

Magnolol suppressed cell migration and invasion and induced cell apoptosis via inhibition of the NF- κ B signaling pathway by upregulating microRNA-129 in multiple myeloma

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Received September 23, 2020 / Accepted November 30, 2020

Multiple myeloma (MM) is incurable cancer in the blood system. Magnolol is an effective component against various cancers. This study tried to investigate the effect and mechanism of magnolol on MM via regulating miR-129. Human normal plasma cells (nPCs) and MM cells U266 and LP1 were used in this study, accompanied by treatment of magnolol. The miR-129 inhibitor was transfected into U266 and LP1 cells during experiments. Cell viability was detected by Cell Counting Kit-8 assay. Cell migration and invasion were tested by wound healing assay and Transwell assay. And Annexin-V-FITC/PI assay was utilized to assess cell apoptosis. miR-129, miR-1271-5p, miR-342-3p, and miR-124-3p expressions were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and western blot was adopted to evaluate Cyclin D1, matrix metalloprotein (MMP)-7, MMP-9, phosphorylation (p)-I κ B α , p-p65, and p65 protein levels. In U266 and LP1 cells, with magnolol concentration increasing, cell viability, migration, and invasion rates, Cyclin D1, MMP-7, and MMP-9 expressions decreased, while cell apoptosis rose. And magnolol increased the miR-129 expression in MM cells. Besides, miR-129 inhibitor antagonized the above-mentioned effect of magnolol and partly offset the magnolol-induced decrease of p-I κ B α and p-p65 expression, as well as the ratio of p-p65 to p65 in U266 and LP1 cells. Magnolol suppressed cell migration and invasion and induced cell apoptosis via inhibiting NF- κ B pathway activation, by upregulating miR-129 in MM.

Key words: magnolol, multiple myeloma, miR-129, apoptosis, NF- κ B

Multiple myeloma (MM) is a hematological malignancy characterized by abnormal proliferation of monoclonal plasma cells in the bone marrow [1], the clinical manifestation of which includes anemia, renal failure, hypercalcemia, and bone lesions (CRAB) [2]. The pathogenesis of MM is associated with multiple mechanisms of molecules and cells such as genetic changes and the unbalance of stromal and plasma cells in the bone marrow microenvironment [3, 4]. In spite of medical progress in immunotherapy, chemotherapy, radiotherapy, and hematopoietic autologous stem cell transplant, MM remains an incurable disease with a 10-year survival rate of no more than 30% [5, 6], partly due to the lack of precision therapies targeting specific oncogene involved in the pathogenesis [7]. In recent years, researches on MM have revealed the functions of several molecules and compounds like long noncoding RNA XLOC_013703, PCAT-1, and

resveratrol, offering limited help to the treatment of MM [8–10]. Besides, some agents such as thalidomide, lenalidomide, and bortezomib have the potential of extending the overall survival of MM patients, however, MM remains an incurable disease, and eventually almost all patients relapse and become resistant to the chemical treatment [11]. Therefore, it is of importance in theory and practice to search for innovative therapeutic targets as well as effective drugs against MM to improve the survival of MM patients.

Traditional Chinese medicines have the advantages of limited adverse effects, significant efficiency, and active-target diversity [12]. Magnolol is a bioactive component extracted from root and bark of which is used as a traditional Chinese herbal medicine. And it has been reported that magnolol fulfills a function on muscle relaxation, anti-anxiety, anti-depressant, anti-oxidation, anti-inflammatory,

and anti-atherosclerosis [13, 14]. Additionally, previous studies showed magnolol promoted cell apoptosis and suppressed cell development to reduce the malignance of various cancers including prostate cancer, osteosarcoma, breast, and colorectal cancer [14–18]. However, few studies elucidated the effect of magnolol on MM and its mechanism of action.

microRNA (miRNA), a group of small non-coding endogenous RNA implicated in regulation after transcription, has been regarded as a crucial point in cell differentiation, multiplication, migration, and carcinogenesis [19, 20]. As a member of the miRNA family, miR-129 is also related to tumors and has been found dysregulated in cancers. Besides, miR-129 has been proven to play a negative part in the cell development of multiple cancers. For instance, miR-129 inhibits cell proliferation, migration, and invasion in prostate cancer as well as suppresses cell growth and progression in glioma and glioblastoma multiforme [21–23]. What's more, a significant reduction of miR-129 has been observed in MM tissue and overexpression of miR-129 disturbs cell growth and proliferation while induces cell apoptosis in MM [10]. Consequently, from all above, we try to study whether magnolol functions on MM by regulating miR-129.

This paper firstly explored the role of magnolol in MM and then investigated the specific mechanism of magnolol through targeting miR-129 during MM, attempting to provide a possible therapeutic strategy of MM.

Materials and methods

Cell culture. The MM cell lines U266 and LP1 were obtained from Shanghai Yaji Biological Technology Co., Ltd. (YS302C; YS2346C, Shanghai, China, <http://www.yajimall.com/>) and human normal plasma cells (nPCs) were bought from Fenghui Biotechnology Co., Ltd (Changsha, China). Cells were cultured in RPMI-1640 medium (31800, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) supplemented with 10% fetal bovine serum (FBS; 11011-8611, Beijing Solarbio Science & Technology Co., Ltd., China) in an incubator at 37°C with 95% humidity as well as 5% CO₂.

Cell transfection. Cells in the logarithmic phase were trypsinized (T1350, Beijing Solarbio Science & Technology Co., Ltd., China), seeded at a density of 1×10^5 cells/well in a 24-well plate, and cultured until 70–90% confluence. miR-129 inhibitor (B03001, Shanghai GenePharma Co., Ltd., Shanghai, China) and inhibitor control (B04003, Shanghai GenePharma Co., Ltd., China) were transfected into U266 and LP1 cells using Lipofectamine 3000 (L30000001, Thermo Fisher Scientific, Waltham, MA, USA). Lipofectamine 3000 (0.75 and 1.5 μ l) and DNA (1 μ g) were diluted in Opti-MEM medium (25 μ l; 31985062, Thermo Fisher Scientific, USA), respectively. Then P3000 reagent (2 μ l) was added into diluted DNA and mixed well. Next, a DNA-reagent mixture was added to dilute Lipofectamine 3000 (1:1) for incuba-

tion at room temperature for 10 min. Finally, a DNA-lipid complex (50 μ l/well) was added to cells and the plate was incubated for 24 h at 37°C.

Cell Counting Kit-8 (CCK-8) assay. Cells in the logarithmic growth were trypsinized and seeded in 96-well plates at 4×10^3 /well, followed by incubation containing 5% CO₂ at 37°C for 24 h. Then, the cells were treated with different concentrations of magnolol (5, 10, 20, 40 μ M; M3445, Sigma-Aldrich, Ontario, Canada) for another 24 or 48 h. Subsequently, 10 μ l CCK-8 solution (CA1210, Beijing Solarbio Science & Technology Co., Ltd., China) was added into each well and cells were further incubating for 4 h at 37°C. The absorbance was measured at 450 nm by a microplate reader (SpectraMax iD5, Molecular Devices, Sunnyvale, CA, USA).

Wound healing assay. Cells were seeded at 5×10^5 /well of 6-well plates and cultured until 80–100% confluence. A pipette tip was used for a straight scratch. Then cells were rinsed 3 times with PBS to remove those detached cells and replaced with serum-free medium for 48 h at 37°C. An inverted biological microscope (magnification: 100 \times ; XSP-19C, Shanghai Optical Instrument Co., Ltd., Shanghai, China, <https://www.shoif.com/>) was used for image capture at 0 and 48 h and a migrated distance was measured by ImageJ, version 1.48 (National Center for Biotechnology Information, Bethesda, MD, USA). Cell migration rates were performed as the percentage of the scratch area filled by migrating cells at 48 h post scratch: migration rate = (scratch width at 0 h – scratch width at 48 h)/scratch width at 0 h \times 100%.

Transwell invasion assay. Cells were trypsinized, rinsed with PBS, and resuspended in the serum-free medium at a concentration of 3×10^5 /ml. The cells then were seeded in the upper filter of a 24-well Transwell chamber (351184, Corning Incorporation, Corning, NY, USA), which was pre-coated with diluted Matrigel (1:8; 354234, Corning Incorporation, USA). The medium with 10% FBS in the lower chamber was used as a source of chemoattractants. Incubated at 37°C for 48 h, cells passing through the membrane were fixed with 4% paraformaldehyde (P0099, Beyotime Biotechnology, Shanghai, China) at 4°C for 30 min and then stained with 0.1% crystal violet (C0121, Beyotime Biotechnology, China) for 30 min at room temperature. Six visual fields were randomly selected from each room for counting by XSP-19C inverted biological microscope (magnification: 200 \times).

Cell apoptosis assay. Annexin-V-FITC Apoptosis Detection Kit (C1062S, Beyotime Biotechnology, China) was employed to evaluate the cell apoptosis. After trypsin digestion, cells centrifuged at $1,000 \times g$ for 5 min were harvested and resuspended in PBS. Afterward, cells (5×10^5 cells) were centrifuged at $1,000 \times g$ for another 5 min, following which cells were resuspended in the binding buffer. Annexin V-FITC (5 μ l) and PI (10 μ l) were added to the cell solution and mixed well in a dark room for 15 min at room temperature. The apoptosis rate was analyzed through a flow cytometer (CytoFLEX, Beckman Coulter, Inc., Brea, CA, USA).

Western blot. With total protein isolated through radio-immunoprecipitation assay (RIPA) lysis buffer (R0010, Beijing Solarbio Science & Technology Co., Ltd., China), cells were centrifuged at 12,000×g at 4°C for 10 min and the supernatant was collected. Total protein concentration was assessed by Pierce[™] Rapid Gold BCA Protein Assay Kit (A53227, Thermo Fisher Scientific, USA). Equal contents of protein and ColorMixed Protein Marker (11-180KD, PR1910, Beijing Solarbio Science&Technology Co., Ltd., China) were separated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with SDS-PAGE Gel Preparation Kit (P0012AC, Beyotime Biotechnology, China), subsequent to which cells were transferred to polyvinylidene fluoride (PVDF) membranes (88585, Thermo Fisher Scientific, USA) blocked in 5% non-fat milk (D8340, Beijing Solarbio Science & Technology Co., Ltd., China) at room temperature for 1 h and then incubated at 4°C with anti-Cyclin D1 (1:10,000; ab134175, Abcam, Cambridge, MA, USA), anti-matrix metalloprotein (MMP)-7 (1:1,000; ab207299, Abcam, USA), anti-MMP-9 (1 µg/ml; ab73734, Abcam, USA), anti-GAPDH (1:500; ab8245, Abcam, USA), anti-phosphorylation (p)-IκBα (1:1,000; #2859, CST, Danvers, MA, USA), anti-p-p65 (1:2,000; ab86299, Abcam, USA) and anti-p65 (0.5 µg/ml; ab16502, Abcam, USA). After overnight, membranes were washed four times with Tris-buffered saline containing Tween 20 (TBST; ST673, Beyotime Biotechnology, China), following which the membranes were incubated with the corresponding secondary antibodies conjugated to Horseradish Peroxidase (HRP; ab6721, 1:5,000; Abcam, USA) for 1 h at room temperature and rinsed five times with TBST for 5 min. The proteins were visualized using electrochemiluminescence (ECL) reagent (PE0010, Beijing Solarbio Science&Technology Co., Ltd., China) through a chemiluminescence system (SH-Focus523, Shenhua Bio. Co., Ltd., Hangzhou, China, <http://www.shenhuabio.cn/index.php>). ImageJ software, version 1.48 (National Institutes of Health, Bethesda, MD, USA) was employed for analyzing grey value of protein bands on blots.

Table 1. Primer sequences used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Target gene	Primers
miR-129 (Forward)	CTTTTTCGCGTCTGGGCTTGC
(Reverse)	an universal reverse primer was provided in the kit
miR-1271-5p (Forward)	ACCTAGCAAGCACTCAGTCG
(Reverse)	GTGTCGTGGAGTCGGCAATT
miR-342-3p (Forward)	TCTCACACAGAAATCGCACCCGT
(Reverse)	an universal reverse primer was provided in the kit
miR-124-3p (Forward)	GCGGTGAATGCCAAGTCGTA
(Reverse)	GTATCCAGTGCCTGTCGTGG
U6 (Forward)	GCTTCGGCAGCACATATACTAAAA
(Reverse)	CGCTTCACGAATTTGCGTGTGCAT

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total miRNA was respectively extracted using PureLink[™] miRNA Isolation Kit (K157001, Thermo Fisher Scientific, USA). The cDNAs of miR-129 and miR-342-3p were synthesized through reverse transcription in HG TaqMan miRNA Synthesis Kit (D1803, HaiGene, Harbin, China, <http://www.haigene.cn/>) while cDNAs of miR-1271-5p and miR-124-3p were synthesized using Prime-Script[™] RT reagent Kit (Perfect Real Time) (RR037A, Takara Bio, Inc., Otsu, Japan). The ABI 7300 real-time fluorescence quantitative PCR instrument (Thermo Fisher Scientific, USA) was utilized for the amplification of cDNAs. The quantitative real-time PCR of miR-129 and miR-342-3p was presented using HG TaqMan miRNA PCR Kit (TAP02445, HaiGene, China) with the conditions including 95°C for 15 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. A universal reverse primer used miR-129 and miR-342-3p for was provided in the kit. As for miR-1271-5p and miR-124-3p, Hieff UNICON[™] Universal Blue qPCR SYBR Green Master Mix (11184ES03, Yeasen Biotech Co., Ltd., Shanghai, China) was adopted to trace its real-time PCR following a condition of a thermal cycling program: pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. The primer sequences (Shanghai GenePharma Co., Ltd., China) were listed in Table 1, as the universal reverse primers of miR-129 and miR-342-3p were provided in the kit. U6 served as the internal control for miRNA. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ relative quantification method [24].

Statistical analysis. All experiments were repeated independently at least three times. Statistical analysis was detected through SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Data were presented as means ± standard deviation. One-way ANOVA was utilized to compare multiple groups with Dunnett's post hoc test. A statistically significant difference can be accepted when $p < 0.05$.

Results

Magnolol suppressed the viability and migration of MM cells. With the concentration of magnolol increasing, no significant difference of cell viability in nPCs was observed among groups (Figure 1A), while the cell viability of U266 and LP1 cells at 24 and 48 h decreased compared with the control groups (Figures 2B, 2C, $p < 0.05$), implicating that magnolol suppressed MM cell development in a dose-dependent manner. Additionally, a marked reduction of cell migration rate was observed in magnolol-treated U266 and LP1 cells compared with the control group (Figures 1D, 1E, $p < 0.01$).

Magnolol inhibited the invasion while advanced apoptosis of MM cells. As the magnolol concentration rose, the invasion rates of U266 and LP1 cells declined in comparison with the control group (Figures 2A, 2B, $p < 0.001$), whereas the opposite result was presented during

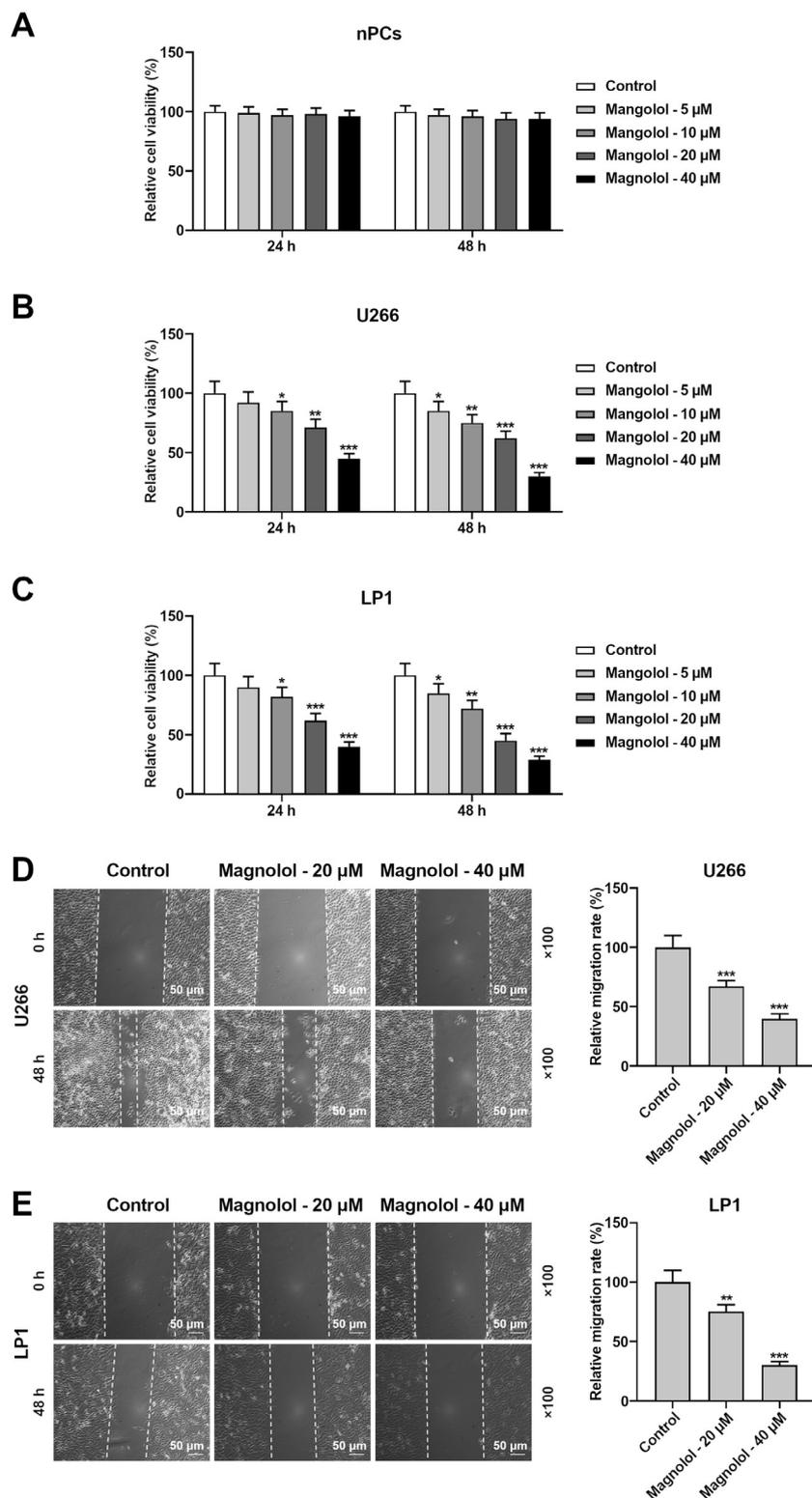


Figure 1. Magnolol suppressed the viability and migration of multiple myeloma (MM) cells. A–C) Cell viability at 24 and 48 h of normal plasma cells (nPCs, A), U266 (B), and LP1 cells (C) was detected by the Cell Counting Kit-8 (CCK-8) assay after the treatment with magnolol. D, E) Migration rate of U266 (D) and LP1 cells (E) was assessed through a wound healing assay after the treatment with magnolol. * $p < 0.05$ vs. control group, ** $p < 0.01$ vs. control group, *** $p < 0.001$ vs. control group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

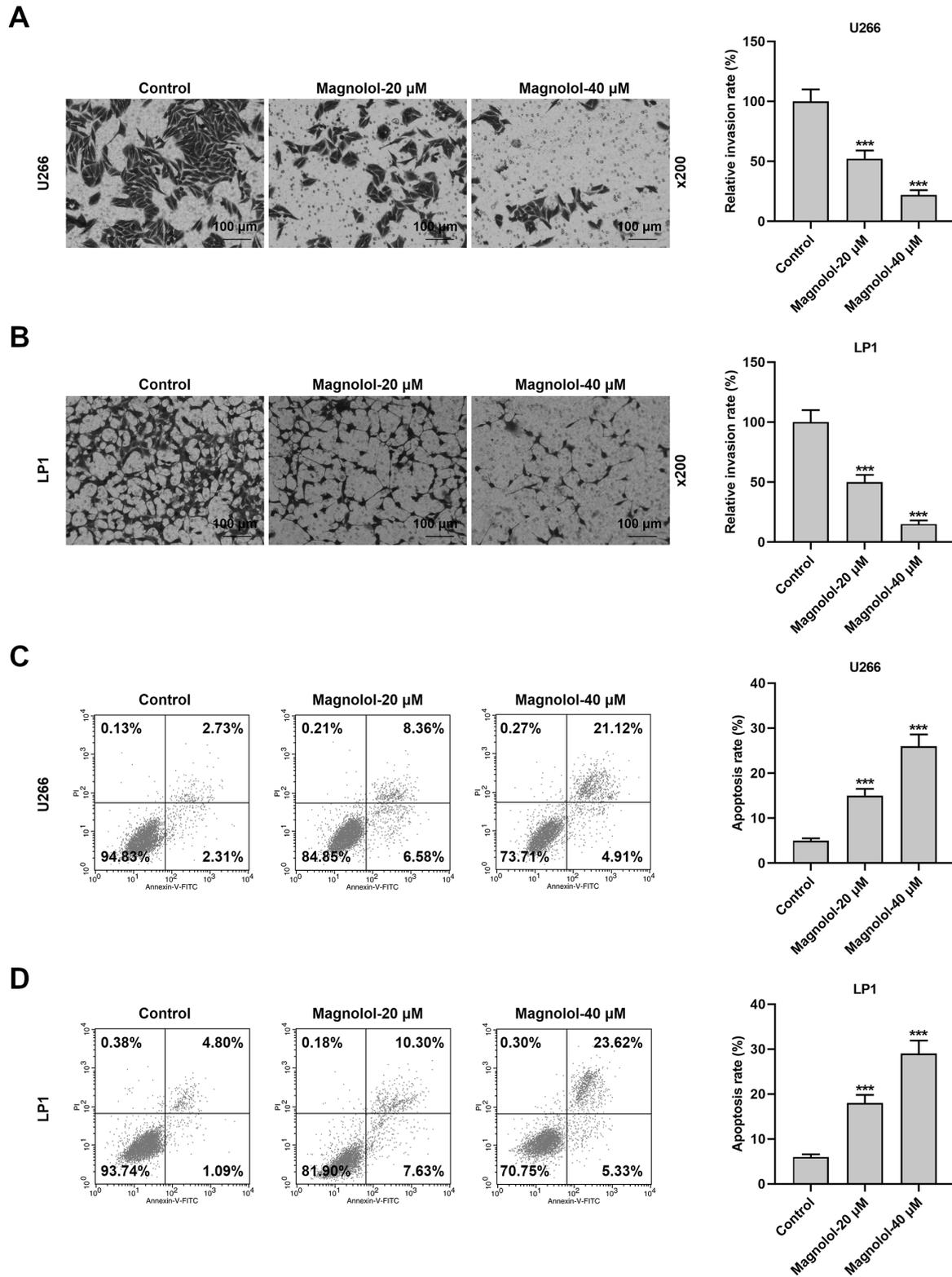


Figure 2. Magnolol inhibited the invasion while advanced apoptosis of multiple myeloma (MM) cells. A, B) Invasion rate of U266 (A) and LP1 cells (B) was assessed through the Transwell invasion assay after the treatment with magnolol. C, D) Apoptosis rate of U266 (C) and LP1 cells (D) was evaluated using Annexin-V-FITC Apoptosis Detection Kit with flow cytometry after the treatment with magnolol. *** $p < 0.001$ vs. control group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

the cell apoptosis assay (Figures 2C, 2D, $p < 0.001$), suggesting magnolol inhibited MM cell invasion and advanced cell apoptosis of MM.

Magnolol repressed Cyclin D1 and MMPs expression of MM cells. When treated with magnolol, U266 and LP1 cells showed lower expression of Cyclin D1, MMP-7, and MMP-9 than the control group (Figures 3A, 3B, $p < 0.05$), with the increased magnolol concentration enhancing inhibitory effect on Cyclin D1, MMP-7, and MMP-9 expression (Figures 3A, 3B, $p < 0.05$).

Magnolol increased the viability of MM cells via upregulating miR-129. miR-129 level of U266 and LP1 cells significantly was increased after treatment of magnolol compared with the control group (Figures 4A, 4B, $p < 0.001$), and higher miR-129 expression was presented in the group treated with a higher concentration of magnolol (Figures 4A, 4B, $p < 0.001$), as the expression level of miR-1271-5p, miR-342-3p, and miR-124-3p showed no significant difference (Figures 4A, 4B), which suggested a possible relation between magnolol and miR-129. In order to further explore the mechanism of magnolol, the miR-129 inhibitor was transfected into U266 and LP1 cells. It was observed that miR-129 expression only slightly reduced in the group of cells transfected with miR-129 inhibitor (Figures 4C, 4D). And a higher miR-129

level of U266 and LP1 cells was found in the magnolol-40 group than the control group (Figures 4E, 4F, $p < 0.001$). Besides, both U266 and LP1 cells had declined cell viability in the magnolol-40 group compared with the control group (Figures 4G, 4H, $p < 0.001$), whereas cells in the magnolol-40 + miR-129 inhibitor group showed higher cell viability than the magnolol-40 + inhibitor control group (Figures 4G, 4H, $p < 0.001$).

Magnolol suppressed the migration and invasion of MM cells via upregulating miR-129. In comparison with the control group, cell migration and invasion rates of U266 and LP1 cells decreased in the magnolol-40 group (Figures 5A, 5D, $p < 0.001$). In addition, cells in the group with the treatment of magnolol-40 and transfection of miR-129 inhibitor greatly increased migration and invasion rates compared with the group treated with magnolol-40 and transfected with inhibitor control (Figures 5A, 5D, $p < 0.001$).

Magnolol repressed Cyclin D1 and MMPs expression of MM cells via upregulating miR-129. Through the analysis of western blot, a lower protein expression of Cyclin D1, MMP-7, and MMP-9 in U266 and LP1 cells was presented in the group magnolol-40 than the control group (Figures 6A, 6B, $p < 0.001$), whereas Cyclin D1, MMP-7, and MMP-9 protein levels were upregulated in magnolol-40 + miR-129

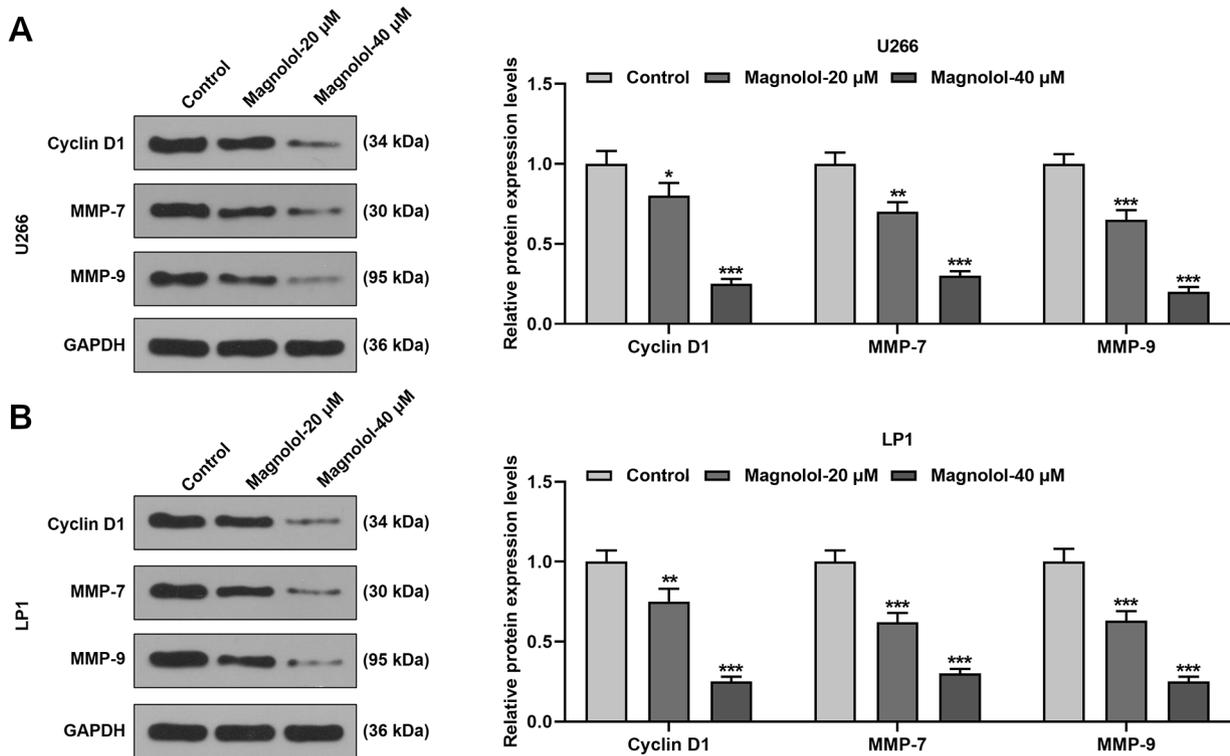


Figure 3. Magnolol repressed Cyclin D1 and matrix metalloproteins (MMPs) expression of multiple myeloma (MM) cells. A, B) Relative protein expression levels of Cyclin D1, MMP-7, and MMP-9 in U266 (A) and LP1 cells (B) were tested by western blot after the treatment with magnolol. ** $p < 0.01$ vs. control group, *** $p < 0.001$ vs. control group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

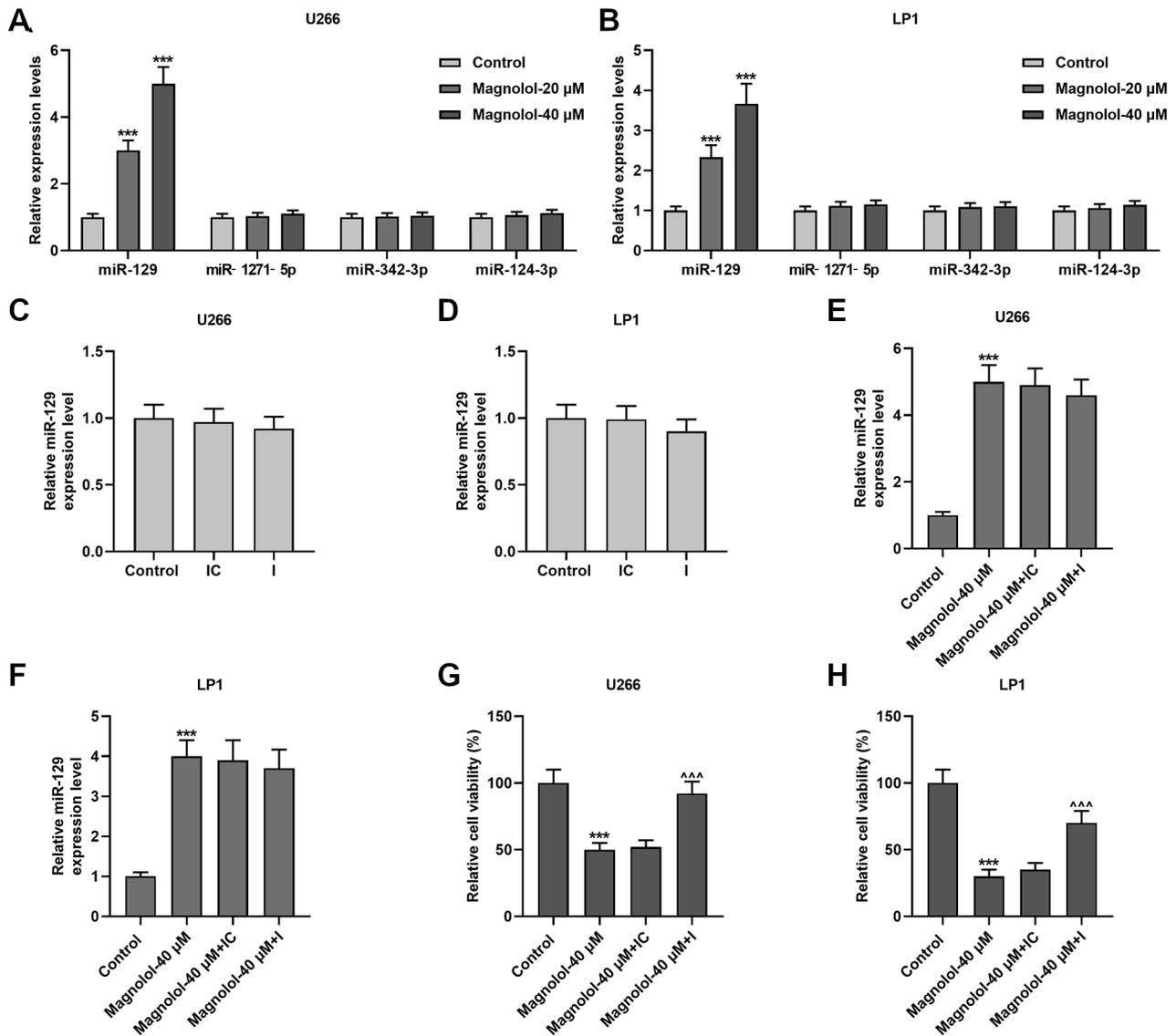


Figure 4. Magnolol increased the viability of multiple myeloma (MM) cells via upregulating miR-129. A, B) Relative expression levels of miR-129, miR-1271-5p, miR-342-3p, and miR-124-3p in U266 (A) and LP1 cells (B) were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) after the treatment with magnolol. C, D) Relative expression levels of miR-129 in U266 (C) and LP1 cells (D) were detected by qRT-PCR after the transfection of the miR-129 inhibitor. E, F) Relative expression levels of miR-129 in U266 (E) and LP1 cells (F) were detected by qRT-PCR after the treatment of magnolol and the transfection of the miR-129 inhibitor. G, H) Cell viability of U266 (G) and LP1 cells (H) was detected by the Cell Counting Kit-8 (CCK-8) assay after the treatment of magnolol and the transfection of the miR-129 inhibitor. *** $p < 0.001$ vs. control group, ^^^ $p < 0.001$ vs. magnolol-40 + inhibitor control (IC) group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

inhibitor group compared with the magnolol-40 + inhibitor control group (Figures 6A, 6B, $p < 0.001$).

Magnolol inhibited the activation of the NF- κ B signaling pathway via upregulating miR-129 in MM cells.

Cells in the magnolol-40 group greatly reduced the protein expression level of p-I κ B α and p-p65 with a declined ratio of p-p65 to p65 compared with the control group (Figures 7A, 7B, $p < 0.001$), as higher p-I κ B α and p-p65 protein expression with an increased ratio of p-p65 to p65 were presented in cells treated with magnolol-40 and transfected with the

miR-129 inhibitor in comparison with the group treated with magnolol-40 and transfected with inhibitor control (Figures 7A, 7B, $p < 0.001$).

Discussion

MM is the second most common hematologic malignancy, which cannot be cured up to now [25]. Accumulating evidence has revealed the pharmacological efficiency of magnolol against various kinds of cancers [14–18]. Never-

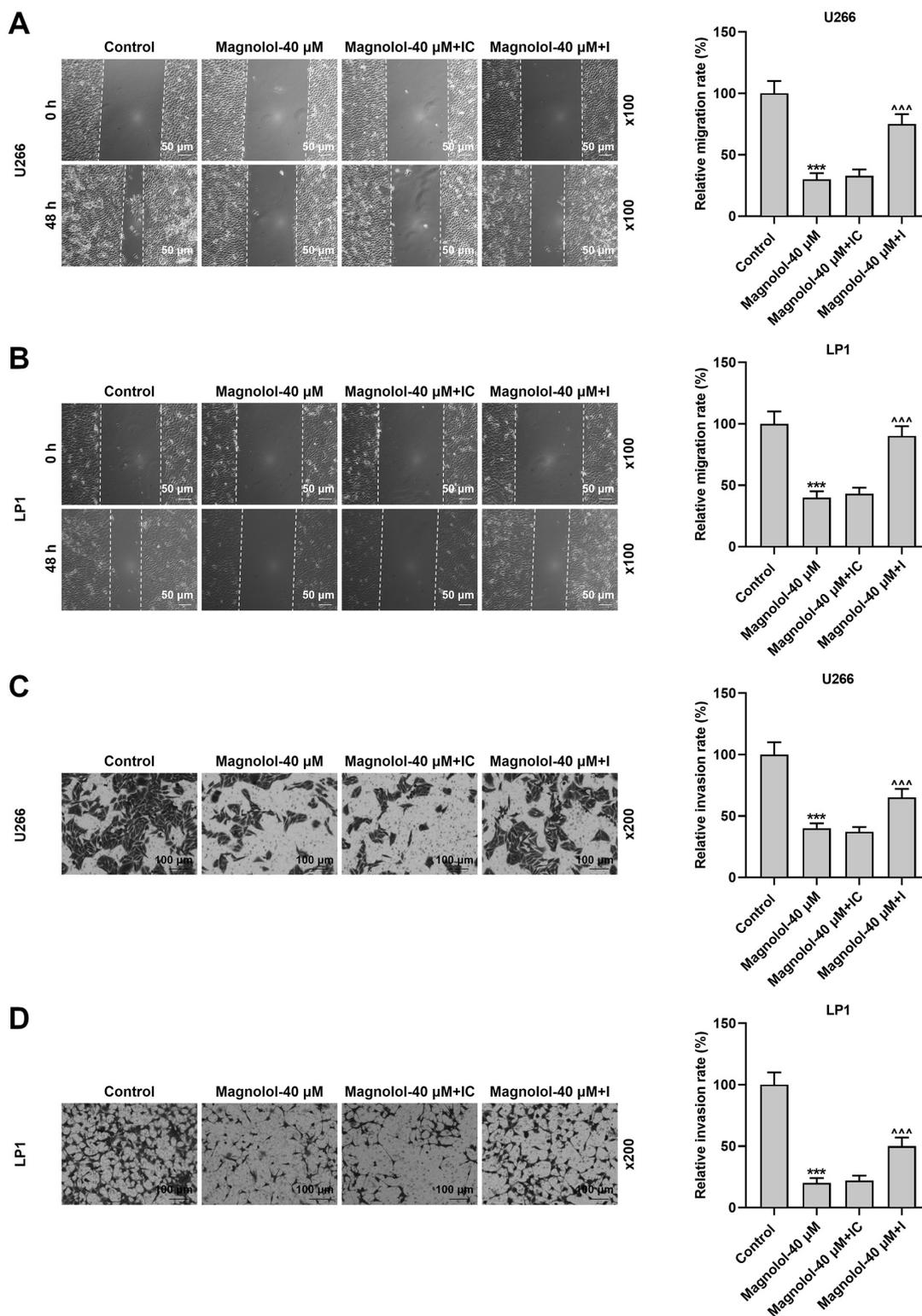


Figure 5. Magnolol suppressed the migration and invasion of multiple myeloma (MM) cells via upregulating miR-129. A, B) Migration rate of U266 (A) and LP1 cells (B) was assessed through a wound healing assay after the treatment of magnolol and the transfection of the miR-129 inhibitor. C, D) Invasion rate of U266 (C) and LP1 cells (D) was assessed through the Transwell invasion assay after the treatment of magnolol and the transfection of the miR-129 inhibitor. *** $p < 0.001$ vs. control group, ^^ $p < 0.001$ vs. magnolol-40 + inhibitor control (IC) group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

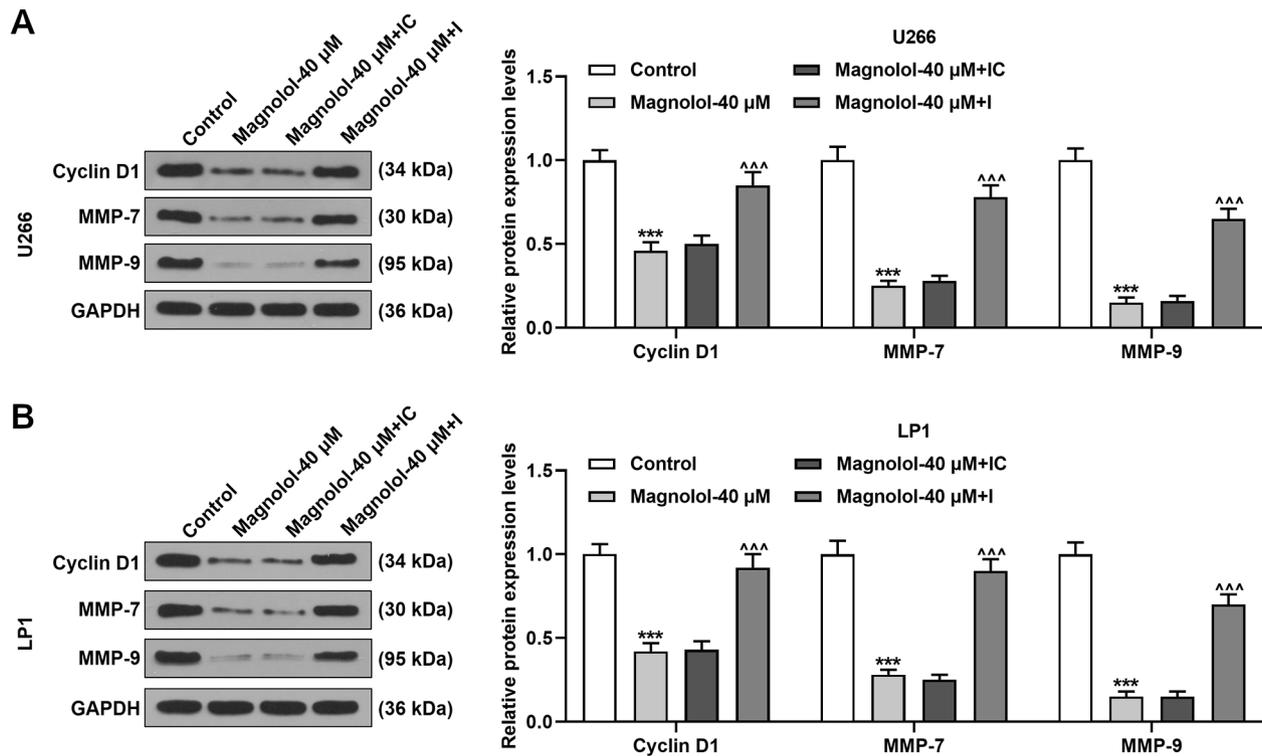


Figure 6. Magnolol repressed Cyclin D1 and matrix metalloproteinases (MMPs) expression of multiple myeloma (MM) cells via upregulating miR-129. A, B) Relative protein expression levels of Cyclin D1, MMP-7, and MMP-9 in U266 (A) and LP1 cells (B) were tested by western blot after the treatment of magnolol and the transfection of the miR-129 inhibitor. *** $p < 0.001$ vs. control group, ^^ $p < 0.001$ vs. magnolol-40 + inhibitor control (IC) group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

theless, the role and mechanism of magnolol on MM is still unknown. miR-129, belonging to the miRNA class, has shown its suppression on cell development of various malignant tumors [21–23]. In addition, downregulation of miR-129 has been observed in MM, with the overexpressed miR-129 inhibiting cell multiplication while promoting cell apoptosis of MM [10]. Hence, we supposed that magnolol might modulate MM cells by regulating miR-129. And this work determined the effect of magnolol on MM and its mechanism of action.

At first, we explored the effect of magnolol on MM cells. In agreement with the study about the influence of magnolol on osteosarcoma [18], our results showed MM cell viability greatly decreased with the concentration of magnolol rising, whereas the cell viability of nPCs was largely unaffected. This suggests a dose-dependent manner during the suppression of magnolol on MM cell viability as well as adds the possibility of magnolol as a safe biological compound for MM therapy in clinics as magnolol was specific for MM cells without harming normal cells. Furthermore, cell migration and invasion rates were negatively correlative to magnolol concentration while cell apoptosis had a positive correlation to magnolol, which was consistent with reports about magnolol functioning on a number of cancers including

Ewing sarcoma, osteosarcoma, cholangiocarcinoma, breast cancer, hepatocellular carcinoma, lung cancer, glioblastoma, prostate cancer, and thyroid carcinoma [14, 17, 18, 26–32].

Cyclin D1, as a cell cycle-related protein regulating cyclin-dependent kinase, participates in cell apoptosis during the progression of many cancers [33, 34]. MMPs are a group of zinc (Zn) containing enzymes and MMP-9 is involved in degradation and remodeling of extracellular matrix (ECM), which connects with epithelial-mesenchymal transition (EMT), implicated in cell metastasis as well as many other progressions of various cancers. As for MMP-7, it has been reported that MMP-7 is capable of destroying basement membrane components, which is a key point of cell invasion and metastasis in cancers. And the degradation of the basement membrane, including the ECM, plays a significant part in invasion [35–40]. In our researches, corresponding to other experiments about the effect of magnolol on osteosarcoma and cholangiocarcinoma cells [18, 31], we discovered that the protein expressions of Cyclin D1, MMP-7, and MMP-9 were inhibited when MM cells treated with magnolol. Therefore, magnolol may inhibit myeloma growth and metastasis through downregulating Cyclin D1, MMP-7, and MMP-9.

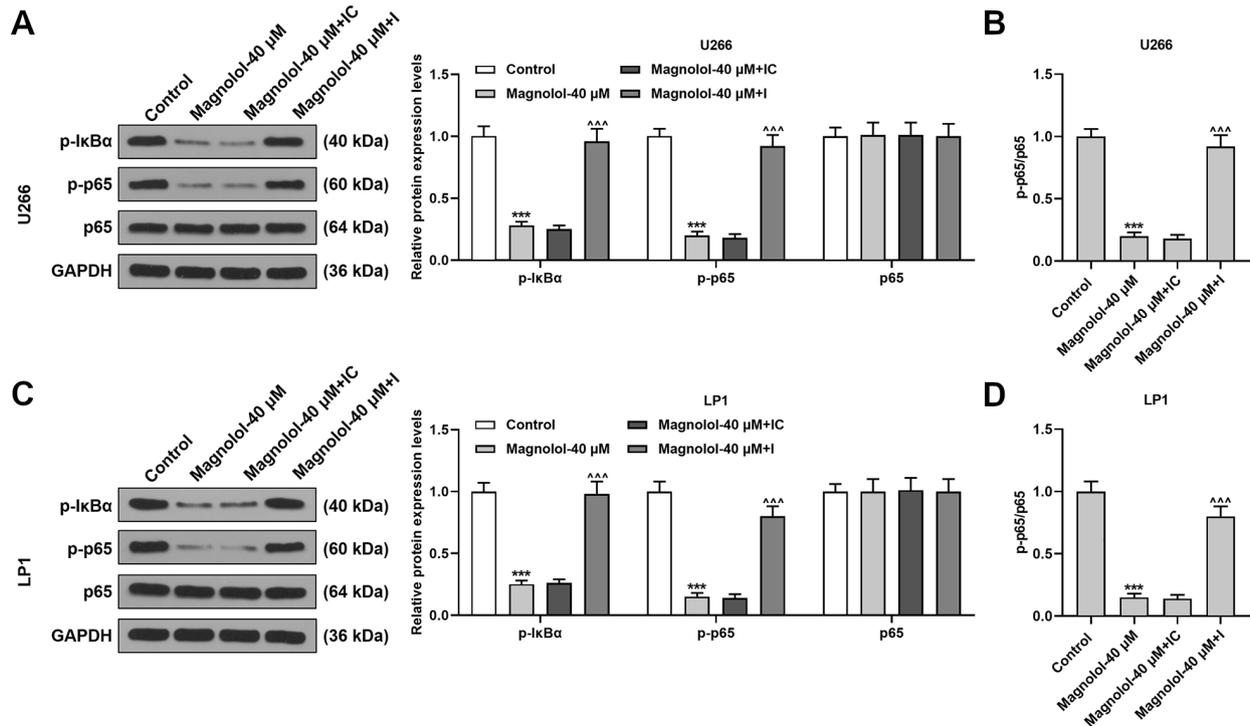


Figure 7. Magnolol inhibited the activation of the NF- κ B signaling pathway via upregulating miR-129 in multiple myeloma (MM) cells. A, B) Relative protein expression levels of phosphorylation (p)-I κ B α , p-p65, and p65 as well as the ratio of p-p65 to p65 in U266 cells were assessed through western blot after the treatment of magnolol and the transfection of the miR-129 inhibitor. C, D) Relative protein expression levels of p-I κ B α , p-p65, and p65, as well as the ratio of p-p65 to p65 in LP1 cells, were assessed through western blot after the treatment of magnolol and the transfection of the miR-129 inhibitor. *** $p < 0.001$ vs. control group, ^^ $p < 0.001$ vs. magnolol-40 + inhibitor control (IC) group. All experiments were repeated independently at least three times. Data are shown as the mean \pm standard deviations.

At second, we looked into the mechanism of magnolol. It has been discovered that miR-1271-5p, miR-342-3p, and miR-124-3p are involved in the suppression of MM [41–46]. The results of qRT-PCR presented a marked upregulation of miR-129 in MM cells treated with magnolol while little difference occurred in the expression of miR-1271-5p, miR-342-3p, and miR-124-3p, indicating magnolol might target miR-129 during MM. In order to further determine whether magnolol realized its function by promoting miR-129 expression, we used the miR-129 inhibitor to transfect into MM cells. During the experiments, we found that magnolol increased miR-129 expression and decreased cell viability while the miR-129 inhibitor raised cell viability. Moreover, magnolol inhibited cell migration and invasion of MM, whereas miR-129 inhibitor counteracted the effect of magnolol. The same results were also performed in the detection of Cyclin D1, MMP-7, and MMP-9 protein expression levels through western blot. NF- κ B is a critical factor whose activation connects with initiation and development in a number of cancers including MM [47, 48]. P-I κ B α and p65 appear to be the major forms of the activated NF- κ B signaling pathway [49]. Consistent with a previous study about magnolol repressing the NF- κ B activation of cholangiocarcinoma cells as well as miR-129 targeting NF- κ B pathway in MM [10, 31], the results showed

that magnolol suppressed activation of NF- κ B signaling pathway, as miR-129 inhibitor antagonized the function of magnolol.

Taken all together, the observation and analysis obtained in our experiments demonstrated the protective effects of magnolol against MM and showed its specific molecular mechanism, providing strong support for the function of magnolol against cancers and offering a promising and potential therapeutic target and drug for intervention and treatment of MM. However, there still exist some problems in our study. Experiments *in vivo* should be done in the following study to further confirm the conclusions of experiments *in vitro*. In the future, we will also investigate the function of magnolol *in vivo* for verification of the conclusion in this paper and continue to search for other targets of magnolol and their concrete mechanisms so that more possible therapies can be provided.

In summary, this work studied the effect of magnolol on MM and its mechanism of action. Magnolol did not suppress harm normal cells, but suppress cell migration and invasion, and induced cell apoptosis in MM via inhibition of the NF- κ B signaling pathway by upregulating miR-129, suggesting magnolol with hypotoxicity might become a novel targeted therapeutic drug for MM.

Acknowledgements: This work was supported by the Clinical Research Fund of Zhejiang Medical Association[2018zyc-A164].

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