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MicroRNA-212 Inhibits Proliferation of Gastric Cancer by Directly Repressing Retinoblastoma Binding Protein 2

Zeng Jiping,[1,2] Fang Ming,[1,3] Wang Lixiang,[1,4] Liang Xiuming,[1] Shen Yuqun,[1] Yu Han,[1] Liu Zhifang,[1,2] Sun Yundong,[1] Liu Shili,[1] Chen Chunyan,[5] and Jia Jihui[1]*

[1]*Department of Microbiology/Key Laboratory for Experimental Teratology of the Chinese Ministry of Education, Shandong University School of Medicine, Jinan, China*

[2]*Department of Biochemistry, Shandong University School of Medicine, Jinan, China*

[3]*Institute of Bacterial Infectious Diseases, Shandong Center for Disease Control and Prevention, Shandong Provincial Key Laboratory of Infectious Disease Control and Prevention, Jinan, China*

[4]*Department of Pharmacology, Shandong University School of Medicine, Jinan, China*

[5]*Department of Hematology, Qilu Hospital, Shandong University School of Medicine, Jinan, China*

ABSTRACT

Retinoblastoma binding protein 2 (RBP2), a newly found histone demethylase, is overexpressed in gastric cancer. We examined the upstream regulatory mechanism of RBP2 at the microRNA (miRNA) level and the role in gastric carcinogenesis. We used bioinformatics to predict that microRNA-212 (miR-212) might be a direct upstream regulator of RBP2 and verified the regulation in gastric epithelial-derived cell lines. Overexpression of miR-212 significantly inhibited the expression levels of RBP2, whereas knockdown of miR-212 promoted RBP2 expression. Furthermore, we identified the putative miR-212 targeting sequence in the RBP2 3′ UTR by luciferase assay. MiR-212 inhibited the colony formation ability of cells by repressing RBP2 expression and increasing that of $P21^{CIP1}$ and $P27^{kip1}$, both critical in cell cycle arrest. In addition, the expression of RBP2 and miR-212 in tumor tissue and matched normal tissue from 18 patients further supported the results in vivo. MiR-212 directly regulates the expression of RBP2 and inhibits cell growth in gastric cancer, which may provide new clues to treatment. J. Cell. Biochem. 114: 2666–2672, 2013.

KEY WORDS: GASTRIC CANCER; MICRORNA-212; RBP2; PROLIFERATION

Gastric cancer (GC) is one of the most common human malignant diseases and the second leading cause of tumor-related deaths worldwide [Ferlay et al., 2010; Petra Hudler, 2012]. The incidence and mortality rate are particularly high in eastern Asia [Brenner et al., 2009]. Despite recent advances in surgical techniques and adjuvant therapy after surgery, the 5-year survival rate is still only 40% [Yamashita et al., 2011]. The prognosis of patients with advanced GC is even worse [Zheng et al., 2004]. Therefore, understanding the molecular mechanisms of GC development and novel therapeutic targets for treatment is urgent.

Retinoblastoma binding protein 2 (RBP2), a member of the JARID protein family, is a newly found epigenetic molecule identified as a histone demethylase. It specifically targets tri- and dimethylated lysine 4 of histone 3 (H3-K4) for demethylation to regulate gene expression [Christensen et al., 2007; Klose et al., 2007; Lopez-Bigas et al., 2008]. Its deregulation may lead to human diseases, especially developmental problems [Lopez-Bigas et al., 2008; Ge et al., 2011; Stratmann and Haendler, 2011]. We found RBP2 overexpressed in GC and triggered senescence of cancer cells by repressing the activity of $P21^{CIP1}$ and $P27^{kip1}$ [Zeng et al., 2010]. However, the upstream

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Zeng Jiping and Fang Ming contributed equally to this work.
*Correspondence to: Jia Jihui, Department of Microbiology/Key Laboratory for Experimental Teratology of the Chinese Ministry of Education, Shandong University School of Medicine, Jinan 250012, PR China. E-mail: jiajihui@sdu.edu.cn.
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regulatory mechanism leading to overexpression of RBP2 in GC is still unknown.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs of about 22 nt that regulate gene expression at the posttranscriptional level [Redis et al., 2012]. MiRNAs mostly bind to the 3′ untranslated region (3′ UTR) of target mRNAs, inducing translation repression and/or mRNA degradation [Chen and Rajewsky, 2007]. MiRNAs play important roles in numerous biological processes, such as cell development, apoptosis, proliferation, and differentiation [Amiel et al., 2012; Hinton et al., 2012; Lee and Shin, 2012; Li et al., 2012a; Leonardo et al., 2012]. Numerous miRNAs regulate tumor suppressors and oncogenes [Su et al., 2010; Hatziapostolou et al., 2011; Png et al., 2011; Ishteiwy et al., 2012; Lee et al., 2012; Pan et al., 2012; Rossi et al., 2012; Zabolotneva et al., 2012; Baer et al., 2013; Okuda et al., 2013]. MiRNA expression profiling in human tumors has revealed that many deregulated miRNAs are closely associated with diagnosis, stage, progression, prognosis, and response to treatment [Esquela-Kerscher and Slack, 2006]. In addition, miRNAs are becoming the new biomarkers for gastric carcinogenesis [Wu et al., 2010; Albulescu et al., 2011; Song and SJ, 2012].

In this study, we aimed to find the miRNA that directly regulates RBP2 expression and reveal its function in GC in vitro and in vivo.

MATERIALS AND METHODS

PATIENTS AND TISSUE SPECIMENS

Resected pairs of cancer tissue and distal normal gastric tissue (>5 cm from the margin of the tumor) from 18 patients with GC were harvested during surgery at Qilu Hospital of Shandong University 2010 to 2011. None of the patients had received adjuvant chemotherapy before surgery. The diagnosis of GC was histopathologically confirmed. The general information for patients is in Supplementary Table I. The study was approved by the ethics committee of Shandong University School of Medicine.

IMMUNOHISTOCHEMISTRY

Resected tissue pairs were embedded with paraffin and sliced into 5-μm pieces, which were deparaffinized and dehydrated with xylene and a graded series of alcohol. Antigen retrieval involved heat treatment performed in 0.1 M citrate buffer at pH 6.0. Then 3% H_2O_2 was used to block the endogenous peroxidase activity. The slides were further incubated with goat serum for 30 min, then with monoclonal rabbit anti-human RBP2 (Sigma, USA) overnight at 4°C. The results were detected with Diaminobenzidine (DAB) staining (Vector Laboratories, USA) analyzed under a microscope (Olympus BX60, Tokyo, Japan) and images were captured for analysis.

CELL LINES AND CULTURE

The gastric epithelial-derived cancer cell lines AGS and BGC-823 were obtained from the cell repository for Academia Sinica (Shanghai). Human gastric epithelial immortalized GES-1 cells were maintained in our laboratory. AGS cells were grown in Ham's F12 (Gibco, USA). BGC-823 and GES-1 cells were grown in RPMI1640 medium (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (Gibco, USA). All cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37°C without antibiotics.

PLASMID CONSTRUCTION, TRANSFECTION AND LUCIFERASE ASSAY

A 370-bp fragment predicted to express miR-212 was amplified by PCR with genomic DNA from AGS cells and cloned into the pSilencer 4.1-CMV vector (Ambion, USA) with the primer sequences sense, 5′-GGATCCTGCGTTGATCAGCACC-3′ (*Bam*HI site underlined), and antisense, 5′-AAGCTTCCCCTCTGGGACATCTTT-3′ (*Hin*dIII site underlined). The corresponding plasmid was named pSilencer-miR-212. Likewise, for construction of the anti-miR-212 interfering plasmid, the miR-212 complementary sequence was synthesized as DNA oligonucleotide sequences with sticking ends, subcloned into the pSuper vector (Oligoengine, USA) and named pSuper-anti-miR-212. The oligonucleotide sequences were sense, 5′-GATCCCCATTGTCAGAGGTCAGTGCCGGTTTTTTGGAAG-3′, and antisense, 5′-TCGACTTCCAAAAAACCGGCACTGACCTCTGACAATGGG-3′. A 329-bp fragment of the RBP2 3′ UTR containing the miR-212 predicted target sites was amplified from cDNA libraries of AGS cells with the primer sequences sense, 5′-ACTAGTGCAGATGCTGTTGAATAA-3′ (SpeI site underlined), and antisense, 5′-AAGCTTCTACAAAGAAGGAATGG-3′ (HindIII site underlined). The PCR fragment was cloned into the multiple cloning sequence of the luciferase reporter pMIR-REPOTR (Applied Biosystems, USA), designated as pMIR-REPORT-RBP2- 3′-UTR, which was also used in PCR to generate pMIR-REPORT-RBP2-3′-UTRmut plasmids with mutation of the binding sites on the 3′ UTR of RBP2: pMIR-REPORT-RBP2-3′-UTRmut1 (mutation of the first binding site at 1208–1214 of RBP2 3′-UTR), pMIR-REPORT-RBP2-3′-UTRmut2 (mutation of the second binding site at 1277–1283 of RBP2 3′-UTR) and pMIR-REPORT-RBP2-3′-UTRmut3 (mutation of both sites). The primer sequences for mutants were MUT1, sense, 5′-GTATAGGCTGTGAAGTAGTCAGAGACA-3′, and antisense, 5′-TGTCTCTGACTACTTCACAGCCTATAC-3′ (mutation of the core sequences underlined); MUT2, sense, 5′-ATATGCTGCTGGATGACTGAAATATA-3′, and antisense, 5′-TATATTTCAGTCATCCAGCAGCATAT-3′ (mutation of the core sequences underlined).

For transient transfection, cells were seeded in 6-well plates (3.0×10^5/well) for 18 to 24 h, then transfected with plasmids by use of Lipofectamine 2000 (Invitrogen, USA).

To examine the direct conjugation of miR-212 to the 3′ UTR of RBP2, pMIR-REPORT-RBP2-3′-UTR, -mut1, -mut2, and -mut3 were co-transfected into GES-1 cells with pSilencer-miR-212, with pMIR-REPORT β-gal plasmid used as a negative control. Luciferase activity in the cell lysates was determined by a single luciferase reporter assay (Promega, USA) 48 h after transfection, and target promoter-driven firefly luciferase activity was normalized to that of β-gal.

RNA EXTRACTION AND QUANTITATIVE RT-PCR (qRT-PCR)

Total RNA from tissue specimens and cells was extracted by use of Trizol reagent (Invitrogen, USA). For qRT-PCR of RBP2, $P27^{kip1}$, and $P21^{CIP2}$, total RNA was reverse transcribed by use of the RevertAid First Strand cDNA synthesis Kit (Fermentas, Canada). Real-time PCR involved the TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) with the ABI Prism 7500 Sequence Detection System. Gene expression was

normalized to that of β-actin. Results were calculated by the $2^{-\Delta\Delta Ct}$ method. The level of mature miR-212 expression was analyzed by TaqMan miRNA Assay (Applied Biosystems). cDNA was synthesized from total RNA samples by use of the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers. The reactions were incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Real-time PCR involved TaqMan miRNA Assay primers with the TaqMan Universal PCR Master Mix. The reactions were run in the ABI Prism 7500 Sequence Detect System at 95°C, 10 min for 1 cycle, then 95°C, 15 s, 60°C, 1 min for 40 cycles. The relative level of miRNA expression was normalized to that of U6 small noncoding RNA and the fold change for miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences for $p21^{CIP1}$ were sense 5′-GCGACTGTGATGCGCTAAT-3′, and antisense 5′-TAGGGCTTCC-TCTTGGAGAA-3′; $p27^{kip1}$, sense, 5′-ATGTCAAACGTGCGAGTGTC-TAA-3′, and antisense, 5′-TTACGTTTGACGTCTTCTGAGG-3′; and β-actin, sense, 5′-AGTTGCGTTACACCCTTTCTTG-3′, and antisense, 5′-CACCTTCACCGTTCCAGTTTT-3′.

WESTERN BLOT ANALYSIS

Cells were washed twice with ice-cold phosphate buffered saline and lysed in RIPA buffer. The lysate was spun down and the supernatant was harvested. Equal amounts of proteins were separated by SDS polyacrylamide gels and transferred onto membranes (Millipore), which were blocked with 5% non-fat milk protein for 1 h, then incubated with primary antibodies overnight at 4°C. The antibodies used were for RBP2 (1:1,000, Sigma), $p27^{kip1}$ (1:300), $p21^{CIP1}$ (1:500, both Santa Cruz Biotechnology), and β-actin (1:5,000, Sigma) and horseradish peroxidase-conjugated goat-anti-mouse or -rabbit IgG secondary antibody (1:5,000, Santa Cruz Biotechnology). Immune complexes were detected by use of the Chemiluminescent HRP Substrate Kit (Millipore Corp).

COLONY FORMATION ASSAY

Cells were incubated in 6-well plates for 18–24 h, then transfected with the corresponding vectors for 48 h. Single cells were seeded on 6-well plates (300 or 500 cells/well). After 10–14 days of incubation, plates were stained with Giemsa for 20 min. The number of colonies with more than 50 cells was counted.

PROMOTER ACTIVITY ASSAY

The plasmids pSilencer-miR-212 and pSuper-anti-miR-212 were transfected into cells with the promoter reporter plasmids for $p21^{CIP1}$ and $P27^{kip1}$, respectively. The Renilla luciferase-containing plasmid controlled by the thymidine kinase (TK) promoter was co-transfected as a control. Luciferase activity in the cell lysates was determined by dual luciferase reporter assay (Promega) 48-h after transfection, and the target promoter-driven firefly luciferase activity was normalized to that of TK renilla.

STATISTICAL ANALYSIS

Quantitative data are expressed as mean ± SEM. Statistical analysis involved use of SPSS 13.0 (SPSS, Inc., Chicago, IL) by two-tailed Student's *t*-test or one-way ANOVA for experiments with more than two subgroups. Statistical significance was set at $P < 0.05$.

RESULTS

Hsa-miR-212 DOWNREGULATED RBP2 EXPRESSION IN VITRO

Our bioinformatics analysis with miRanda (http://www.microrna.org/microrna), TargetScan (http://www.targetscan.org), and miRBase (http://www.mirbase.org) predicted miR-212 as an upstream regulator of RBP2 that directly bound to the 3′ UTR of RBP2 at two different sites (Fig. 1A).

To determine whether miR-212 induced RBP2 mRNA degradation, we transiently transfected the plasmid pSilencer-miR-212 into AGS, BGC-823, and GES-1 cells for 48 h. The high expression of has-miR-212 induced by pSilencer-miR-212 (Fig. 1B) repressed both the mRNA and protein levels of RBP2 (Fig. 1C,D). To further determine whether hsa-miR-212 regulated RBP2 expression, pSuper-anti-miR-212 was used to knock down hsa-miR-212 expression, which was verified by TaqMan qRT-PCR (Fig. 1E). Accordingly, the low expression of has-miR-212 strengthened both the mRNA and protein levels of RBP2 to a certain extent (Fig. 1F,G). Therefore, hsa-miR-212 inhibited RBP2 expression in all three types of cells by degrading the mRNA level of RBP2.

Hsa-miR-212 TARGETED THE 3′ UTR OF RBP2

To determine whether the putative miR-212 targeting sequence in the RBP2 3′ UTR could mediate the repression of RBP2, we co-transfected the pMIR-REPORT luciferase plasmid containing the putative hsa-miR-212 binding sites of the RBP2 3′ UTR into GES-1 cells with the pSilencer-miR-212 plasmid, with the pMIR-REPORT β-gal control plasmid as a control. Co-transfection of pMIR-REPORT-RBP2-3′-UTR with pSilencer-miR-212 significantly decreased luciferase activity as compared with the negative control (Fig. 2A), so miR-212 modulated RBP2 expression by directly binding to its 3′ UTR.

As noted before, RBP2 contains two predicted binding sites for hsa-miR-212 in its 3′ UTR. Normal plasmid and the three mutant plasmids were co-transfected with pSilencer-miR-212 and pMIR-REPORT β-gal into GES-1 cells, respectively. The mutation of either predicted binding sites attenuated hsa-miR-212-mediated repression of luciferase activity to some extent, whereas the mutation of both binding sites almost restored the hsa-miR-212-mediated repression of activity (Fig. 2B). Therefore, both of the predicted binding sites in the 3′ UTR of RBP2 contributed to the miRNA–mRNA interaction.

Hsa-miR-212 INHIBITED THE PROLIFERATION OF GC CELLS BY REGULATING THE EXPRESSION OF $p21^{CIP1}$ AND $p27^{kip1}$

Colony formation assay in AGS and GES-1 cell lines revealed that enforced expression or knockdown of miR-212 affected cloning of cells (Fig. 3A,B). Transfection of pSilencer-miR-212 reduced and anti-miR-212 transfection increased the number of colonies respectively (Fig. 3C,D). Therefore, miR-212 inhibited the proliferation of human gastric cells in vitro.

The promoters of $p21^{CIP1}$ and $p27^{kip1}$ can be bonded by RBP2 and are involved in the proliferation of gastric cell lines. We examined the expression of $p21^{CIP1}$ and $p27^{kip1}$ and their promoter activities to further confirm the role of miR-212 in the repression of cell proliferation. AGS and GES-1 cells transfected with pSilencer-miR-212 showed increased mRNA levels of $p21^{CIP1}$ and $p27^{kip1}$ (Fig. 4A,B). Co-transfection of pSilencer-miR-212 and the promoter reporter plasmids for $p21^{CIP1}$ and $p27^{kip1}$ increased the promoter activity of

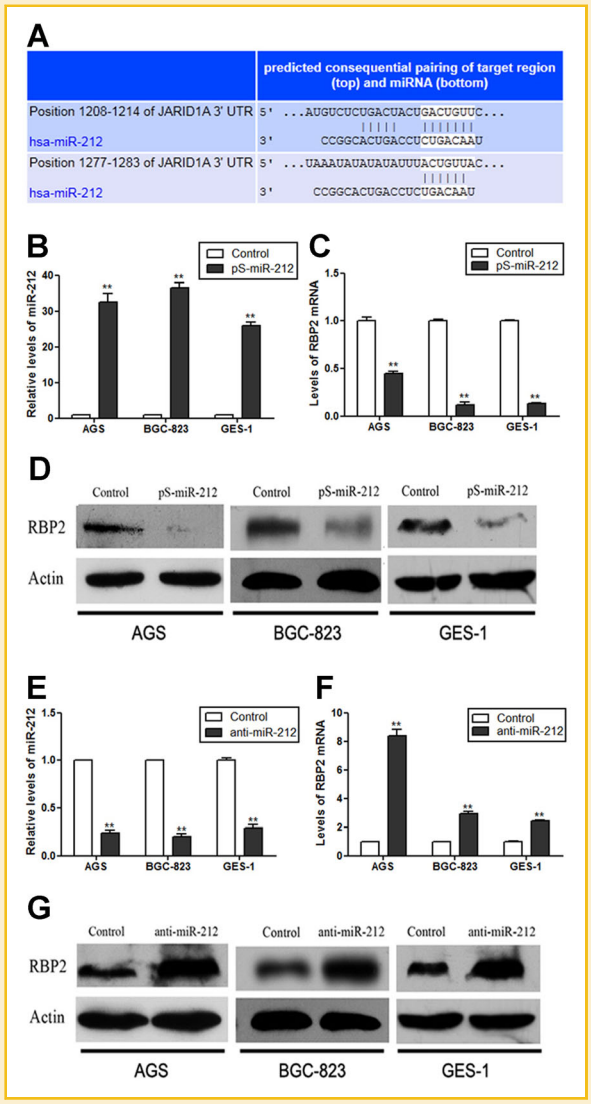

Fig. 1. MiR-212 downregulated RBP2 expression at both mRNA and protein levels. A: The binding sites for miR-212 on the 3′ UTR of RBP2 (from miRanda). B: Quantitative RT-PCR analysis of overexpression of miR-212 in AGS, BGC-823, and GES-1 gastric cancer cells. $^{**}P < 0.01$ versus control (con). C,D: qRT-PCR and Western blot analysis of overexpression of miR-212 on mRNA and protein levels of RBP2 in AGS, BGC-823, and GES-1 cells. $^{**}P < 0.01$ versus con. E: qRT-PCR analysis of repression of miR-212 inhibited by pSuper-anti-miR-212 transfection. $^{**}P < 0.01$ versus con. F,G: qRT-PCR and Western blot analysis of RNA and protein levels of RBP2 with inhibition of miR-212 in AGS, BGC-823, and GES-1 cells. $^{**}P < 0.01$ versus con. Data are mean ± SEM. (n = 3 experiments).

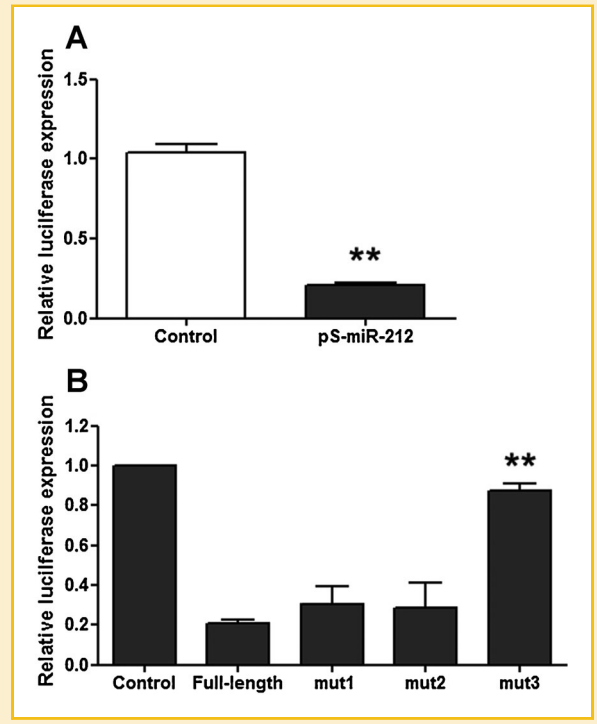

Fig. 2. Hsa-miR-212 directly bound to the 3′ UTR of RBP2. A: Luciferase activity assay with pMIR-REPORT-RBP2-3′-UTR co-transfected with pSilencer-miR-212 or the negative control in GES-1 cells. $^{**}P < 0.01$ versus con. B: Luciferase activity assay of the wild-type and 3 mutant plasmids for 2 binding sites of miR-212 on the 3′ UTR of RBP2 co-transfected with pSilencer-miR-212 or the negative control in GES-1 cells, respectively. $^{**}P < 0.01$ versus con. Data are mean ± SEM. (n = 5 experiments).

$p21^{CIP1}$ and $p27^{kip1}$ respectively (Fig. 4C,D). However, cells transfected with pSuper-anti-miR-212 showed reduced mRNA level of $p21^{CIP1}$ and $p27^{kip1}$ (Fig. 4E,F) and repressed promoter activity of $p21^{CIP1}$ and $p27^{kip1}$ (Fig. 4G,H). The effect of overexpression of miR-212 on the expression of $P21^{CIP1}$ and $P27^{kip1}$ proteins was determined in GES-1 cells by transfection of pSilencer-miR-212 and pSuper-anti-miR-212, respectively. Similarly, miR-212 overexpression upregulatated the expression of $P21^{CIP1}$ and $P27^{kip1}$, whereas the inhibition of miR-212 downregulated them (Supplementary Fig. 1).

Hsa-miR-212 EXPRESSION WAS INHIBITED IN HUMAN PRIMARY GC

We determined the expression of hsa-miR-212 in human primary GC specimens and matched normal tissues from 18 patients. As compared with normal tissue, GC specimens showed inhibited expression of miR-212 (Fig. 5A), which supports the anti-proliferation role of miR-212 seen in vitro in GC. RBP2 was overexpressed at the mRNA and protein levels in human primary GC specimens (Fig. 5B,C). Therefore, miR-212 and RBP2 may be negative covalents in vivo (Fig. 5D). We found no association of miR-212 or RBP2 expression and patient age or gender (Supplementary Table I).

DISCUSSION

GC is one of the most common malignant tumors worldwide. Epigenetic molecules involved in gastric carcinogenesis include histone demethylase [Gigek et al., 2012]. RBP2, also called KDM5A and JARID1A, is a histone demethylase for H3K4me3 and H3K4me2 [Christensen et al., 2007; Klose et al., 2007]. RBP2 plays an important role in the process of cell proliferation, survival, differentiation, and aging by regulating specific gene transcription. We first found RBP2 overexpressed in GC and that its inhibition triggers gastric cell senescence by affecting the promoter activity of $p21^{CIP1}$ and $p27^{kip1}$

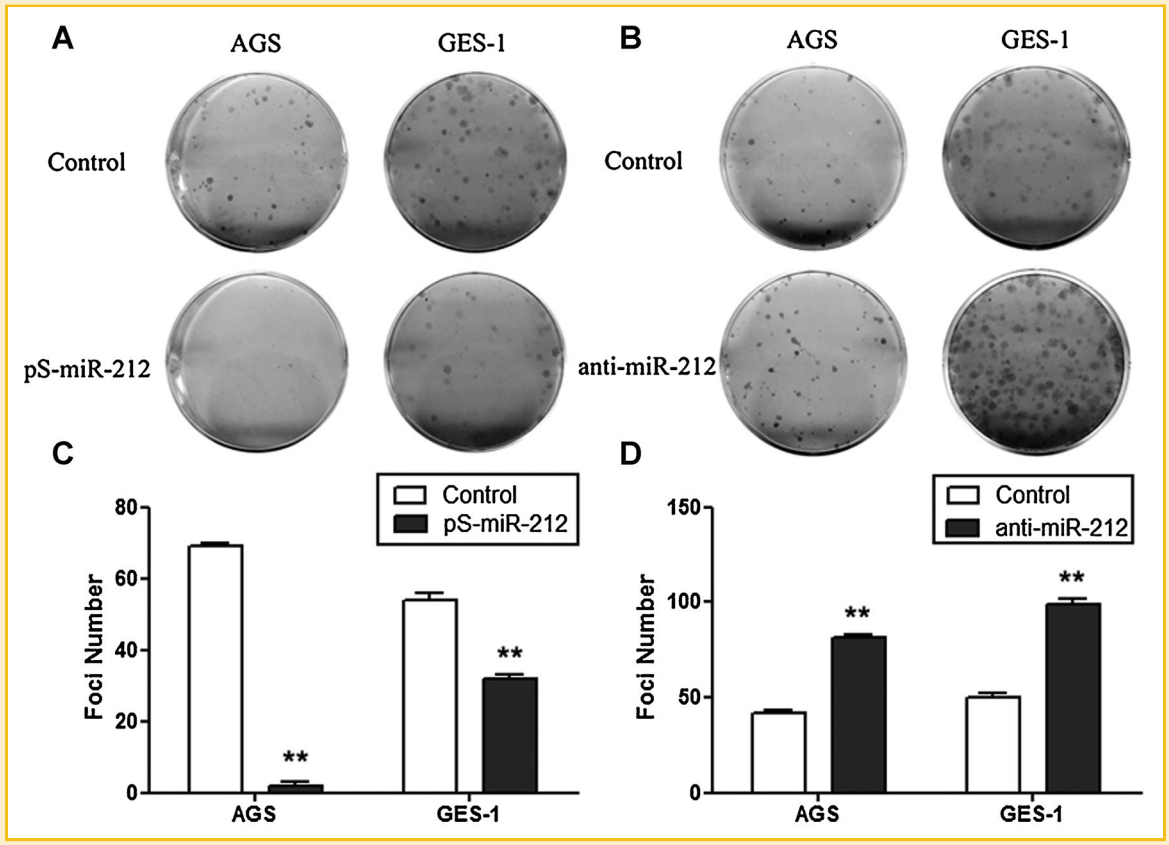

Fig. 3. Hsa-miR-212 is involved in gastric cell proliferation. A,B: Colony formation ability in AGS and GES-1 cells with overexpression and knockdown of miR-212 and (C,D) quantification. $^{**}P < 0.01$, $^{*}P < 0.05$ versus con. Data are mean ± SEM. (n = 3 experiments).

[Zeng et al., 2010]. In the current research, we detected the mechanism of RBP2 overexpression in GC. We found that the level of miR-212, which directly targets RBP2, was inhibited in GC, which led to the upregulation of RBP2 and the downregulation of $p21^{CIP1}$ and $p27^{kip1}$, thus resulting in gastric cell proliferation.

In general, miRNAs can affect the expression of multiple genes and thus exert their different function. Bioinformatics has been used to predict several miRNAs targeting RBP2. Among these miRNAs, miR-212 was highly revealed in 2 independent databases and bound to the 3′ UTR of RBP2 at 2 sites. Therefore, miR-212 was a candidate target of RBP2. MiRNAs are involved in various stages of carcinogenesis. Their abnormal expression plays critical roles in tumor genesis by modulating the expression of oncogenes or tumor suppressor genes during cancer progression [Farazi et al., 2013]. By pairing and targeting the 3′ UTR of mRNAs, miRNAs induce carcinogenesis-related mRNA degradation or translation disruption or both [Chen and Rajewsky, 2007]. However, the mechanism of miR-212 on RBP2 expression and the corresponding function in GC remained unknown.

We found that miR-212 was repressed and its expression inversely related to RBP2 expression in both gastric cell lines and primary GC human tissue. Transfection of plasmids to overexpress or inhibit miR-212 regulated the expression of RBP2 at both mRNA and protein levels. Luciferase reporter assay revealed that miR-212 directly targeted the 2 binding sites on the RBP2 3′ UTR. Therefore, RBP2 may be a functional target of miR-212.

MiRNAs implicated in GC proliferation and metastasis including miR-15, miR-21, and miR-150 [Link et al., 2012]. MiR-212 has different roles in different kinds of tumors. It may be downregulated in GC and upregulated in pancreatic cancer and non-small-cell lung cancer, respectively [Park et al., 2011; Xu et al., 2011; Li et al., 2012b]. MiR-212 may be involved in drug resistance and transformation of gastritis into mucosa-associated lymphoid tissue lymphoma [Thorns et al., 2012; Turrini et al., 2012]. We found that miR-212 was repressed in gastric carcinogenesis and participated the inhibition of cell proliferation by downregulating RBP2. $P21^{CIP1}$ and $P27^{kip1}$ are direct targets of RBP2. As important cyclin-dependent kinase inhibitors, $P21^{CIP1}$ and $P27^{kip1}$ are the main checkpoint proteins for the G1/S and G2/M stages [Wang et al., 2009]. $P21^{CIP1}$ and $P27^{kip1}$ take part in cell cycle arrest and are downregulated in GC for cell proliferation, which have been the hallmarks of cancers [Xia et al., 2012]. We found that restoring miR-212 expression could inhibit GC cell growth and repressing miR-212 expression could accelerate cell growth. In this process, both the expression and promoter activity of $P21^{CIP1}$ and $P27^{kip1}$ were regulated by miR-212. When miR-212 was overexpressed in gastric cells, the expression and promoter activity of $P21^{CIP1}$ and $P27^{kip1}$ were upregulated, respectively. In contrast, with the inhibition of miR-212, the expression and promoter activity of $P21^{CIP1}$ and $P27^{kip1}$ were downregulated. Thus, the miR-212-RBP2 axis may modulate the expression of $P21^{CIP1}$ and $P27^{kip1}$ and repress cell proliferation in GC.

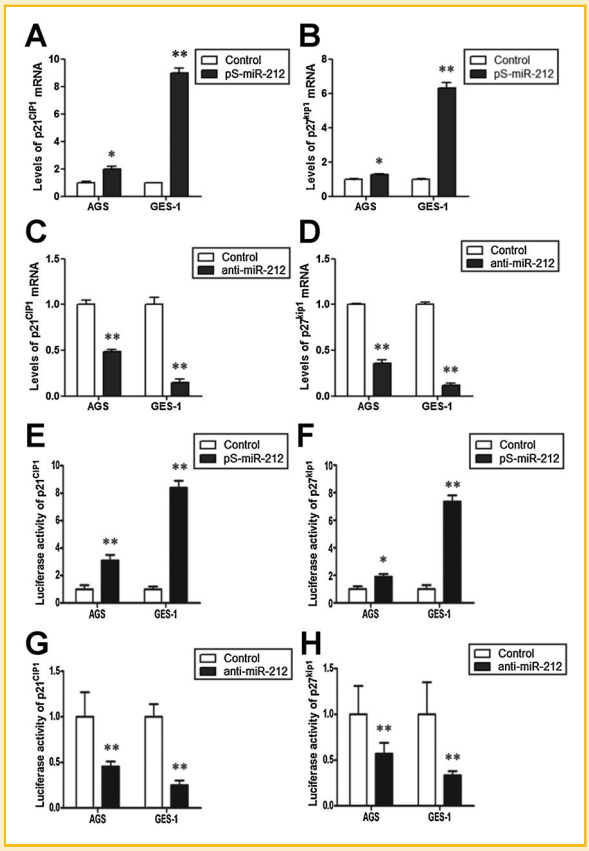

Fig. 4. Hsa-miR-212 regulated the expression of $p21^{CIP1}$ and $p27^{kip1}$. A,B: Overexpression of hsa-miR-212 with transfection of pSilencer-miR-212 upregulated the mRNA expression of $p21^{CIP1}$ and $p27^{kip1}$. C,D: Downregulation of $p21^{CIP1}$ and $p27^{kip1}$ promoter activity with pSliencer-miR-212 transfection. E,F: Inhibition of hsa-miR-212 by transfection with pSuper-anti-miR-212 repressed the expression of $p21^{CIP1}$ and $p27^{kip1}$. G,H: Downregulation of $p21^{CIP1}$ and $p27^{kip1}$ promoter activity with pSuper-anti-miR-212 transfection. $^{**}P < 0.01$, $^{*}P < 0.05$ versus con, respectively. Data are mean ± SEM. (n = 3 experiments).

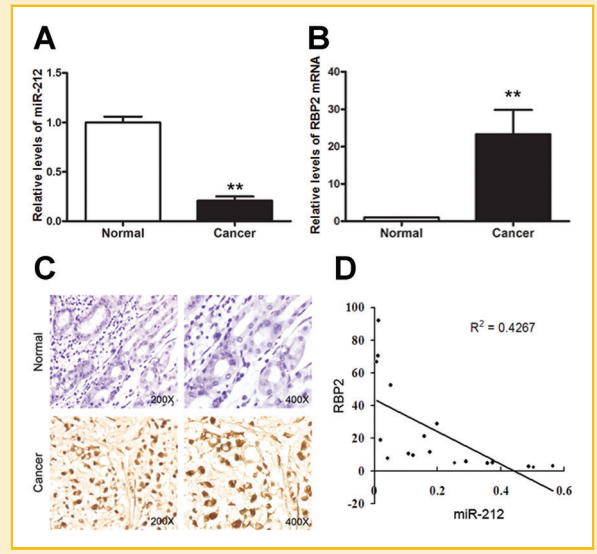

Fig. 5. Hsa-miR-212 was lowexpressed and RBP2 was overexpressed in human primary gastric cancer tissue. QRT-PCR analysis of the mRNA expression of (A) miR-212 and (B) RBP2 in normal and gastric cancer tissue and (C) immunohistochemistry images of the protein expression of RBP2 ($^{**}P < 0.01$, paired *t*-test). Data are shown as mean ± SEM. (n = 3 experiments). D: Correlation of RBP2 and miR-212 levels in gastric cancer tissue after standardization with matched normal tissues.

In summary, miR-212 expression was significantly downregulated in human GC tissue and negatively covalent with the expression of RBP2 as compared with distal normal gastric mucous tissues. MiR-212 may be a tumor suppressor in GC growth and responsible for RBP2 inhibition at both mRNA and protein levels by directly binding to the 3′ UTR of RBP2 at 2 sites. MiR-212 inhibited GC growth by affecting the expression of $P21^{CIP1}$ and $P27^{kip1}$, which was directly regulated by RBP2. This miR-212–RBP2–$P21^{CIP1}$/$P27^{kip1}$ signaling axis may explain the overexpression of the epigenetic molecule RBP2 in GC and provide insights into the mechanisms of gastric carcinogenesis.

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