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MiR-320a was highly expressed in postmenopausal osteoporosis and acts as a negative regulator in MC3T3E1 cells by reducing MAP9 and inhibiting PI3K/AKT signaling pathway

Yao Kong[a], Zhi-Kui Nie[a,1], Feng Li[b,1], Hong-Min Guo[a,2], Xing-Lin Yang[b,2], Shao-Feng Ding[b,*]

[a] Department of Osteoarticular Surgery, Jining NO.1 People's Hospital, China
[b] Department of Endocrinology, Jining NO.1 People's Hospital, China

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ABSTRACT

Background: Postmenopausal osteoporosis (PMO), as a frequent disease in postmenopausal women, is mainly caused by the lack of estrogen. MiR-320a has been found to abate osteoblast function and induce oxidative stress before osteoporosis. However, studies on the downstream target gene and related signaling pathway of miR-320a in PMO are still obscure. This study aims to deal with these problems.

Methods: The expression levels of miR-320a and microtubule-associated protein 9 (MAP9) in patients with osteoporosis were analyzed on the basis of the GEO database. The cells viability was determined by methylthiazolyl tetrazolium bromide (MTT). Flow cytometry and western blot were used to detect the cells apoptosis and the expression of apoptosis-related proteins, respectively. The cells differentiation-related proteins were detected by qRT-PCR and western blot. The interaction between miR-320a and MAP9 was predicted by biological software and verified by dual luciferase reporter assay and rescue assay. The expression levels of PI3K, p-PI3K, AKT and p-AKT in MC3T3-E1 cells were assessed by western blot.

Results: We observed that miR-320a was over-expressed in PMO patients and exhibited inhibitory effects on MC3T3-E1 cells activity and differentiation, as well as promoting effects on MC3T3-E1 cells apoptosis. MAP9 was verified as a target gene of miR-320a and was negatively regulated by miR-320a. Based on the GEO database, MAP9 was found to be lower expressed in PMO patients. Rescue assay demonstrated that down-regulation of MAP9 could alleviate the promoting effects of miR-320a inhibitor on MC3T3-E1 cells activity and differentiation and the inhibitory effects of miR-320a inhibitor on MC3T3-E1 cells apoptosis. Mechanically, miR-320a/MAP9 possibly took part in MC3T3-E1 cells viability, differentiation and apoptosis via mediating PI3K/AKT signaling pathway.

Conclusions: Our outcomes demonstrated that miR-320a promoted MC3T3-E1 cells apoptosis, suppressed MC3T3-E1 cells viability and differentiation through targeting MAP9 and modulating PI3K/AKT signaling pathway, which provided theoretical support for miR-320a/MAP9 as promising targets for the treatment and prevention of PMO.

1. Introduction

Postmenopausal osteoporosis (PMO) is a common age-related disease accompanied by the characteristics of decreased bone mass and bone structure changes, usually attributed to estrogen deficiency (Lin et al., 2017). PMO is an insidious disease which occurs generally without any symptoms. However, fractures has already been occurred when a hunchback, shortened figure or bone pain is found. Bone formation and resorption are mediated by osteoblast and osteoclast, respectively. Disorders of these two processes can lead to osteoporosis, which reduces bone strength and improves the risk of fracture (Wang et al., 2018). Currently, therapeutic drugs intend for remedying osteoporosis including menopausal hormone therapy (MHT), selective estrogen receptor modulators (SERMs) (Tella and Gallagher, 2014),

* Corresponding author at: Department of Endocrinology, Jining NO.1 People's Hospital, NO.6 Health Road, Rencheng District, Jining City, Shandong Province 272000, China.
E-mail address: ding_shao_feng@163.com (S.-F. Ding).
[1] Zhi-Kui Nie and Feng Li contributed equally.
[2] Hong-Min Guo and Xing-Lin Yang contributed equally.

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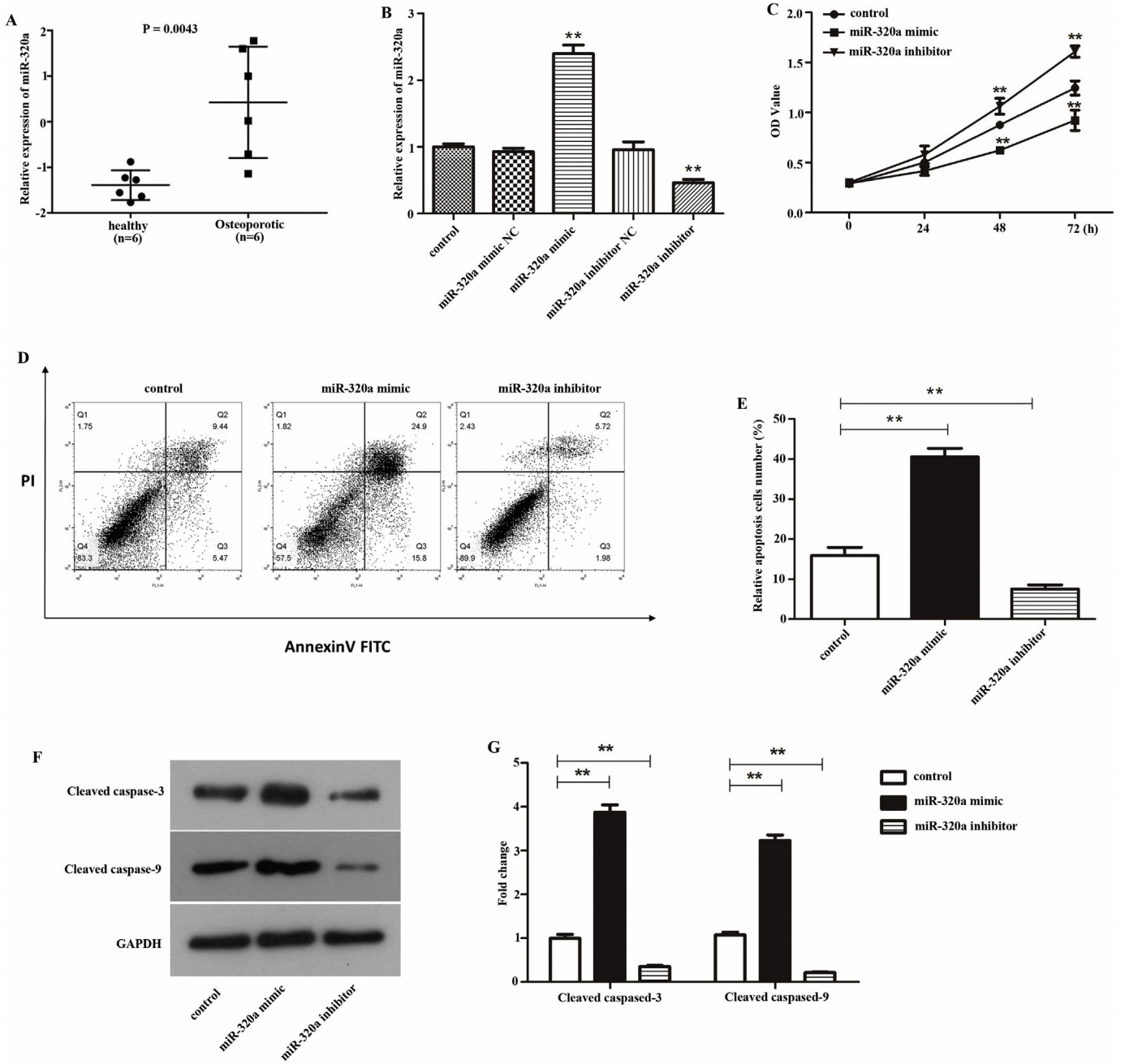

Fig. 1. MiR-320a had an inhibitory effect on MC3T3-E1 cells activity and a promoting effect on MC3T3-E1 cells apoptosis. A: The miR-320a expression level in PMO patients was analyzed based on GEO database, P = .0043. B: The miR-320a expression level was measured by qRT-PCR in MC3T3-E1 cells after transfected with miR-320a mimic/inhibitor/NC. C: The proliferation of MC3T3-E1 cells transfected with miR-320a mimic/inhibitor was detected by MTT. D-E: Apoptosis of MC3T3-E1 cells transfected with miR-320a mimic/inhibitor was determined by flow cytometry. F-G: The expression of pro-apoptotic proteins Cleaved caspase-3 and Cleaved caspase-9 were determined by western bolt after transfected with miR-320a mimic/inhibitor, $^{**}P < .01$.

calcition, bisphosphonates, vitamin D_3 (Gambacciani and Levancini, 2014), parathyroid hormone (PTH) analogs(Finkelstein et al., 2003), etc. While, long run of these drugs have been found to pose safety risks, such as increased risk of several diseases including breast cancer, endometrial cancer, cardiovascular disease and venous thromboembolism (Lin et al., 2017). Consequently, safer and more efficient alternative treatments for PMO are urgently required.

MicroRNAs (miRNAs), as small non-coding RNAs, control gene expression at the post-transcriptional level via immediately inhibition of target mRNA (Schetter et al., 2012). Emerging evidences hinted that miRNAs took vital roles in multifarious physiological and pathological procedures. More momentously, miRNAs might be promising targets for the treatment of dozens of diseases, including PMO. A recent study exposed that miR-133a participated in the regulation of PMO by stimulating osteoclast differentiation (Li et al., 2018). In the analysis of Zhang et al., miR-221 acted as a vital role in the modulation of osteoporosis by controlling Runx2 protein expression and osteoblast differentiation (Zhang et al., 2017). Recent work by Wang et al. has established that depletion of miR-144-3p in serum and bone regulated the pathogenesis of osteoporosis via targeting RANK (Wang et al., 2018). MiR-320a, as a pivotal miRNA, has been identified to act as a key regulator in various diseases. It has previously been observed that miR-320a played a crucial role in mediating doxorubicin–induced cardiotoxicity (Yin et al., 2016). Recent research has shown that miR-320a

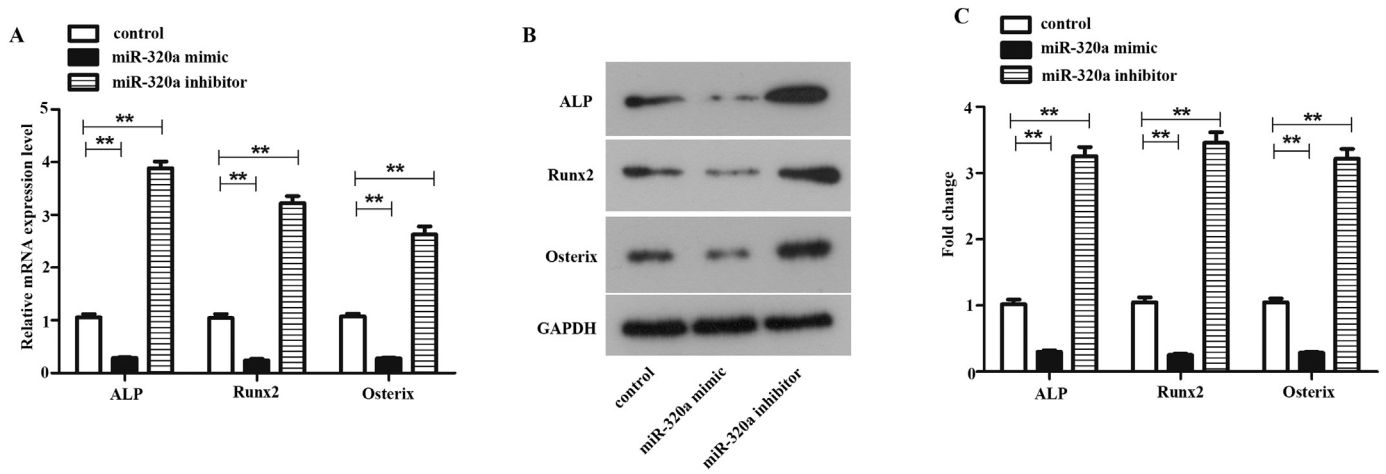

Fig. 2. MiR-320a suppressed MC3T3-E1 cells differentiation. The mRNA and protein expression levels of ALP, Runx2 and Osterix were measured by qRT-PCR (A) and western blot (B—C), $^{**}P < .01$.

inhibited biological behaviors of breast cancer cells by reducing RAB11A (Wang et al., 2015). Furthermore, miR-320a has been illustrated to be highly expressed in patients with osteoporosis (De-Ugarte et al., 2015) and to damage the function of osteoblast as well as to induce oxidative stress before osteoporosis (De-Ugarte et al., 2018). However, the downstream target gene and signaling pathway of miR-320a in PMO have been poorly investigated.

In the present study, we attempted to explore the function of miR-320a on osteoblast viability, differentiation and apoptosis, as well as the downstream target gene and the relevant signal pathway of miR-320a in PMO. Our results revealed that miR-320a suppressed MC3T3-E1 cells viability and differentiation, accelerated MC3T3-E1 cells apoptosis by reducing MAP9 and mediating PI3K/AKT signaling pathway, suggesting that miR-320a/MAP9 might be an effective target for the diagnosis and prevention of PMO.

2. Materials and methods

2.1. Data collection

To observe the expression patterns of miR-320a and MAP9 in PMO, datasets from the GEO database with accession number GSE74209 and GSE56116 were applied, respectively. 12 cases were used to analyze the expression of miR-320a, fresh femoral neck trabecular bone was obtained from 12 postmenopausal women undergoing hip replacement due to either osteoporotic fracture ($n = 6$) or osteoarthritis in the absence of osteoporosis ($n = 6$). MAP9 expression was analyzed with 13 cases, containing 10 patients with PMO (osteoporosis) and 3 healthy postmenopausal women (healthy).

2.2. Cell culture and transfection

Murine osteoblastic MC3T3-E1 cells were purchased from the Shanghai cell bank of the Chinese academy of medical sciences (Shanghai, China), maintained in a α minimum essential medium (MEM) containing 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, USA). 10 μmol/mL β-soldium glycerophosphatate and 50 μg/mL ascorbic acid were added into the medium to induce differentiation of MC3T3-E1 cells, and the medium was changed every 3 days.

MiR-320a mimic/inhibitor, miR-320a mimic/inhibitor NC, si-con and si-MAP9 were synthesized by GenePharma Co. (Shanghai, China) and transfected into MC3T3-E1 cells using Lipofectamine 3000 transfection reagent (Carlsbad, CA, USA). The transfection concentrations were 100 nM for miR-320a mimic/inhibitor and miR-320a mimic/inhibitor NC, 50 nM for si-con and si-MAP9. The sequences were presented as follows:

miR-320a mimic: 5′-AAAAGCUGGGUUGAGAGGGCGA-3′,

miR-320a inhibitor: 5′-UCGCCCUCUCAACCCAGCUUUU-3′,

miR-320a mimic/inhibitor NC: 5′-UUGUCCUACACCUCACUCCUG-3′,

si-con: 5’-GACUCUUAGUUCAAUUGUACU-3′,

si-MAP9: 5’-UUCUCCGAACGUGUCACGU-3′.

2.3. Cell viability test

Cell viability was assessed through MTT assay. First, the MC3T3-E1 cells were implanted into 96-well plates with the density of 1×10^3 cells/well. Then, the MC3T3-E1 cells were treated with 10 μmol/mL β-soldium glycerophosphatate and 50 μg/mL ascorbic acid, and transfected with miR-320a mimic/inhibitor for 48 h. Before detection, added 20 μL of MTT solution into each well and cultured for 4 h under the condition of 37 °C and 5% CO_2. Subsequently, discarded the supernatant, added 200 μL of DMSO into each well and incubated for 10 min. Eventually, the optical density (OD) values were acquired at 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Detection of apoptosis

The MC3T3-E1 cells apoptosis was measured by flow cytometry. The treated cells were gathered and suspended with pre-cooling binding buffer. Then 5 μL of Annexin V-fluorescein isothiocyanate (V-FITC) and 10 μL of propidium iodide (PI) were added to the cell suspension, and then the suspension hatched on ice for 5 min in the darkness. The apoptosis proportion was analyzed by FACScanto (BD Biosciences, California, USA).

2.5. Dual luciferase reporter assay

Wild Type (WT) or Mutant Type (MUT) of MAP9 3’-UTR was cloned into pmiR-RB-REPORTTM vector, and co-transfected into HEK293 cells with miR-320a mimic and miR-320a mimic NC through Lipofectamine 2000 (Invitrogen, USA). After 48 h, gathered and lysed the cells, and the luciferase activity was calculated using the dual luciferase reporter assay kit (Promega, Wisconsin, USA).

2.6. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was abstracted by TRIzol reagent and then inverse transcribed using PrimeScript RT Reagent Kit and Mir-X™ miRNA First

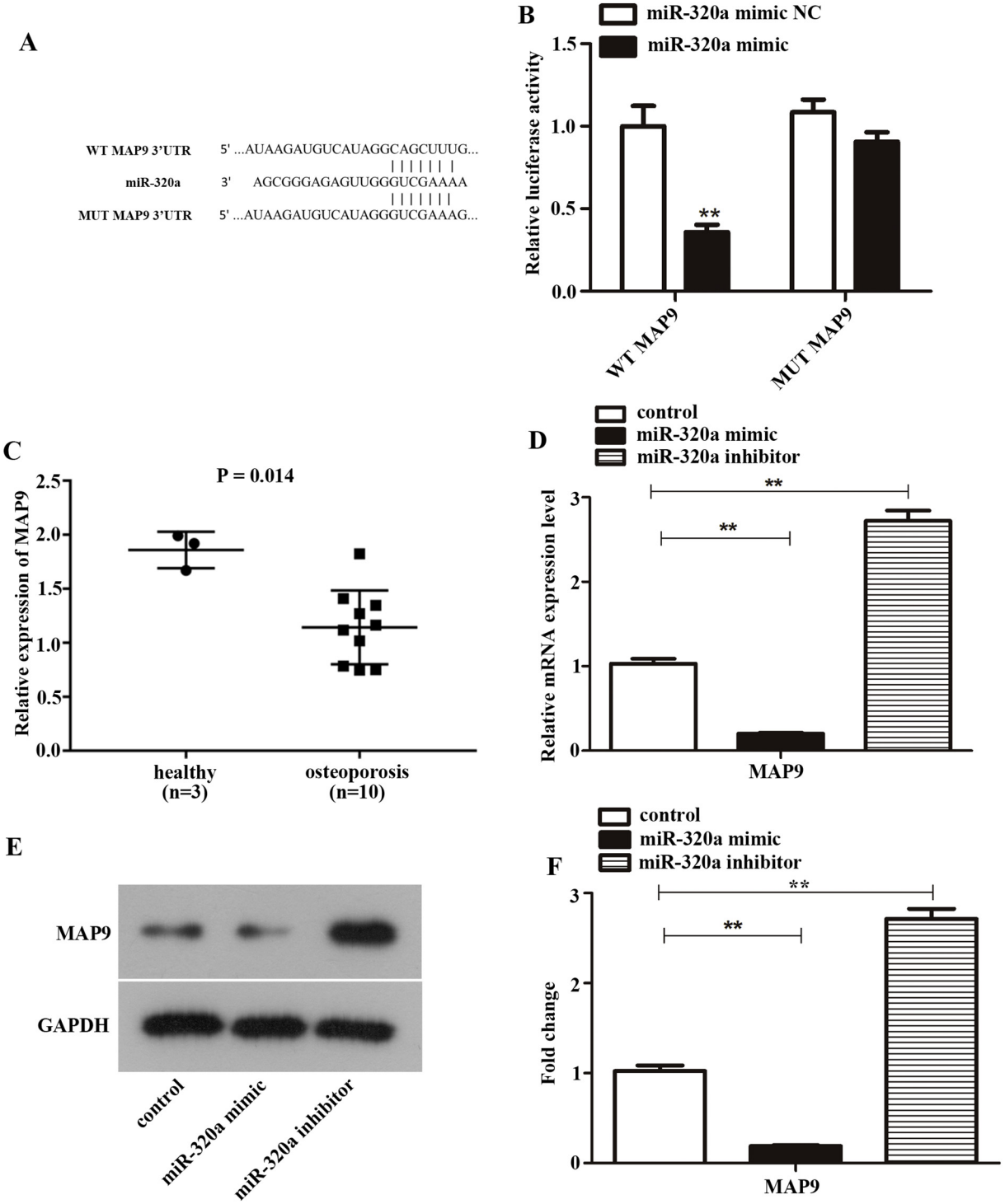

Fig. 3. MAP9 was a target gene of miR-320a and was negatively regulated by miR-320a. A: The sequences of WT MAP9 3'UTR, MUT MAP9 3'UTR and miR-320a. B: The relative luciferase activity was detected after transfected with miR-320a mimic and NC, $^{**}P < .01$. C: The expression of MAP9 in PMO patients was analyzed based on the data from GEO database, P = .014. D-F: The mRNA and protein expression of MAP9 in MC3T3-E1 cells after transfected with miR-320a mimic and inhibitor, $^{**}P < .01$.

Strand Synthesis Kit (Thermo Scientific, Waltham, USA). QRT-PCR was conducted by SYBR Premix Ex Taq II and SYBR PrimeScriptTM miRNA RT-PCT Kit (TaKaRa, Japan) to detect mRNA and miRNA expression with a real-time thermal cycler, according to the supplier's instructions. Real-time PCR was performed on a 7900HT real-time PCR system followed by 40 circles consist of 95 °C for 5 min, 95 °C for 30 s, then 60 °C for 45 s, 72 °C for 30 min. GAPDH and U6 were regarded as internal references for mRNA and miRNA calculation, respectively. $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative expression of mRNA and miRNA. The primers were shown below:

MiR-320a: F: 5'-GGGCTAAAAGCTGGGTTGA-3',
R: 5'-CAGTGCGTGTCGTGGAGT-3'.
U6: F: 5'-CTCGCTTCGGCAGCACA-3',
R: 5'-AACGCTTCACGAATTTGCGT-3'.
MAP9: F: 5'-GCCCTCCAAGCAGAACTGTG-3',
R: 5'-TCAGCAGGAGTGTCTGGCATT-3'.

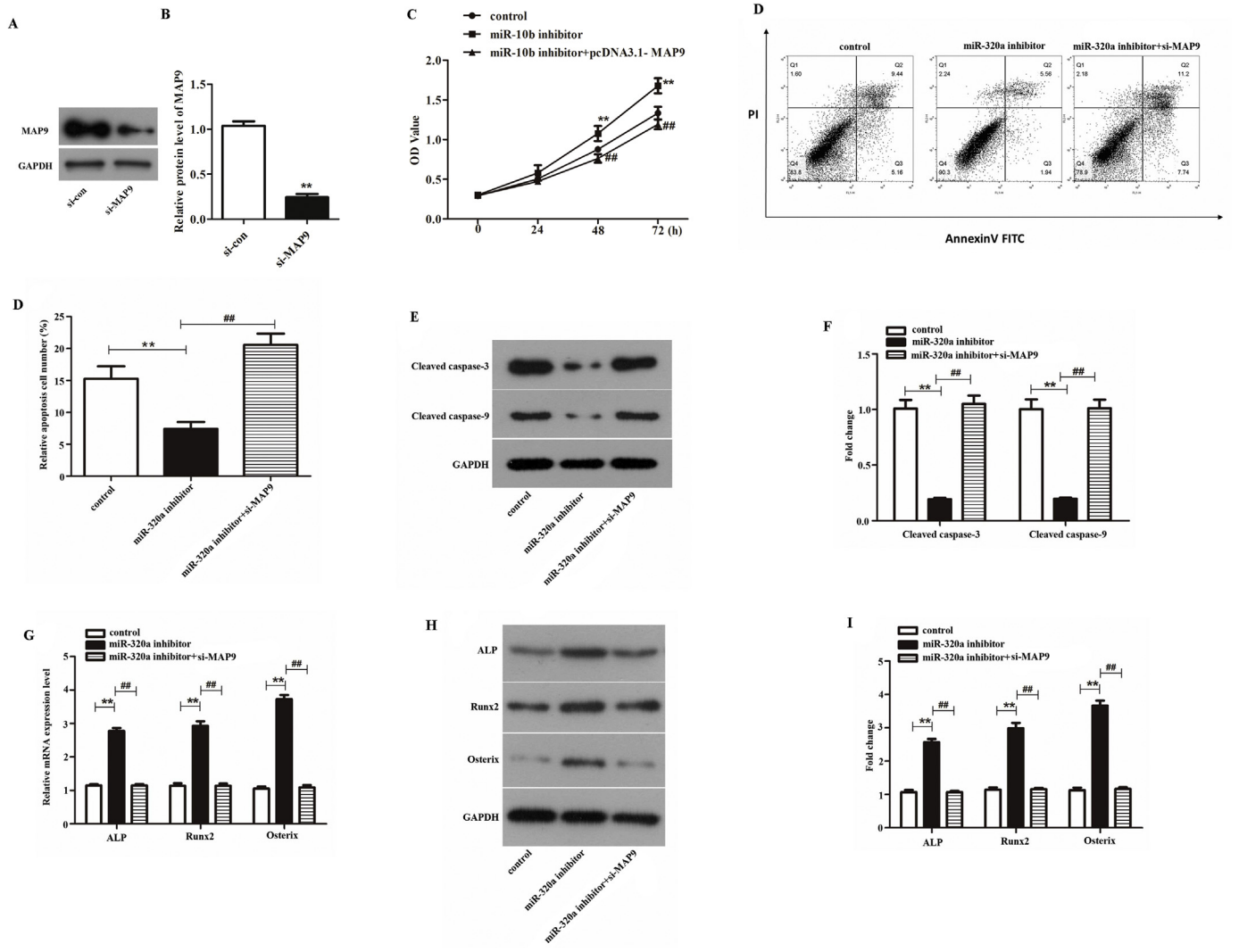

Fig. 4. MAP9 had negative effects on inhibition of MC3T3-E1 cells apoptosis, promotion of MC3T3-E1 cells viability and differentiation induced by miR-320a inhibitor. A-B: Detection of MAP9 interference efficiency, $P < .01$. C: The MTT assay showed the viability of MC3T3-E1 cells after treated with miR-320a inhibitor and miR-320a inhibitor/si-MAP9. D-E: The flow cytometry exhibited the MC3T3-E1 cells apoptosis. F-G: Western blot was used to analyze the expression levels of pro-apoptotic protein Cleaved caspase-3 and Cleaved caspase-9. H-J: The mRNA and protein expression levels of ALP, Runx2 and Osterix were measured by qRT-PCR and western blot. $^{**}P < .01$, $^{\#\#}P < .01$. ** represented miR-320a inhibitor vs control, $^{\#\#}$ represented miR-320a inhibitor/si-MAP9 vs miR-320a inhibitor.

Runx2: F: 5'-GACTGTGGTTACCGTCATGGC-3′,
R: 5'-GACTGTGGTTACCGTCATGGC-3′.
ALP: F: 5'-CAGCGGGTAGGAAGCAGTTTC-3′,
R: 5'-CCCTGCACCTCATCCCTGA-3′.
Osterix: F: 5'-CCTTAGCCCTCCGTGTTTTGT-3′,
R: 5'-CCAGTCGTTTTCCTTGTGGAAG-3′.
GAPDH: F: 5'-ACACCCACTCCTCCACCTTT-3′,
R: 5'-TTACTCCTTGGAGGCCATGT-3′.

2.7. Western blot

The treated cells were gathered via centrifugation and dissolved by RIPA regent with protease inhibitor (PMSF). The concentration of the protein was measured by BCA method. Then the protein samples (20 μg) were isolated with sodium dodecyl sulfate-polyacrylamide gels and electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with 5% skimmed milk at about 25 °C for 1 h, cultured overnight with primary antibodies including MAP9 (1 μg/mL), Cleaved caspase-3 (1 μg/mL), Cleaved caspase-9 (1 μg/mL), ALP (1 μg/mL), Runx2 (1:1000), Osterix (0.5 μg/mL), PI3K (1:1000), AKT (1:1000), p-PI3K (1:5000), p-AKT (1:5000), GAPDH (1:10000) (Abcam, Cambridge, UK) at 4 °C, then mixed with secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rat (1:2000; Sigma-Aldrich, St. Louis, USA) for 1 h at about 25 °C. Finally, added enhanced chemiluminescence (ECL) solution in the dark room to develop the signals. The proteins bands were scanned by QUANTITY ONE software.

2.8. Statistical analysis

The experimental data were processed through SPSS22.0 and GraphPad Prism 5.0 softwares. Each assay was repeated at least three times in this study. Data were showed as mean value ± standard deviation (SD). Student's *t*-test was used for the comparison between two groups, but the comparison between multiple groups was analyzed by one-way analysis of variance (ANOVA) following Dunnett's post hoc test (compare all groups vs. control group) and Bofferornie 's post hoc test (compare all pairs of groups). $P < .05$ was regarded as statistically significant.

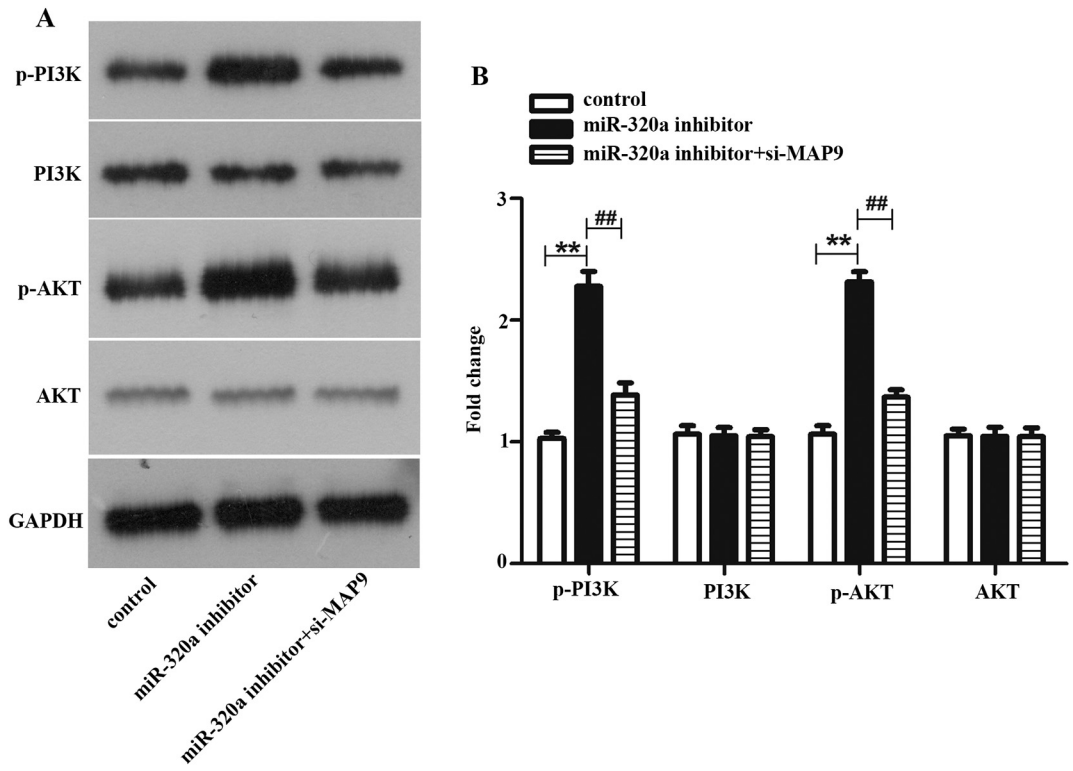

Fig. 5. MiR-320a/MAP9 maintained the MC3T3-E1 cells biological behaviors through PI3K/AKT signaling pathway. A-B: The protein expression levels of PI3K, p-PI3K, AKT and p-AKT were analyzed by qRT-PCR and western blot, $^{**}P < .01$, $^{\#\#}P < .01$. ** represented miR-320a inhibitor vs control, $^{\#\#}$ represented miR-320a inhibitor/si-MAP9 vs miR-320a inhibitor.

3. Results

3.1. MiR-320a showed significant high expression in patients with PMO and led to decreased MC3T3-E1 cells viability and increased MC3T3-E1 cells apoptosis

To observe the influence of miR-320a on PMO, we analyzed the expression level of miR-320a in PMO with GEO database. As presented in Fig. 1A, miR-320a was highly expressed in patients with PMO fracture (osteoporotic, $n = 6$) in contrast with patients with osteoarthritis (healthy, $n = 6$) ($P = .0043$). Then miR-320a mimic/inhibitor was applied to regulate the expression of miR-320a. The result showed that miR-320a was significant up/down-regulated after transfected with miR-320a mimic/inhibitor (Fig. 1B, $P < .01$). Moreover, to explore the effect of miR-320a on MC3T3-E1 cells viability and apoptosis, MTT, flow cytometry and western blot were applied, respectively. The MTT assay discovered that over-expression of miR-320a inhibited the viability of MC3T3-E1 cells, while down-regulation of miR-320a promoted the viability of MC3T3-E1 cells (Fig. 1C, $P < .01$). The data from flow cytometry manifested that the apoptosis of MC3T3-E1 cells was remarkably increased/decreased after transfected with miR-320a mimic/inhibitor (Fig. 1D-E, $P < .01$). In further experiments, we discovered that the expression of pro-apoptotic proteins Cleaved caspase-3 and Cleaved caspase-9 were significant raised/reduced after treated with mimic/inhibitor (Fig. 1F-G, $P < .01$). The above results demonstrated that miR-320a was over expressed in patients with PMO and MC3T3-E1 cells, and resulted in increasing apoptosis and decreasing viability of MC3T3-E1 cells.

3.2. Over-expression of miR-320a suppressed MC3T3-E1 cells differentiation

Based on the above results, the key proteins which were involved in MC3T3-E1 cells differentiation were determined through qRT-PCR and western blot. A significant decrease/increase in the expression of ALP, Runx2 and Osterix were observed in MC3T3-E1 cells after transfected with miR-320a mimic/inhibitor (Fig. 2A-C, $P < .01$). This results stated that overexpression of miR-320a could suppress the differentiation of MC3T3-E1 cells, however, depletion of miR-320a exhibited the opposite effect.

3.3. MAP9 was lower expressed in PMO and was a target gene of miR-320a

It is well known that miRNAs performed their functions by inhibiting the expression of their target genes. In our study, MAP9 was predicted as a target gene of miR-320a through miRNA target analysis tools including TargetScan, MiRanda, miRDB and miRWalk. Subsequently, this consequence was confirmed by dual luciferase reporter assay. The sequences of WT MAP9 3'UTR, MUT MAP9 3'UTR and miR-320a were presented in Fig. 3A. As the luciferase assay illustrated, miR-320a significant inhibited the activity of WT MAP9 3'UTR, but have no effect on the MUT MAP9 3'UTR luciferase activity (Fig. 3B, $P < .01$). The data from GEO database revealed that MAP9 was poorly expressed in PMO (osteoporosis, $n = 10$, Fig. 3C, $P = .014$) compared with that in healthy postmenopausal women (healthy, $n = 3$). By qRT-PCR and western blot assays, we found that the mRNA and protein expression of MAP9 were significantly decreased/increased after treated with miR-320a mimic/inhibitor (Fig. 3D-F, $P < .01$). These results illustrated that MAP9, which worked in PMO, was a target gene of miR-320a and was negatively regulated by miR-320a.

3.4. Depletion of MAP9 attenuated the promoting effects of miR-320a inhibitor on the biological behaviors of MC3T3-E1 cells

Based on the experimental results of Fig. 1 and Figure2, and the high expression of miR-320a in PMO, we used miR-320a inhibitor and si-MAP9 for co-transfection of MC3T3-E1 cells in subsequent functional experiments. The results showed that the expression of MAP9 was

reduced significantly after transfected with si-MAP9 (Fig. 4A-B, $P < .01$). As the results illustrated, a significant decrease of MC3T3-E1 cells vitality was occurred after transfected with miR-320a inhibitor and si-MAP9 compared with that only transfectd with miR-320a inhibitor (Fig. 4C, $P < .01$). The flow cytometry data revealed that the apoptosis numbers of MC3T3-E1 cells were significant up-regulated after transfected with miR-320a inhibitor and si-MAP9 in contrast with just transfectd with miR-320a inhibitor (Fig. 4D-E, $P < .01$). The protein expression levels of pro-apoptotic proteins Cleaved caspase-3 and Cleaved caspase-9 were remarkably raised after transfected with miR-320a inhibitor and si-MAP9 compared with that only transfectd with miR-320a inhibitor (Fig. 4F-G, $P < .01$). And the mRNA and protein expression of the differentiation related proteins (ALP, Runx2 and Osterix) have a rapid decline after transfected with miR-320a inhibitor and si-MAP9 in contrast with just transfected with miR-320a inhibitor (Fig. 4H-J, $P < .01$). The rescue assay demonstrated that depletion of MAP9 could attenuate the promoting effect of miR-320a inhibitor on MC3T3-E1 cells activity and differentiation, as well as the inhibitory influences of miR-320a inhibitor on MC3T3-E1 cells apoptosis.

3.5. MiR-320a/MAP9 perform biological function in MC3T3-E1 cells partly via modulating PI3K/AKT signaling pathway

It is well known that PI3K/AKT signaling pathway took a significant role in cell growth, apoptosis and differentiation (Chen et al., 2017; Shen et al., 2019; Zhu et al., 2019). Based on the above research and the high expression of miR-320a in PMO, miR-320a inhibitor was selected for relevant experiments in subsequent functional experiments. Hence, in Fig. 5, the results from miR-320a mimic were not test. In the present research, we revealed that the protein expression levels of the phosphorylated PI3K and AKT were significant up-regulated in MC3T3-E1 cells after treated with miR-320a inhibitor. However, the upward trend was clearly reversed when cells were treated with miR-320a inhibitor and si-MAP9 together. The protein expression levels of PI3K and AKT were almost unchanged no matter treated with miR-320a inhibitor or miR-320a inhibitor and si-MAP9 (Fig. 5A-C, $P < .01$). The findings demonstrated that miR-320a acted as a positive modulator in reducing MC3T3-E1 cells viability and differentiation and raising MC3T3-E1 cells apoptosis through regulating PI3K/AKT signaling pathway.

4. Discussion

Postmenopausal osteoporosis is not valued by people and is not easy to detect. There are various effective drug therapeutics for PMO, but long-term use of these drugs is more or less safety hazards (Diab and Watts, 2013). Therefore, it is urgent to search novel treatment to defeat PMO. In our study, we perceived that miR-320a was over-expressed in patients with PMO and up-regulation of miR-320a inhibited MC3T3-E1 cells activity and differentiation, and promoted MC3T3-E1 cells apoptosis. Moreover, MAP9, which confirmed as a target gene of miR-320a, was down-regulated in PMO. Rescue assay demonstrated that miR-320a could impair the functions on MC3T3-E1 cells biological behaviors by reducing MAP9 and PI3K/AKT signaling pathway. In a word, our findings demonstrated that up-regulated miR-320a promoted the development of MC3T3-E1 cells by attenuating MAP9 expression through inhibition of PI3K/AKT signaling pathway.

Abnormal expression of miRNAs is concerned in numerous diseases processes. MiR-320a has been found to suppress the growth of multiple myeloma cells (Lu et al., 2016), colon cancer cells (Sun et al., 2012) and breast cancer cells (Wang et al., 2015). Whilst, miR-320a was implicated in the occurrence of diabetic nephropathy by suppressing MafB (He et al., 2019). In addition, miR-320a has also been reported to play a vital role in the process of ischemia reperfusion injury in rats (Li et al., 2016). Although miR-320a has been illustrated to be highly expressed in osteoporosis (De-Ugarte et al., 2015), can slash osteoblast activity, and induce oxidative stress (De-Ugarte et al., 2018), the downstream target gene and related signaling pathway of miR-320a in osteoblast have not been identified. In our study, we investigated that miR-320a was highly expressed in PMO, which reduced the viability of osteoblast, promoted the apoptosis of osteoblast and inhibited the differentiation of osteoblast. Whilst, we discovered the target gene of miR-320a and the signaling pathway involved in osteoblast.

As far as we know, miRNAs exert their biological functions through regulating downstream target genes. In this study, several biological softwares including TargetScan, MiRanda, miRDB and miRWalk were used to predict miR-320a target gene. And this prediction was confirmed by dual luciferase assay. Eventually, MAP9 was verified as a target gene of miR-320a. MAP9, as a mitotic associated protein, is crucial for the normal cycling of cells (Venoux et al., 2008). Some studies demonstrated that MAP9 acted as a crucial part in early development of zebrafish by modulating different MT-based processes (Fontenille et al., 2014). Emerging evidence have discovered that MAP9 was involved in numerous cancers, including gastric carcinoma (Liu et al., 2018), colorectal and breast cancers (Rouquier et al., 2014). However, the function of MAP9 on PMO has been poorly established. In our research, the data from dual luciferase reporter assay indicated that MAP9 was a target gene of miR-320a. However, the relationship between miR-320a and MAP9 was confirmed in HEK293 cells, more experiments in vivo and MC3T3-E1 cells were needed in the future study. Moreover, we noticed that MAP9 was lower expressed in patients with PMO, and was negatively regulated by miR-320a. Furthermore, MAP9 can regulate the viability, differentiation and apoptosis of MC3T3-E1 cells together with miR-320a. These findings illustrated that miR-320a/MAP9 may function as key regulators to suppress MC3T3-E1 cells viability and differentiation, to promote MC3T3-E1 cells apoptosis. Rescue assay discovered that exhausting of MAP9 could weaken the promoting effects of miR-320a inhibitor on MC3T3-E1 cells activity and differentiation, and the inhibitory effects of miR-320a inhibitor on MC3T3-E1 cells apoptosis. So, we conjectured that miR-320a could inhibit MC3T3-E1 cells viability and differentiation, promote MC3T3-E1 cells apoptosis with reducing MAP9.

PI3K/AKT, as an important signaling pathway, regulates cell survival, proliferation, differentiation in several pathophysiological process (Yu and Cui, 2016). Numerous studies have shown that PI3K/AKT signaling pathway was involved in the process of osteoporosis. Recent literature has demonstrated that miR-210 promoted the expression of vascular endothelial growth factor and osteoblasts differentiation through PI3K/AKT signaling pathway, and played an active regulatory role in alleviating PMO caused by estrogen deficiency (Liu et al., 2015). Extensive researches have shown that Si-Wu-tang (traditional Chinese medicine) extracts promoted bone formation via PI3K/AKT signaling pathway in osteoblast (Wu et al., 2013). Data from several studies suggested that inhibition of miR-148a protected against ovariectomy-induced osteoporosis through PI3K/AKT signaling pathway by estrogen receptor α (Xiao et al., 2018). Moreover, plenty of researches have stated that miR-320a affected the activation of the PI3K/AKT signaling pathway. Zhao et al. investigated that miR-320a-3p regulated cell metastasis and aggressiveness in non-small cell lung cancer via PI3K/AKT pathway (Zhao et al., 2018). And several studies have shown that up-regulation of miR-320a inhibited glioma cells biological behaviors and tumorigenesis by targeting IGF-1R and regulating the downstream signaling pathway PI3K/AKT (Guo et al., 2014). However, very little was found in the literature about the relation among PI3K/AKT, miR-320a and PMO. In our research, we observed that high-expression of miR-320a promoted the apoptosis of osteoblast and suppressed the activity and differentiation of osteoblast through inhibiting PI3K/AKT signaling pathway. Although we have obtained some experimental results with MC3T3-E1 cells, MC3T3-E1 cells do not fully reflect the status of real osteoblast in PMO. Therefore, more in vivo experiments were need in the further study.

In summary, our outcomes illustrated that miR-320a could affect the apoptosis and differentiation of osteoblast by attenuating the

expression of MAP9 and inhibiting the PI3K/AKT signaling pathway, probably regulating the progression of PMO. The study provided theoretical supports for selecting miR-320a/MAP9 as a target for the diagnosis and treatment of PMO. However, more in vivo studies are needed to confirm miR-320a/MAP9 clinical application.

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