGC803 cells were digested and centrifuged. Total RNA extraction was performed using TRIzol reagent (Qiagen). Reverse transcription was performed using the extracted RNA, and mRNA expressions of IRF1 and PPP2R5E gene were detected using real-time PCR based on SYBR green I fluorescence (ABI 7500). The primers for IRF1 and PPP2R5E are shown in **Table 1**. PCR conditions: 94°C 4 min, 94°C 1 min, 56°C 1 min, 72°C 1 min, 40 cycles; 3 replicates for each sample. Data processing was carried out using Excel 2007 software.

Table 2. siRNA primers of IRF1 and PPP2R5 gene

Primer	Sequence (5'-3')
IRF1-siR-Top	GATCCGCTGAGGACATCATGAAGCTTTCAAGAGAAGCTTCATGATGTCCTCAGTTTTTTGGAAA
IRF1-siR-Bot	AGCTTTTCCAAAAAACTGGGACATCATGAAGCTTCTCTTGAAAGCTTCATGATGTCCTCA
PPP2R5E-siR-Top	GATCCGCAGAAGAAGATGAACCTACTTCAAGAGAGTAGGTTCATCTTCTTCTGTTTTTT GGAAA
PPP2R5E-siR-Bot	AGCTTTTCCAAAAAACAGAAGAAGATGAACCTACTCTCTTGAAGTAGGTTCATCTTC TTCTGCG

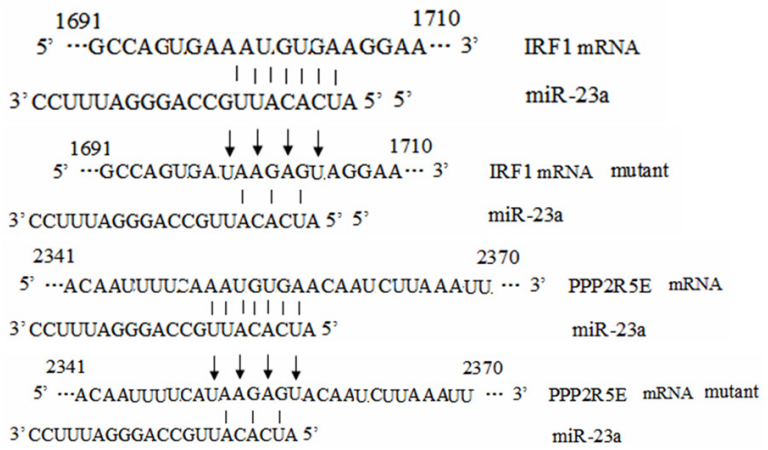

Figure 1. Binding sites of 3'UTR of IRF1, PPP2R5E and IL-6R and their mutants with miR-23a.

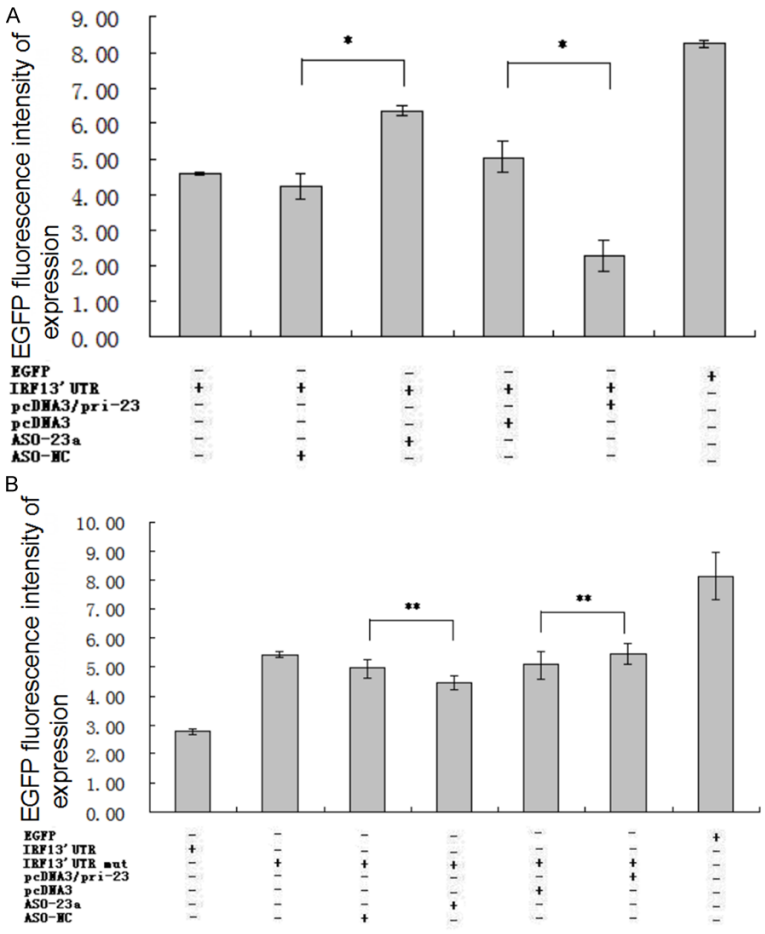

Figure 2. Fluorescent protein expression after transfection with IRF1 3'UTR and the mutants. *P<0.05 compared with the control; **P>0.05 compared with the control.

Silencing of genes and evaluation

The sequence of siRNA was designed according to the sequence of human IRF1 and PPP2R5E using Ambion software (**Table 2**). The specificity was ensured by sequence alignment using Blast program on-line. Stop codon TTTTTT was attached to the 3' terminal, and the sticky end of restriction enzyme was attached to both two terminals. Double strands obtained after annealing were ligated to the linearized plasmid pSilencer, and the plasmid was used to transform the competent *E. coli* cells. The positive clones were picked, sequenced and named as pSilencer/IRF1-siRNA and pSilencer/PPP2R5E-siRNA, respectively. The plasmid was extracted and used to transfect the MGC803 cells. mRNA expressions of IRF1 and PPP2R5E gene were detected to evaluate gene silencing. The effect of silencing of IRF1 and PPP2R5E gene on the proliferation of MGC803 cells was observed by MTT method and clone formation assay.

Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{x}$ ±s). One-way ANOVA was per-

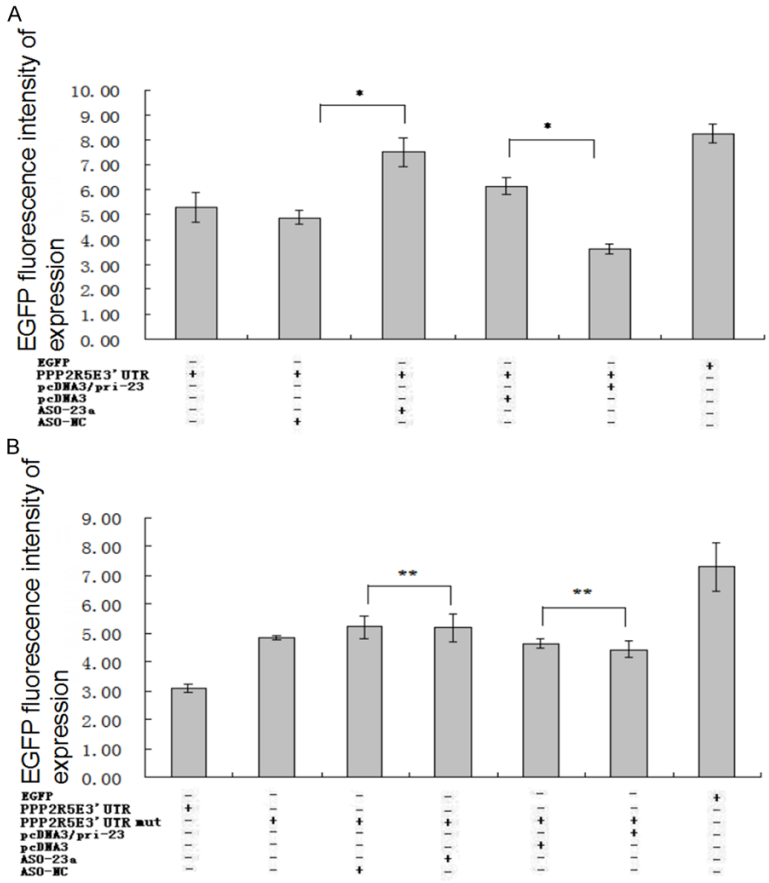

Figure 3. Fluorescent protein expression after transfection with PPP2R5E 3'UTR and the mutants. *P<0.05 compared with the control; **P>0.05 compared with the control.

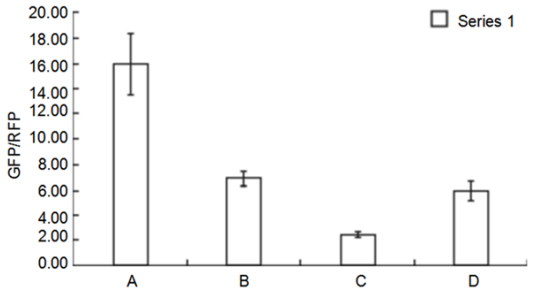

Figure 4. Fluorescent protein expression after transfection with IL-6R 3'UTR and the mutants.

formed using SPSS 13.0 software, and q test rate was compared by χ^2 test. P<0.05 indicated significant difference between the groups.

Results

miR-23a inhibited the activity of 3'UTR of IRF1, PPP2R5E and IL-6R gene

The recomibnant plasmids were successfully constructed as confirmed by sequencing. The binding sites of 3'UTR of IRF1, PPP2R5E and IL-6R and their mutants with miR-23a are shown in **Figure 1**.

The MGC803 cells were co-transfected with pcDNA3/EGFP-IRF1 3'UTR (**Figure 2**) or pcDNA3/EGFP-PPP2R5E 3'UTR (**Figure 3**) and miR-23a ASO. After transfection, the fluorescent protein expression of the group transfected with ASO-23a was obviously higher than that of other groups. Thus, after the silencing of miR-23a with ASO-23a, the inhibition on the expression of IRF1 or PPP2R5E 3'UTR was reduced, leading to upregulation of EGFP. MGC803 cells were co-transfected with pcDNA3/EGFP-IRF1 3'UTR or pcDNA3/EGFP-PPP2R5E 3'UTR and pcDNA3/pri-23a to induce the overexpression of miR-23a. As a result, the fluorescent protein expression decreased significantly. It was indicated that miR-23a had a greater inhibitory effect on IRF1 or PPP2R5E 3'UTR, leading to downregulation of EGFP. Site-directed mutagenesis was performed for the binding site between IRF1 (**Figure 2**) or PPP2R5E (**Figure 3**) 3'UTRandthemiR-23aseedsequence.ThenMGC803 cells were co-transfected with the mutant plasmids and miR-23a ASO or pcDNA3/miR-23a to enhance the expression of fluorescent protein.

The fluorescent protein expression was the highest in the group only transfected with pcDNA3/EGFP. After the transfection with pcDNA3/EGFP-IL-6R-3'UTR, the fluorescent protein expression was downregulated due to the action of endogenous miR-23a alone. After co-transfection with pcDNA3/EGFP-IL-6R-3'UTR and pcDNA3- pri-miR-23a, the fluorescent protein expression was the lowest due to the action of both endogenous and exogenous miR-23a. The fluorescent protein expression after co-transfection with pcDNA3/EGFP-IL-6R-3'UTR and pcDNA3 was similar to that after transfection with pcDNA3/EGFP-IL-6R-3'UTR (**Figure 4**).

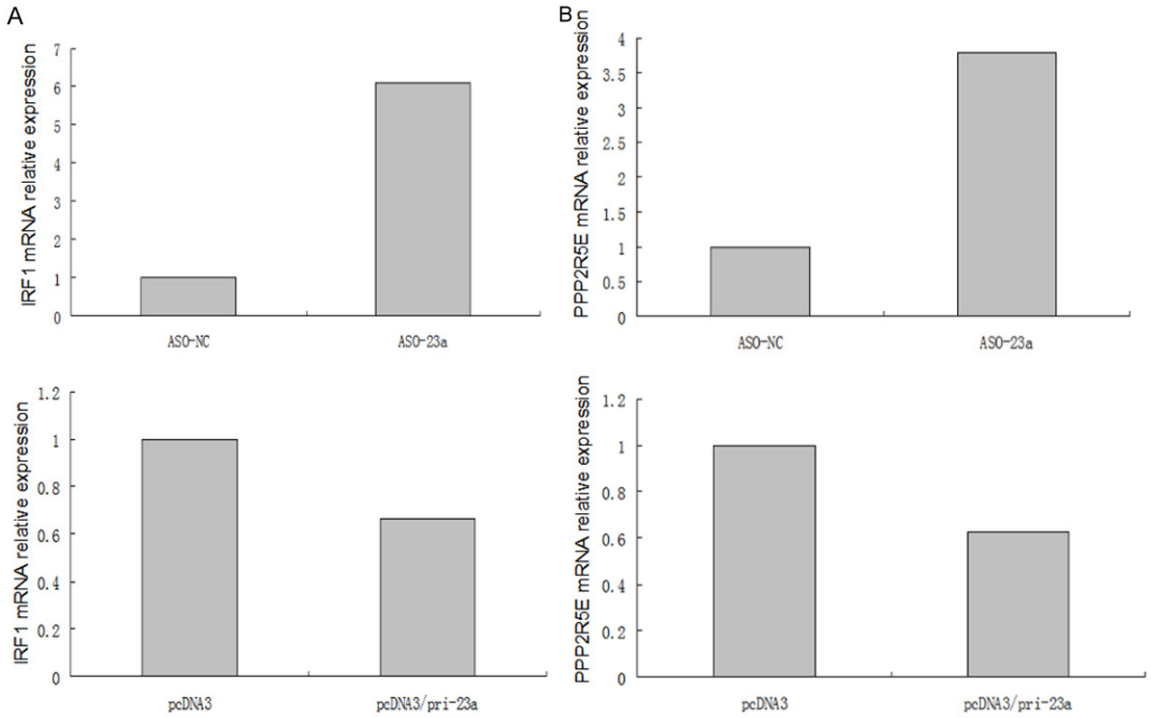

Figure 5. mRNA expression of IRF1 and PPP2R5E. Note: A is mRNA expression of IRF1; B is mRNA expression of PPP2R5E.

mRNA expressions of IRF1 and PPP2R5E

The mRNA expressions of IRF1 and PPP2R5E in the group transfected with ASO-23a were obviously higher than those of other groups. This indicated that miR-23a was effectively silenced by ASO-23a, leading to the upregulation of mRNA of IRF1 and PPP2R5E. Since miR-23a was effectively expressed by pcDNA3/pri-23a, the mRNA expressions of IRF1 and PPP2R5E decreased (**Figure 5**). The mRNA expressions of IRF1 and PPP2R5E in the gastric adenocarcinoma were significantly lower than those in the normal gastric tissues (data were not shown).

Enhanced cell activity after silencing of IRF1 and PPP2R5E

mRNA expressions of IRF1 and PPP2R5E were detected by fluorescent quantitative PCR after transfection of MGC803 cells with IRF1 or PPP2R5E siRNA plasmid. MTT method and clone formation assay indicated that the cell activity and clone-forming ability were both enhanced after the silencing of IRF1 or PPP2R5E gene (**Figures 6** and **7**).

Discussion

miRNAs are widely present in organisms and involved in the regulation of various physiological and pathological process. As a newly discovered regulatory factor of gene expression, miRNA can negatively regulate the expression of target genes by specifically binding to 3'UTR [10]. It is found that the abnormal expression of miRNAs is involved in the occurrence and progression of several malignant tumors. Therefore, research efforts have been made to understand the working mechanism of tumor-related miRNAs [11, 12]. Fluorescent protein reporter gene vector is an easy, sensitive and reliable way to identify the target genes [13].

By combining gene chip assay and bioinformatics prediction, we predicted the target genes of miR-23a from the genes with lower expression. Since miR-23a is overexpressed in gastric adenocarcinoma, miR-23a may regulate the genes with antiproliferative activity, invasiveness or apoptosis-inducing effect. miR-23a may play a role in enhancing the proliferative activity and invasiveness and reducing the apoptosis of tumor cells, thus facilitating malignant transformation or increasing malignancy [14-17]. Of 5 candidate target genes of miR-23a during preliminary screening (IL-6R, Genbank: NM_000565; PPP2R5E, Genbank: NM_006246; IRF1, Genbank: NM_002198; CAPN6, Genbank: NM_014289; CLCN3, Genbank: NM_173872),

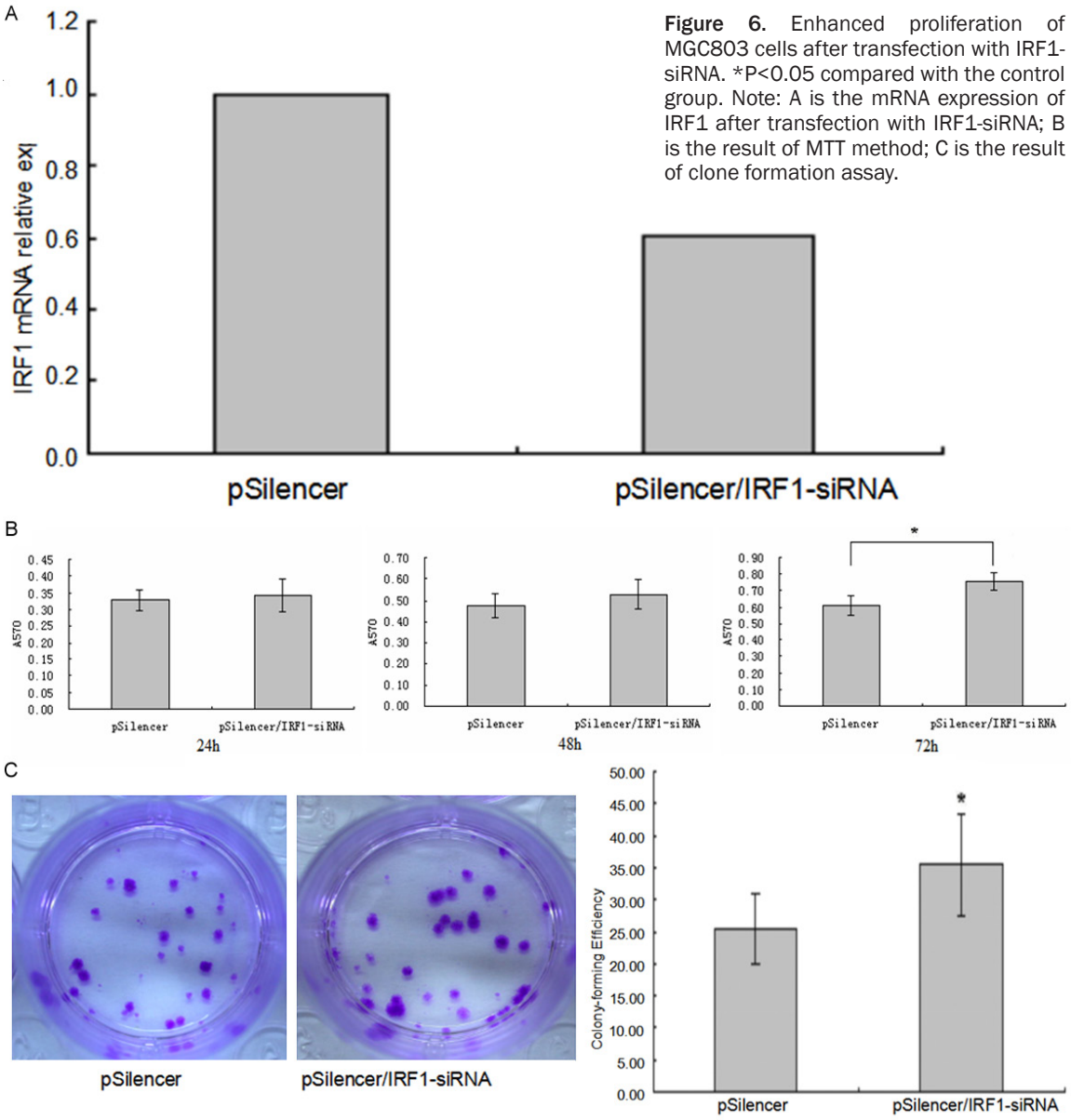

Figure 6. Enhanced proliferation of MGC803 cells after transfection with IRF1-siRNA. *P<0.05 compared with the control group. Note: A is the mRNA expression of IRF1 after transfection with IRF1-siRNA; B is the result of MTT method; C is the result of clone formation assay.

3'UTR of proliferation-related genes IL-6R, PPP2R5E and IRF1 all contains the binding site of miR-23a. So these genes evoked special interest. According to the existing studies, IRF1 is a tumor suppressor gene in gastric adenocarcinoma [18] and shows tumor suppression effect in mice. Overexpression of IRF-1 can lead to cell cycle arrest and therefore promote gastric cancer [19]. PPP2R5E is related to cell growth, but it is uncertain whether PPP2R5E is the tumor suppressor gene in gastric adenocarcinoma. IL-6, a multi-potential cytokine, is the mediator of various immune response and inflammatory response. Many researchers have been devoted to the role of IL-6 and its receptor (IL-6R) in the occurrence of tumors. IL-6 can either stimulate or inhibit the growth of cells, depending on the target cells. We found through the current study that IRF1, PPP2R5E and IL-6R may be the target genes of miR-23a in MGC803 cells. miR-23a can negatively regulate these genes and promote the proliferation and invasiveness, and reduce the apoptosis of the tumor cells. Thus miR-23a may be involved in the occurrence and progression of tumors by inhibiting IRF1, but the specific mechanism is unknown.

The occurrence and progression of malignant tumors are associated with abnormal expres-

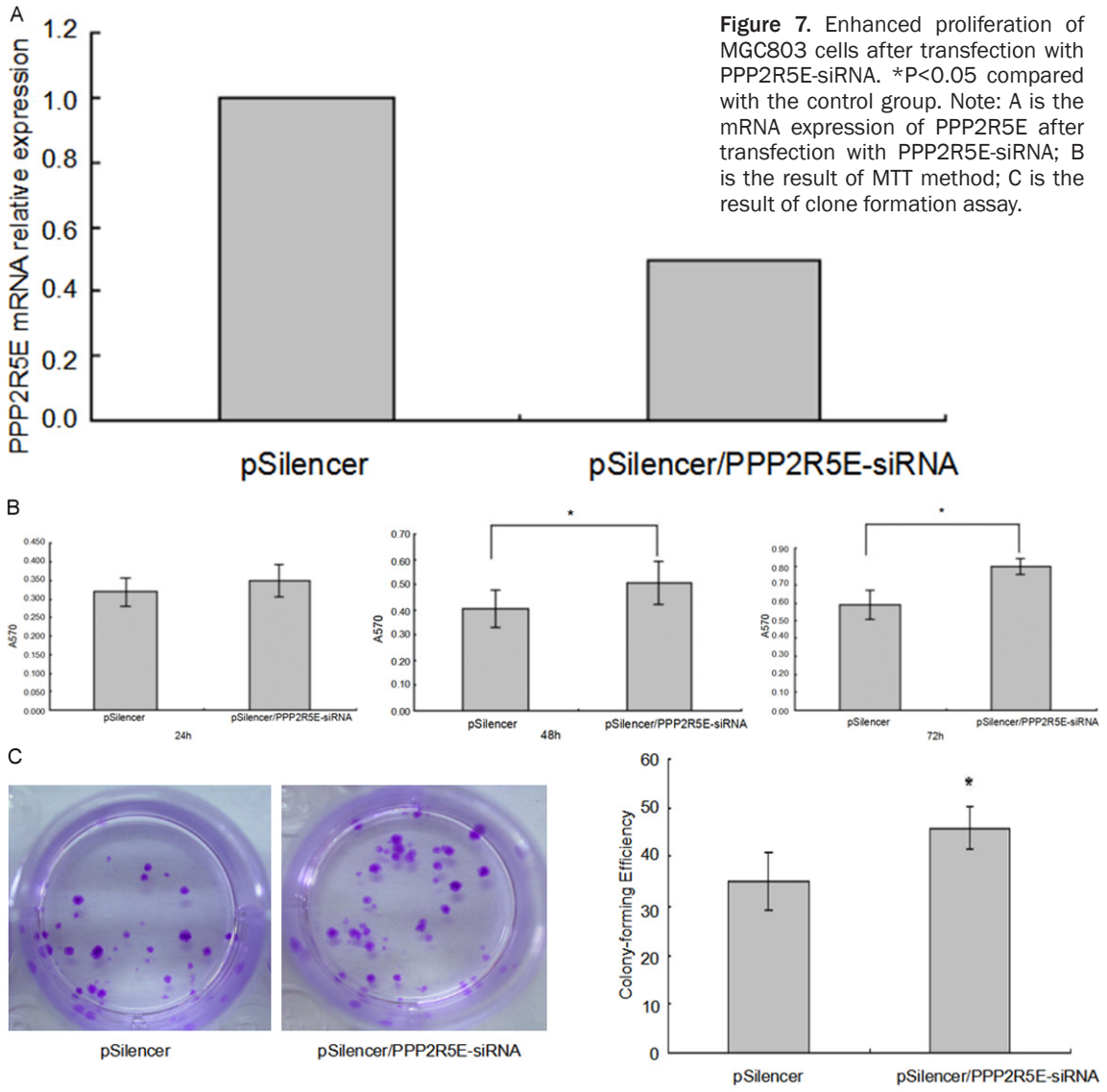

Figure 7. Enhanced proliferation of MGC803 cells after transfection with PPP2R5E-siRNA. *P<0.05 compared with the control group. Note: A is the mRNA expression of PPP2R5E after transfection with PPP2R5E-siRNA; B is the result of MTT method; C is the result of clone formation assay.

sions of several genes. There may exist several regulatory pathways involving miRNAs that target the epithelial cells of the gastric mucosa. By studying tumor-related miRNAs and their working mechanism, we can find clues for tumor prevention and treatment.

Acknowledgements

This work was supported by the Science and Technology of Hebei Province of China (162-77782D).

Disclosure of conflict of interest

None.

Address correspondence to: Li-Hua Zhu, Department of Pathogeny Biology, School of Basic Medical, North China University of Science and Technology, Jianshe Road 57#, Tangshan 063000, Hebei, China. Tel: +86-13932554697; Fax: +86-13932554697; E-mail: zlhuadr@163.com

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