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LncRNA RMRP knockdown promotes proliferation and inhibits apoptosis in osteoarthritis chondrocytes by miR-206/CDK9 axis

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The etiology of osteoarthritis (OA) has been discussed widely, but the molecular mechanisms beneath OA aggravation have not yet been investigated in detail. This study focused on the role of lncRNA RMRP (RMRP) on OA progression. We found that the expression of RMRP was significantly increased in cartilage tissues of patients with OA. CCK-8 and colony formation assays showed that RMRP knockdown promoted proliferation of chondrocytes treated with IL-1β. Flow cytometry and caspase-3 activity analysis indicated that RMRP silence inhibited apoptosis of chondrocytes treated with IL-1β. Moreover, luciferase reporter, RNA pull-down and RIP assays showed that RMRP competing with miR-206. Additionally, CDK9 acted as a direct target of miR-206. Moreover, rescue assays indicated that miR-206 inhibitor or pcDNA-CDK9 reversed the effects of RMRP suppression on the proliferation and apoptosis of chondrocytes. Taken together, our results indicated that RMRP knockdown could promote proliferation and inhibit apoptosis in OA chondrocytes via the miR-206/CDK9 axis.

1. Introduction

Osteoarthritis (OA) is a common degenerative disease of joints which could result in serious pain and disability (Durg et al. 2019). As the only cell type in cartilage tissues, chondrocytes dysfunction, such as abnormal proliferation, apoptosis, and extracellular matrix occurs during osteoarthritis (Theocharis et al. 2019; Pap and Korb-Pap 2015). Therefore, exploring the underlying molecular mechanisms of chondrocytes dysfunction is key for us to understand the pathogenesis of OA.

Long noncoding RNAs (lncRNAs) are little protein-coding potential RNA molecules that are longer than 200 nucleotides (nt) (Fatica and Bozzoni 2014). Several recent reports have shown that lncRNAs are associated with OA progression (Wang et al. 2018). Li et al. (2017) found that lncRNA-CIR overexpression significantly increased the expression of MMP13 by acting as a sponge of miR-27 in OA. LncRNA-MSR, the TMSB4 pseudogene, was increased in the damaged cartilage and promoted cartilage degradation by regulated the miR-152/TMSB4 axis in human OA (Liu et al. 2016). The overexpression of lncRNA GAS5 alleviated LPS-induced inflammatory injury and apoptosis in ATDC5 chondrocytes through the regulation of KIF12-mediated NF-κB and Notch pathways (Li et al. 2018). MEG3 was reported to contribute to cell proliferation and inhibit cell apoptosis and degradation of extracellular matrix (ECM) *via* the miR-361-5p/FOXO1 axis in OA chondrocytes (Wang et al., 2019).

LncRNA RMRP (RMRP) has been reported to play important roles in inflammation-related diseases. For example, Moharamoghli et al. (2019) suggested that the expression of RMRP increased in T cells of patients with rheumatoid arthritis and showed a positive correlation with disease duration in rheumatoid arthritis. Moreover, RMRP prevented mitochondrial dysfunction and cardiomyocyte apoptosis in LPS-induced sepsis mice via the miR-1-5p/hsp70 axis (Han et al. 2020). However, the role and underlying mechanism of RMRP on chondrocytes dysfunction during OA remain unclear.

This present study was devised to determine the role and underlying mechanism of RMRP on chondrocytes dysfunction during OA. Herein, we found that the expression of RMRP was significantly increased in cartilage tissues of patients with OA. Furthermore, RMRP knockdown promoted proliferation and inhibited apoptosis of OA chondrocytes by the miR-206/CDK9 axis.

2. Investigations and results

2.1. The expression level of RMRP was upregulated in cartilage tissues of OA patients

To investigate the role of RMRP in OA, we collected 24 OA cartilage tissue samples and 24 normal cartilage tissue samples. The expression level of RMRP in cartilage tissues of OA patients was detected by RT-qPCR. As shown in Fig. 1, the expression level of RMRP in cartilage tissue of OA patients was much higher than that in normal cartilage tissue.

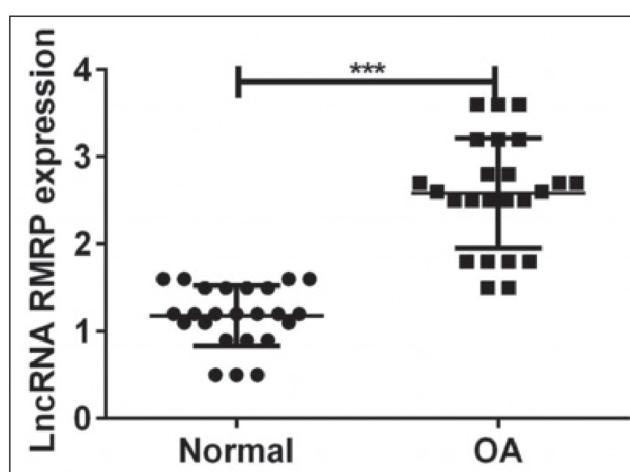

Fig. 1: The expression level of RMRP was upregulated in OA tissue. The level of RMRP in (OA) tissues (n=24) and normal samples (n=24) was analyzed by RT-qPCR. *P<0.05.

2.2. RMRP knockdown promoted chondrocyte proliferation

To further explore the effects of RMRP on the biological function of chondrocytes, we transfected C28/I2 cells with si-RMRP-1, si-RMRP-2 or si-NC, followed via the stimulation of IL-1β. The RT-qPCR results showed that both si-RMRP-1 and si-RMRP-2

treatment significantly reduced the expression level of RMRP in C28/I2 cells compared with si-NC treatment (Fig. 2A). The CCK-8 assay indicated that si-RMRP-1 and si-RMRP-2 treatment markedly promoted C28/I2 cell proliferation compared with si-NC treatment (Fig. 2B). Furthermore, RMRP knockdown significantly reduced the number of C28/I2 cell clones (Fig. 2C and 2D).

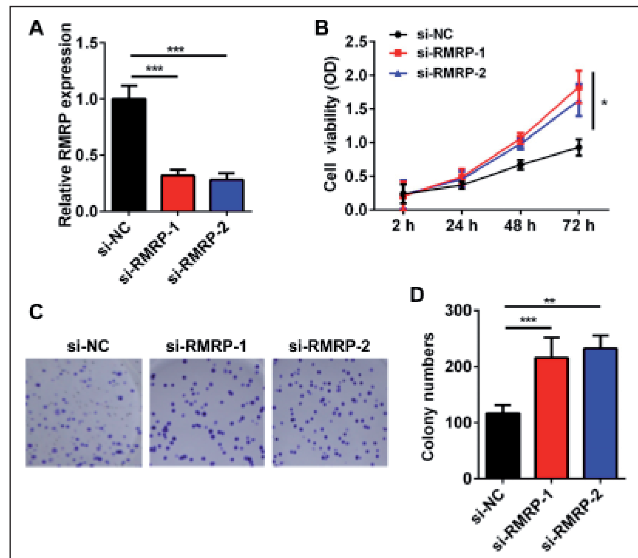

Fig. 2: RMRP knockdown promoted the proliferation of chondrocytes treated with IL-1β. (A) The expression of RMRP in C28/I2 cells after si-NC, si-RMRP-1 or si-RMRP transfection was analyzed by RT-qPCR. (B) The cell viability of C28/I2 cells after si-NC, si-RMRP-1 or si-RMRP transfection was analyzed by CCK8 analysis. (C) The proliferation viability of C28/I2 cells after si-NC, si-RMRP-1 or si-RMRP transfection was analyzed by colony formation analysis. (D) Statistical summary of colony formation results in fig C. *$P<0.05$; ***$P<0.001$.

2.3. RMRP knockdown inhibited chondrocytes apoptosis

As shown in Fig. 3A and 3B, both si-RMRP-1 and si-RMRP-2 treatment significantly reduced the apoptosis rate of C28/I2 cells compared with si-NC treatment, followed by the stimulation of IL-1β. Moreover, the results of the caspase-3 activity assay indicated that C28/I2 cells transfected with si-RMRP-1 or si-RMRP-2

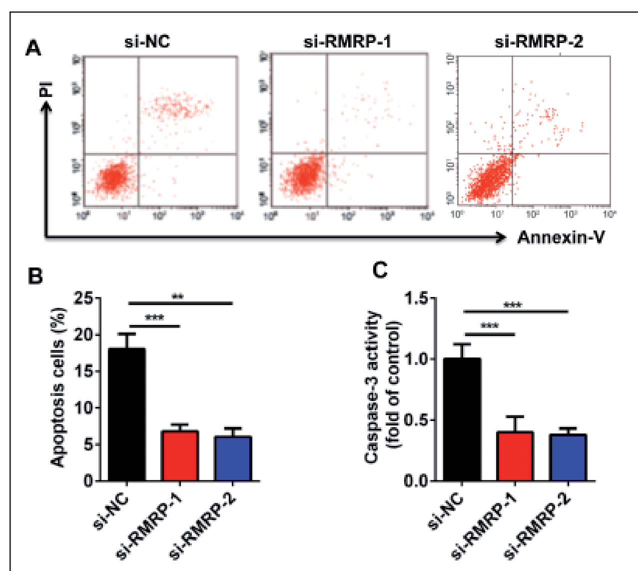

Fig. 3: RMRP knockdown inhibited the apoptosis of chondrocytes treated with IL-1β. (A) Flow cytometry analysis showed that si-RMRP-1 or si-RMRP transfection reduced C28/I2 cell apoptosis. (B) Statistical summary of flow cytometry results in fig B. (C) Caspase-3 activity in C28/I2 cells after si-NC, si-RMRP-1 or si-RMRP transfection were analyzed. ***$P<0.001$.

showed downregulated caspase-3 activity level compared with C28/I2 cells transfected with si-NC (Fig. 3C). These results indicated that RMRP knockdown inhibited chondrocytes apoptosis.

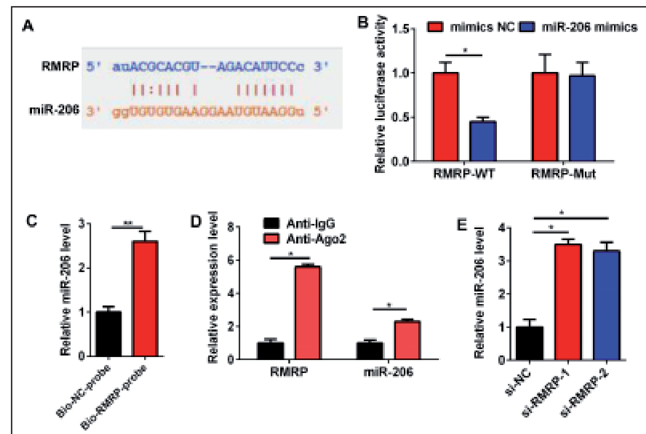

Fig. 4: RMRP was regulated by miR-206. (A) Starbase 3.0 prediction of conserved miR-206 binding sites in the RMRP. (B) Luciferase assay was conducted in HEK293T cells co-transfected with PmirGLO vectors overexpress of RMRP and miR-206 mimics. (C) The direct interaction between miR-206 and RMRP was analyzed by RNA pull-down assay. (D) RIP assay indicated that RMRP and miR-206 were enriched in Ago2 immunoprecipitates. (E) The expression of miR-206 in C28/I2 cells after si-NC, si-RMRP-1 or si-RMRP transfection was analyzed by RT-qPCR. *$P<0.05$; **$P<0.01$.

2.4. MiR-206 was a target of RMRP

The binding site of RMRP and miR-206 was predicted using Starbase (Fig. 4A). The results of the luciferase activity assay showed that RMRP-WT and miR-206 mimics co-transfection significantly reduced the luciferase activity compared with RMRP-WT and mimics NC transfection (Fig. 4B). Moreover, miR-206 mimics transfection had no effect of luciferase activity after miR-206 mimics and RMRP-Mut co-transfection (Fig. 4C). RNA pull-down assay indicated that the miR-206 level was significantly increased in the Bio-RMRP-probe group (Fig. 4D). RIP assay showed that both RMRP and miR-206 were enriched in Ago2 group (Fig. 4E). In addition, RMRP knockdown significantly promoted the expression level of miR-206 (Fig. 4F).

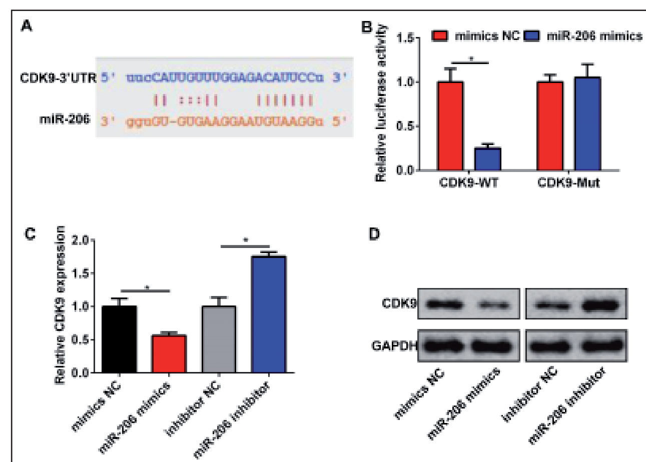

Fig. 5: CDK9 is regulated by miR-206. (A) Starbase 3.0 prediction of conserved miR-206 binding sites in the CDK9. (B) Luciferase assay was conducted in HEK293T cells co-transfected with PmirGLO vectors overexpressing CDK9 and miR-206 mimics. (C, D) The mRNA (C) and protein (D) expression of CDK9 in C28/I2 cells after miR-206 mimics or inhibitor transfection. *$P<0.05$.

2.5. CDK9 was a target of miR-206

The binding site of miR-206 and CDK9 3'-UTR was predicted using Starbase (Fig. 5A). The results of luciferase activity assay showed that CDK9-WT and miR-206 mimics co-transfection

significantly reduced the luciferase activity compared with CDK9-WT and mimics NC transfection (Fig. 5B). Moreover, miR-206 mimics transfection had no effect of luciferase activity after miR-206 mimics and CDK9-Mut co-transfection (Fig. 5C). Moreover, miR-206 mimics transfection significantly downregulated the mRNA and protein expression levels of CDK9 (Fig. 5C and 5D). In contrast, miR-206 inhibitor transfection significantly increased the mRNA and protein expression levels of CDK9 (Fig. 5C and 5D).

2.6. RMRP regulated the proliferation and apoptosis of chondrocytes by the miR-206/CDK9 axis

To examine whether RMRP-regulated the proliferation and apoptosis of chondrocytes was miR-206/CDK9 axis dependent, we transfected C28/I2 cells with si-NC, si-RMRP-1, si-RMRP-1+miR-206 inhibitor or si-RMRP-1+pcDNA-CDK9 followed via the stimulation of IL-1β. si-RMRP-1 transfection markedly reduced the protein level of CDK9 (Fig. 6A). Meanwhile, miR-206 inhibitor or pcDNA-CDK9 transfection abolished the inhibitory effect of si-RMRP-1 on CDK protein expression (Fig. 6A). In addition, miR-206 inhibitor or pcDNA-CDK9 transfection abolished the effect of si-RMRP-1 transfection on C28/I2 cell proliferation (Fig. 6B). Moreover, miR-206 inhibitor or pcDNA-CDK9 transfection reversed the effect of si-RMRP-1 transfection on Caspase-3 activity (Fig. 6C).

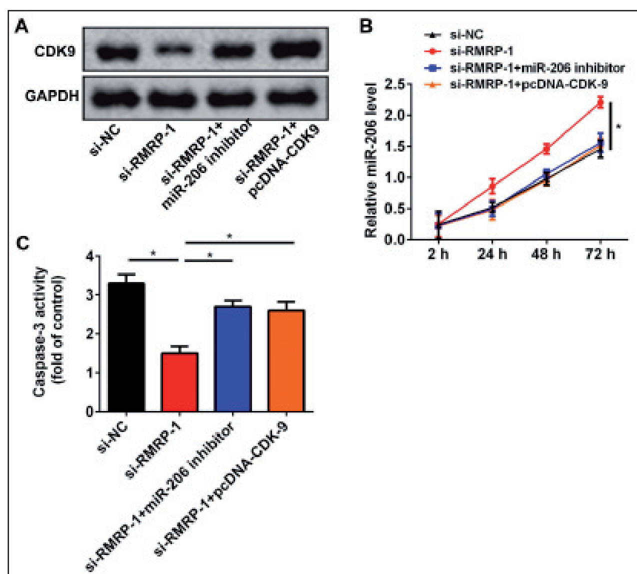

Fig. 6: RMRP regulated cell activity and apoptosis via the miR-206/CDK9 axis. C28/I2 cells were transfected with si-NC, si-RMRP-1, si-RMRP-1+miR-206 inhibitor or si-RMRP-1+pcDNA-CDK9 followed via the stimulation of IL-1β. (A) The protein expression of CDK9 in different transfection content was analyzed by Western blot. (B) The cell viability of C28/I2 cells in different transfection content was analyzed by CCK8 analysis. (C) Caspase-3 activity in C28/I2 cells in different transfection content was analyzed. *$P<0.05$.

3. Discussion

Although the etiology of OA has been discussed widely (Guilak et al. 2018), the molecular mechanisms beneath OA aggravation have not yet been investigated in detail. In the current study, we found that the expression level of RMRP was upregulated in cartilage tissues of OA patients. RMRP knockdown significantly promoted chondrocyte proliferation and inhibited chondrocyte apoptosis.

Previous studies indicated that RMRP played a key role in various diseases. For example, RMRP knockdown suppressed hepatocellular carcinoma cell proliferation, invasion, and migration by the regulation of miRNA-206/TACR1 (Hongfeng et al. 2020). Knockdown of RMRP by small-interfering RNA inhibited cardiac fibroblast proliferation, differentiation and collagen accumulation by regulating miR-613 (Zhang et al. 2019). RMRP suppression ameliorated neural cell injury induced by oxygen-glucose deprivation/re-oxygenation through inhibiting autophagy and PI3K/Akt/mTOR-mediated apoptosis (Zhou et al. 2019). In addition, RMRP overexpression inhibited LPS-induced apoptosis of cardiomyocytes and mitochondrial damage by suppressing the post-transcriptional regulatory function of miR-1-5p on HSPA4 (Han et al, 2020). Although the abovementioned study indicated RMRP exerts a key role in various diseases, the role of RMRP in OA remains unclear. In this study, we found that RMRP knockdown significantly promoted proliferation and inhibited apoptosis of chondrocytes treated by IL-1β by regulating miR-206.

The importance of the ceRNA network mechanism mediated by lncRNA and miRNA has been reported in OA (Chen et al. 2019). For example, miR-142-5p protected against OA through competing with lncRNA XIST (Sun et al. 2020). LncRNA GAS5 participated in the development of OA by regulating the biological behavior of chondrocytes via competing with the miR-34a/Bcl2 axis (Ji et al. 2019). LncRNA PVT1 knockdown increased cell viability and autophagy but inhibited apoptosis and inflammatory response in chondrocytes treated by IL-1β via competing with miR-27b-3p (Lu et al. 2019). Given this notion, we predicted potential lncRNA-miRNA interactions by bioinformatics analysis and found that miR-206 was identified as the target of RMRP. MiR-206 was significantly increased in human OA tissues and chondrocytes (Ni et al. 2018). Moreover, MiR-206 overexpression significantly inhibited the proliferation but promoted apoptosis of chondrocytes by decreasing the expression of RUNX2 and MMP13 (Ni et al. 2018). Yu et al. reported that miR-206 promoted OA development through regulating articular chondrocyte apoptosis and autophagy via modulating the phosphoinositide 3-kinase/protein kinase B-mTOR pathway by targeting insulin-like growth factor-1 (Yu et al. 2019). In the current study, we found that RMRP-WT and miR-206 mimics co-transfection significantly reduced the luciferase activity. RNA pull-down assay and RIP assay showed indicated that RMRP could compete with miR-206 in chondrocytes. More importantly, miR-206 inhibitor transfection abolished the effect of si-RMRP-1 transfection on C28/I2 cell proliferation and caspase-3 activity. These results indicated that RMRP regulates the proliferation and apoptosis of chondrocytes via miR-206.

CDK9 has been reported to act as an important regulator in OA progression. For example, LDC000067, a specific inhibitor of CDK9, could prevent IL-1β-induced production of MMPs and inflammatory cytokines in chondrocytes, including MMP3, MMP9, MMP13, IL-6, IL-8 and TNF-α (Xue et al., 2019). Hu et al. (2016) found that CDK9 inhibition attenuated chondrocyte apoptosis and reduced cartilage matrix degradation in cartilage explants. A previous study indicated that miR-206 could target CDK9 and inhibit the growth of hepatocellular carcinoma cells (Pang et al. 2017). In this study, we found that CDK9-WT and miR-206 mimics co-transfection significantly reduced the luciferase activity. MiR-206 mimics transfection significantly downregulated the mRNA and protein expression levels of CDK9. In contrast, miR-206 inhibitor transfection significantly increased the mRNA and protein expression levels of CDK9. More importantly, pcDNA-CDK9 transfection abolished the effect of si-RMRP-1 transfection on C28/I2 cell proliferation and Caspase-3 activity. These results indicated that RMRP regulates the proliferation and apoptosis of chondrocytes via the miR-206/CDK9 axis.

In sum, RMRP knockdown may promote cell proliferation, and impair cell apoptosis via the miR-206/CDK9 axis in OA chondrocytes, indicating that RMRP might be used as a novel target for the treatment of OA.

4. Experimental

4.1. OA tissue samples

Twenty-four OA cartilage tissue samples were obtained from OA patients who underwent total knee arthroplasty at The Second Affiliated Hospital of Soochow University. 24 normal cartilage tissue samples were obtained from trauma patients without OA or rheumatoid arthritis. All clinic experiments were approved by the Ethics Committee of The Second Affiliated Hospital of Soochow University. And informed consent was obtained from each patient.

4.2. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

We extracted total RNA from tissues or cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was reversely transcribed to cDNA by PrimeScript RT kit (Takara). RT-qPCR was performed with the miScript SYBR-Green PCR Kit (Qiagen). β-actin or U6 was used as the reference gene. The primers (Table) for amplification were synthesized by Shanghai Sangon Biological Engineering Technology and Services Ltd. (Shanghai, China).

Table: Primer sequences used in the present study

Primer Name	Primer Sequence(5'-3')
RMRP-Forward	ACTCCAAAGTCCGCCAAGA
RMRP-Reverse	TGCGTAACTAGAGGGAGCTGAC
miR-206-Forward	ACACTCCAGCTGGGTGGAATGTAAGGAAGT
miR-206-Reverse	CTCAACTGGTGTCGTGGAGTCGGCAAT-TCAGTTGAGCCACACAC
U6-Forward	CTCGCTTCGGCAGCACA
U6-Reverse	AACGCTTCACGAATTTGCGT
CDK9-Forward	ATGGCAAAGCAGTACGACTCG
CDK9-Reverse	GCAAGGCTGTAATGGGGAAC
β-actin-Forward	AAGGAGCCCCACGAGAAAAAT
β-actin-Reverse	ACCGAACTTGCATTGATTCCAG

4.3. Chondrocyte culture, treatment, and transfection

Human chondrocyte C28/I2 and the HEK293T cells were purchased from American Type Culture Collection (ATCC, Walkersville, MD, USA). The C28/I2 cells were cultured in DMEM/F-12 medium (Thermo Fisher Scientific, Wilmington, DE, USA) containing 10% fetal bovine serum (FBS, Gibco, Norwalk, CT) at 37 °C with 5% CO_2. HEK293T cells were cultured in DMEM medium (Thermo Fisher Scientific) containing 10% FBS.

For the establishment of the OA model *in vitro*, C28/I2 cells were incubated with 5 ng/mL of IL-1β (Sigma, St. Louis, MO, USA) for 12 h.

Cells were transfected with specific siRNAs against RMPR, miR-206 mimics, miR-206 inhibitor, pcDNA-CDK9, and their corresponding negative control (Gene-Pharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

4.4. Cell counting kit 8 (CCK-8) assay

C28/I2 cells (1×10^3) were first plated into 96-well plates and treated with RMPR siRNAs, miR-206 inhibitor or pcDNA-CDK-9. After 2, 24, 48, and 72 hours, each well was added 10 uL CCK-8 reagents for cultivation for an additional 4 h. The absorbance of each well at 450 nm was examined.

4.5. Colony formation assay

C28/I2 cells (500) were treated with 0.3% soft agar and incubated in 6-well plates for 10-14 days. We fixed the colonies with methanol, stained the colonies with 0.1% crystal violet (Merck), and counted the number of colonies manually.

4.6. Cell apoptosis analysis

Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (Thermo Fisher Scientific) was used to assess cell apoptosis. Briefly, C28/I2 cells were stained with 5 μL Annexin V-FITC and 5 μL PI in the dark for 15 min. The percentage of apoptosis cells was detected using a FACS Caliber system (Beckman).

4.7. Caspase-3 activity assay

The specific caspase-3 activity kit (R&D Systems) was used to determine the caspase-3 activity of C28/I2 cells according to the manufacturer's instructions.

4.8. Western Blot

Western Blot was performed as described previously (Lu 2019). The antibodies used in this study were as follow rabbit anti-CDK9 antibody (Abcam) and mouse anti-GAPDH antibody (Abcam).

4.9. RNA immunoprecipitation (RIP)

The Magna RIP kit was used for the RIP experiment (Millipore) following the manufacturer's instructions. Briefly, C28/I2 cells were lysed in complete RIP lysis buffer and incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody (Millipore) or normal mouse IgG (negative control) for 8 h at 4 °C. Next, immunoprecipitated RNA was isolated using TRIzol reagent.

4.10. RNA pull-down analysis

C28/I2 cell lysates were harvested with RIPA lysis buffer and incubated with Bio-RMRP-probe and Bio-NC-probe (Thermo Fisher Scientific) at room temperature for 60 min. Each binding reaction was then added to Streptavidin agarose beads (Sigma-Aldrich) and incubated with RNase-free DNase I (Takara) at 37 °C for 15 min and proteinase K at 45 °C for 30 min. Finally, RNA was isolated using TRIzol reagent.

4.11. Luciferase reporter assay

The potential target sites of miR-206 and RMRP or CDK9 were predicted by starbase 3.0 (http://starbase.sysu.edu.cn/). The luciferase reporter vectors containing the wild-type (WT) or mutant (Mut) 3'-UTR of RMRP and CDK9 were obtained from GenePharma. The luciferase reporter vectors were co-transfected with miR-206 mimics or control into HEK293T cells and a dual-luciferase reporter gene assay was performed after 48 h transfection using the dual-luciferase reporter gene assay (Promega, USA).

4.12. Statistical analysis

We used SPSS 22.0 (Chicago, IL, USA) software for data analysis. Student's t-test or a one-way ANOVA was used for significant differences. $P<0.05$ was considered statistically significant.

Conflict of Interest: The authors did not report any conflict of interest.

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