

Circ_0007841 promotes the progression of multiple myeloma via miR-338-3p/BRD4 axis

Yan Wang (✉ wy1782@126.com)

The Fifth Affiliated Hospital of Zhengzhou University <https://orcid.org/0000-0002-4694-9014>

Quande Lin

Henan Cancer Hospital

Chunge Song

The Fifth Affiliated Hospital of Zhengzhou University

Ruojin Ma

The Fifth Affiliated Hospital of Zhengzhou University

Xiaojie Li

The Fifth Affiliated Hospital of Zhengzhou University

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Abstract

Background

The pathogenesis of multiple myeloma (MM) is not completely known. Herein, we explored the function and the working mechanism of circular RNA circ_0007841 in MM progression.

Methods

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to detect the expression of circ_0007841, microRNA-338-3p (miR-338-3p) and bromodomain containing 4 (BRD4). The proliferation and metastasis of MM cells were examined by cell counting kit-8 (CCK8) assay and transwell assays. Flow cytometry was conducted to assess the cell cycle and the apoptosis of MM cells. The targets of circ_0007841 and miR-338-3p were predicted by circinteractome and targetscan softwares, and these predictions were confirmed by dual-luciferase reporter assay and RNA-pull down assay. The protein levels of BRD4, phosphorylated-phosphatidylinositol 3-kinase (p-PI3K), PI3K, p-AKT serine/threonine kinase (p-AKT) and AKT were measured by Western blot assay. Exosomes were extracted using Exosome isolation kit.

Results

Circ_0007841 was highly expressed in bone marrow (BM)-derived plasma cells of MM patients and MM cells than that in healthy volunteers and normal plasma cells nPCs. Circ_0007841 promoted the proliferation, cell cycle and metastasis and impeded the apoptosis of MM cells. MiR-338-3p was a direct target of circ_0007841 in MM cells. Circ_0007841 accelerated the progression of MM through targeting miR-338-3p. BRD4 could directly bind to miR-338-3p in MM cells. MiR-338-3p exerted an anti-tumor role through targeting BRD4. Circ_0007841 promoted the activation of PI3K/AKT signaling via miR-338-3p/BRD4 axis. Exosomes generated from mesenchymal stromal cells (MSCs) elevated the malignant behaviors of MM cells via circ_0007841.

Conclusion

Circ_0007841 acted as an oncogene to promote the proliferation, cell cycle and motility and restrain the apoptosis of MM cells through sequestering miR-338-3p to up-regulate the expression of BRD4.

Background

Multiple myeloma (MM) is a kind of hematologic cancer that derived from plasma cells. Normal plasma cells were transformed into malignant myeloma cells in MM patients [1]. The treatment methods for MM patients include chemotherapy, radiotherapy and target therapy [2–4]. Uncovering the molecular mechanism behind the progression of MM and intercellular interaction is important to find more effective treatment methods for MM patients.

Non-coding RNAs (ncRNAs) are a class of RNAs that unable to code proteins, and they are abundant in human genome to regulate the cellular processes including proliferation, metastasis and apoptosis [5]. Circular RNAs (circRNAs) are a kind of ncRNAs that characterized by covalently closed loop structure [6]. CircRNAs are more stable than linear RNAs and they are resistant to exonuclease due to their loop structure [7]. CircRNAs engaged in the pathogenesis of cancers through serving as microRNAs (miRNAs) sponges to modulate the abundance of downstream genes linked to proliferation, metastasis and apoptosis [8, 9]. The roles of circRNAs in hematological cancers have been reported before [10, 11]. However, the functions of circRNAs in MM remain to be uncovered.

MiRNAs belong to another class of ncRNAs that involved in the progression of cancers through inducing degradation or translational repression of target messenger RNAs (mRNAs) [12]. The dysregulation of miRNAs was involved in the pathogenesis of MM [13, 14]. MiR-338-3p suppressed the development of many cancers [15–18]. As for MM, Cao et al. reported that miR-338-3p suppressed the proliferation and accelerated the apoptosis of MM cells via CDK4 [19]. Nevertheless, the function of miR-338-3p in MM is largely unexplored.

Bromodomain containing 4 (BRD4) is a crucial epigenetic protein, and it has been reported to elevate the levels of oncogenic proteins and accelerate the progression of cancers [20]. Zheng et al. claimed that H19 accelerated the development of MM through up-regulating BRD4 via sponging miR-152-3p [21]. Here, the direct interaction between miR-338-3p and BRD4 was first found in MM, and the function of BRD4 in MM was illustrated.

In this study, circ_0007841 was abnormally up-regulated in MM. The biological functions and the underlying mechanism of circ_0007841 in MM were further explored.

Materials And Methods

Patients

Plasma cells from MM patients (n = 41) and healthy volunteers (n = 41) in The Fifth Affiliated Hospital of Zhengzhou University were collected to detect the expression of circ_0007841, miR-338-3p and BRD4.

Cell Culture

MM cell lines (H929 and OPM2) and normal plasma cell line nPCs were purchased from BeNa Culture Collection (Beijing, China) and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS; Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin. Cell culture plates were placed in a 5% CO₂ incubator at 37°C.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

After measuring the concentration using NanoDrop 2000 (Invitrogen, Carlsbad, CA, USA), RNA sample (2 ng) was used to synthesize complementary DNA (cDNA) with ReverTra Ace qPCR RT Kit (Takara,

Dalian, China) and All-in-One™ miRNA First stand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). Housekeeping genes served as the controls in this study. The quantification of circ_0007841, miR-338-3p and BRD4 was carried out with the $2^{-\Delta\Delta Ct}$ method. The specific primers enrolled in this study were listed as below: circ_0007841 (Forward, 5'-CTAACATCTGTGAAACCATCGT-3'; Reverse, 5'-TCATCACATACACGATAGACTGG-3'), miR-338-3p (Forward, 5'-UCCAGCAUCAGUGAUUUUGUUG-3'; Reverse, 5'-CAACAAAAUCACUGAUGCUGGA-3'), BRD4 (Forward, 5'-GTGGTGACATCATCCAGTC-3'; Reverse, 5'-CCGACTCTGAGGACGAGAAG-3'), U6 (Forward, 5'-CTCGCTTCGGCAGCACA-3'; Reverse, 5'-AACGCTTCACGAATTTGCGT-3'), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Forward, 5'-GCGACACCCACTCCTCCAC-3'; Reverse, 5'-TCCACCACCCTGTTGCTGTAG-3').

Cell Transfection

Three small interfering RNAs (siRNAs) targeting circ_0007841, including si-circ_0007841 #1, si-circ_0007841 #2 and si-circ_0007841 #3, its negative control (si-NC), circ_0007841 overexpression plasmid (circ_0007841), its control (Vector), BRD4 overexpression plasmid (BRD4), its control (pcDNA), miR-338-3p mimics (miR-338-3p), its control miR-NC, miR-338-3p inhibitor (in-miR-338-3p) and its control in-miR-NC were obtained from Genepharma (Shanghai, China).

Cell Counting Kit-8 (CCK8) Assay

MM cells were plated in 96-well plates and cultured overnight. After transfection for indicated time points (0 h, 24 h, 48 h or 72 h), MM cells were incubated with 10 μ L CCK-8 (Sigma, St. Louis, MO, USA) for 4 h. The absorbance at 450 nm was detected by a microplate reader.

Flow Cytometry For Cell Cycle And Apoptosis Detection

For cell cycle analysis, MM cells were collected using cold phosphate buffer saline (PBS) and then immobilized using 70% cold ethanol solution. Prior to propidium iodide (PI; Solarbio, Beijing, China) staining, RNase was used to remove RNA in the samples. The percentage of MM cells in different phases was detected on a flow cytometer.

For apoptosis analysis, MM cells were collected with PBS, and then these cells were suspended in binding buffer. Annexin V combined fluorescein isothiocyanate (Annexin V-FITC; Solarbio) and PI (Solarbio) were used to identify apoptotic MM cells on a flow cytometer.

Transwell Assays

In transwell migration assay, cell suspension (MM cells suspended in 100 μ L serum-free medium) was added into the upper chambers (Costar, Corning, NY, USA). A total of 600 μ L culture medium with 10% FBS was added into the lower chambers. FBS acted as the chemotactic factor in this study. After 24-h incubation, MM cells remained in the upper surface were removed, and the migrated MM cells were fixed and counted.

In transwell invasion assay, the upper chambers were pre-coated with 50 μ L Matrigel (Sigma) to mimic the extracellular matrix. The other steps were similar as mentioned above.

Bioinformatic Prediction And Dual-luciferase Reporter Assay

The targets of circ_0007841 and miR-338-3p were predicted by circinteractome and targetscan software, respectively.

The wild-type sequence in circ_0007841 predicted to bind to miR-338-3p, along with the mutant-type sequence, was amplified and cloned into pGL3 luciferase reporter vector (Promega, Madison, WI, USA), termed as circ_0007841 WT or circ_0007841 MUT. MM cells were co-transfected with miR-NC or miR-338-3p and circ_0007841 WT or circ_0007841 MUT. After 48-h transfection, MM cells were harvested and the luciferase activity was detected with the dual-luciferase reporter assay system (Promega).

The confirmation of the target relationship between miR-338-3p and BRD4 was conducted according to the similar procedures.

RNA-pull Down Assay

Biotin RNA Labeling Mix (Roche, Shanghai, China) was used in this study. The wild-type and mutant-type binding sites of circ_0007841 that were predicted to bind to miR-338-3p were biotinylated to obtain Bio-circ_0007841 WT and Bio-circ_0007841 MUT. MM cells were disrupted and incubated with Bio-NC, Bio-circ_0007841 WT or Bio-circ_0007841 MUT. The abundance of miR-338-3p was measured by qRT-PCR.

Western Blot Assay

Proteins were obtained using whole cell lysis buffer (Roche, Basel, Switzerland). Proteins were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking, primary antibodies were used to probe the indicated proteins followed by incubation with the secondary antibody (ab205718; Abcam, Cambridge, MA, USA). The protein bands were measured using the enhanced chemiluminescent (ECL) system (Beyotime, Shanghai, China). Gray analysis was conducted to quantify the expression of proteins. Primary antibodies, including anti-BRD4 (ab128874), anti-phosphorylated-phosphatidylinositol 3-kinase (anti-p-PI3K; ab70912), anti-PI3K (ab32089), anti-p-AKT serine/threonine kinase (p-AKT; ab38449), anti-AKT (ab64148), anti-CD63 (ab59479), anti-CD81 (ab79559) and anti- β -actin (ab8226), were purchased from Abcam.

Exosome Isolation

Exosome isolation kit (Qiagen, Frankfurt, Germany) was used to extract exosomes from the culture medium of MM cells according to previous studies [22, 23].

Statistical analysis

All statistical data were shown as mean \pm standard deviation (SD). Data were analyzed using GraphPad Prism 7.0. The differences between two groups or among more than two groups were assessed through using Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's test. The comparison between groups was considered significant when P value less than 0.05. Linear correlation was analyzed using Spearman's correlation coefficient.

Results

Circ_0007841 promotes the progression of MM

Circ_0007841 was abnormally up-regulated in bone marrow (BM)-derived plasma cells from MM patients compared with that in healthy individuals (Fig. 1A). Meanwhile, the level of circ_0007841 was higher in MM cells than that in normal plasma cells (nPCs, Fig. 1B). Circ_0007841 specific small interfering RNAs were used to knockdown circ_0007841 to uncover its biological functions in MM cells. As mentioned in Fig. 1C, the level of circ_0007841 was down-regulated with the transfection of si-circ_0007841#1, si-circ_0007841#2 or si-circ_0007841#3. Among these three siRNAs, si-circ_0007841#1 was chose for the following assays due to its highest knockdown efficiency (Fig. 1C). According to the results of CCK8 assay, si-circ_0007841#1 transfection inhibited the proliferation of MM cells (Fig. 1D and 1E). The cell cycle of MM cells was arrested in G1/S transition in si-circ_0007841#1 group than that in si-NC group (Fig. 1F). What's more, circ_0007841 interference notably suppressed the migration and invasion of MM cells (Fig. 1G and 1H). The apoptosis of MM cells was promoted in si-circ_0007841#1 group compared with that in si-NC group (Fig. 1I). Overall, circ_0007841 accelerated the proliferation, cell cycle, metastasis and inhibited the apoptosis of MM cells.

MiR-338-3p Could Directly Interact With circ_0007841 In MM Cells

To address the mechanism by which circ_0007841 functioned in MM cells, circinteractome website was used to seek the targets of circ_0007841. As shown in Fig. 2A, miR-338-3p possessed the complementary sites with circ_0007841. However, the interaction between miR-338-3p and circ_0007841 has never been reported. The luciferase activity was dramatically reduced in circ_0007841 WT group when co-transfecting with miR-338-3p (Fig. 2B and 2C). RNA-pull down assay revealed that miR-338-3p could be pulled-down when using Bio-circ_0007841 WT, proving the target relationship between miR-338-3p and circ_0007841 (Fig. 2D and 2E). An obvious decrease in the level of miR-338-3p was observed in BM-derived plasma cells from MM patients in contrast to that in normal volunteers (Fig. 2F). Additionally, there was a prominent reduction in the expression of miR-338-3p in MM cells than that in nPCs cells (Fig. 2G). The expression of miR-338-3p was negatively correlated with the level of circ_0007841 in BM-derived plasma cells from MM patients (Fig. 2H). The overexpression efficiency of circ_0007841 was high in MM cells, and circ_0007841 accumulation caused a notable decrease in the level of miR-338-3p in MM cells (Fig. 2I and 2J). In summary, circ_0007841 could inversely regulate the expression of miR-338-3p through direct interaction.

Circ_0007841 plays an oncogenic role through targeting miR-338-3p in MM cells

H929 and OPM2 cells were transfected with si-NC, si-circ_0007841#1, si-circ_0007841#1 + in-miR-NC or si-circ_0007841#1 + in-miR-338-3p to disclose the functions of circ_0007841 and miR-338-3p. As mentioned in Fig. 3A, si-circ_0007841#1 transfection increased the level of miR-338-3p, and the introduction of in-miR-338-3p reversed the influence of circ_0007841 silencing in the expression of miR-338-3p. We also detected the proliferation ability, cell cycle, motility and apoptosis of MM cells with same transfection. Si-circ_0007841#1-mediated inhibitory effect on the proliferation of MM cells was counteracted by the interference of miR-338-3p (Fig. 3B and 3C). Additionally, cell cycle of MM cells was arrested at G1/S transition in si-circ_0007841#1 group, and this suppressive impact in the cell cycle of MM cells was attenuated by the addition of in-miR-338-3p (Fig. 3D and 3E). The migration and invasion of MM cells were suppressed by the knockdown of circ_0007841, and the metastasis ability was recovered in si-circ_0007841#1 and in-miR-338-3p co-transfected group (Fig. 3F and 3G). Si-circ_0007841#1-induced apoptosis of MM cells was suppressed by the addition of in-miR-338-3p (Fig. 3H). Overall, circ_0007841 could promote the malignant potential of MM cells through sponging miR-338-3p.

BRD4 is validated as a target of miR-338-3p in MM cells

BRD4 was predicted as a direct target of miR-338-3p by targets can database (Fig. 4A). As exhibited in Fig. 4B and 4C, the luciferase activity was markedly decreased in miR-338-3p and BRD4 3'untranslated region (3'UTR) WT co-transfected group, while miR-338-3p transfection had no effect on the luciferase activity in BRD4 3'UTR MUT group compared with that in miR-NC and BRD4 3'UTR MUT co-transfected group. BRD4 was conspicuously up-regulated in BM-derived plasma cells of MM patients compared with that in healthy individuals (Fig. 4D). Meanwhile, BRD4 was also found to be up-regulated in MM cells than that in nPCs cells (Fig. 4E). The expression correlation between BRD4 and circ_0007841 or miR-338-3p was analyzed using Spearman's correlation coefficient. As shown in Fig. 4F and 4G, there was an inverse correlation between the levels of BRD4 and miR-338-3p, while the expression of BRD4 was positively correlated with the level of circ_0007841. MiR-338-3p overexpression significantly down-regulated the expression of BRD4 in MM cells (Fig. 4H). Circ_0007841 and miR-338-3p were co-transfected into MM cells to uncover the relationship among circ_0007841, miR-338-3p and BRD4. As presented in Fig. 4I, circ_0007841 overexpression up-regulated the level of BRD4, and the expression of BRD4 was decreased in circ_0007841 and miR-338-3p co-transfected group. Collectively, BRD4 was a target of miR-338-3p, and circ_0007841 could elevate the expression of BRD4 through sponging miR-338-3p.

BRD4 overexpression attenuates the effects of miR-338-3p accumulation on MM cells

MiR-338-3p and BRD4 were co-transfected into MM cells to explore whether miR-338-3p exerted an anti-tumor role in MM cells through targeting BRD4. As shown in Fig. 5A, the addition of BRD4 overexpression plasmid recovered the expression of BRD4 in MM cells that was down-regulated by the accumulation of miR-338-3p. MiR-338-3p overexpression inhibited the proliferation, cell cycle and metastasis of MM cells, and these inhibitory effects were attenuated by the addition of BRD4 overexpression plasmid (Fig. 5B-

5G). The apoptosis of MM cells was induced by the transfection of miR-338-3p, and the introduction of BRD4 overexpression plasmid recovered the viability of MM cells (Fig. 5H). In conclusion, miR-338-3p accumulation-mediated influence on MM cells was overturned by the introduction of BRD4.

The activation of PI3K/AKT signal pathway is modulated by circ_0007841/miR-338-3p/BRD4 axis

The activation of PI3K/AKT signal pathway is linked to the promotion of cell proliferation and metastasis and the inhibition of cell apoptosis. Herein, we examined the phosphorylation levels of PI3K and AKT to illustrate the influence of circ_0007841/miR-338-3p/BRD4 axis in the activation of PI3K/AKT signaling. Circ_0007841 silencing down-regulated the level of BRD4, and the level of BRD4 was recovered in si-circ_0007841#1 and in-miR-338-3p co-transfected group (Fig. 6A and 6B). The activation of PI3K/AKT signaling was suppressed with the silencing of circ_0007841, and the addition of in-miR-338-3p recovered the phosphorylation levels of PI3K and AKT (Fig. 6A and 6C). Meanwhile, H929 and OPM2 cells were transfected with miR-NC, miR-338-3p, miR-338-3p + pcDNA or miR-338-3p + BRD4. As mentioned in Fig. 6D and 6E, miR-338-3p overexpression down-regulated the level of BRD4, and the introduction of BRD4 overexpression plasmid regained the level of BRD4 in MM cells. The addition of BRD4 alleviated the inhibitory influence of miR-338-3p overexpression on the activation of PI3K/AKT signaling in MM cells (Fig. 6D and 6F). Taken together, circ_0007841 accelerated the progression of MM through miR-338-3p/BRD4/PI3K/AKT axis.

Mesenchymal stromal cells (MSCs)-generated exosomes accelerate the malignant potential of MM cells via circ_0007841

MSCs exert crucial roles in the progression of MM. Herein, we explored whether exosomes derived from MSCs could regulate the proliferation, cell cycle, metastasis and apoptosis of MM cells via circ_0007841. MSCs were isolated from the adjacent tissues of MM and normal tissues. The expression of circ_0007841 was higher in MSCs and MSCs-derived exosomes from adjacent tissues than that in normal tissues (Fig. 7A and 7B). The markers of exosomes (CD63 and CD81) were notably up-regulated in exosomes of MSCs instead of cell lysate (Fig. 7C). As mentioned in Fig. 7D, we established a working model as previously described to explore if MSCs-derived exosomes could regulate the proliferation, cell cycle, motility and apoptosis of MM cells [24]. In this model, only exosomes could be transmitted through the filter to the upper chambers. As presented in Fig. 7E-7J, si-circ_0007841#1 transfection inhibited the malignant behaviors of MM cells in Mock + si-circ_0007841#1 group compared with that in Mock + si-NC group. Besides, MSCs-derived exosomes (MSCs + si-NC group) promoted the proliferation, cell cycle, metastasis and inhibited the apoptosis of MM cells than that in Mock + si-NC group, and these effects were attenuated by the silencing the circ_0007841, suggested that MSCs-derived exosomes could promote the progression of MM via circ_0007841. What's more, the exosomes generated from MSCs accelerated the activation of PI3K/AKT signaling, while this effect was counteracted with the transfection of si-circ_0007841#1 (Fig. 7K). Collectively, MSCs-derived exosomes could facilitate the progression of MM via circ_0007841.

Discussion

MM is an incurable cancer at present. Because many MM patients were diagnosed at late stage, the treatment outcomes were unsatisfactory [25]. Therefore, finding crucial markers in MM is urgent to improve the therapeutic effects.

CircRNAs featured by closely loop structure and they are widely distributed in human tissues. Due to the stability and the universality of the distribution, circRNAs are identified as ideal biomarkers for cancers and other diseases [26]. For example, the high expression of circ_0004277 was associated with the better prognosis of AML patients [27]. Xia et al. claimed that circ-CBFB was highly expressed in chronic lymphocytic leukemia, and circ-CBFB accelerated the proliferation and suppressed the apoptosis of chronic lymphocytic leukemia cells [10]. Circ_0007841 was found to be overexpressed in BM-derived plasma cells of MM patients and MM cells. Further studies suggested that circ_0007841 promoted the proliferation, cell cycle, metastasis and inhibited the apoptosis of MM cells. These findings demonstrated that circ_0007841 might be an important biomarker for MM patients, which was in agreement with the former article [28]. However, the regulatory mechanism by which circ_0007841 promoted the progression of MM was unclear.

MiRNAs are single-stranded ncRNAs, and they are implicated in cell proliferation, metastasis and apoptosis through base pairing with mRNAs [29]. Additionally, circRNAs could act as miRNAs sponges to function [30]. MiR-338-3p played a tumor suppressor role in multiple cancers. For instance, Sui et al. proved that miR-338-3p suppressed the development of thyroid cancer via AKT3 [31]. Jin et al. found that miR-338-3p played an anti-tumor role in breast cancer via SOX4 [32]. Xue et al. demonstrated that miR-338-3p suppressed the metastasis of colorectal cancer cells through targeting smoothed [33]. MiR-338-3p was also found to be down-regulated in MM, and it suppressed the proliferation and facilitated the apoptosis of MM cells via CDK4 [19]. MiR-338-3p was identified as a novel target of circ_0007841 in MM cells in our study. Subsequently, rescue experiments were performed to explore whether circ_0007841 functioned through sponging miR-338-3p. We found that si-circ_0007841#1-mediated effects in MM cells were alleviated by the transfection of in-miR-338-3p, suggested that circ_0007841 acted as an oncogene in MM through targeting and down-regulating miR-338-3p.

To uncover the potential mechanism that was responsible for the functions of miR-338-3p in MM cells, the downstream genes of miR-338-3p were searched using targets can software. BRD4 was confirmed as a candidate gene of miR-338-3p. BRD4 was regulated by circ_0007841/miR-338-3p axis in MM cells. High expression of BRD4 promoted the progression of high-grade serous ovarian cancer [34]. Besides, BRD4 was found to be a target of H19/miR-152-3p axis to promote the progression of MM [21]. Rescue experiments revealed that BRD4 overexpression attenuated the inhibitory effects of miR-338-3p transfection on the proliferation, cell cycle and metastasis and the promoting effect on the apoptosis of MM cells, proved that BRD4 acted as a target of miR-338-3p to promote the progression of MM.

PI3K/AKT signal pathway was related to cell proliferation, viability, apoptosis and autophagy. Accumulating articles have reported the association between the pro-proliferative influence of circRNAs in

cancer cells and the activation of PI3K/AKT pathway. For example, circ-IGF1R promoted the proliferation and blocked the apoptosis of hepatocellular carcinoma cells through activating PI3K/AKT pathway [35]. Liu et al. found that circ_8073 accelerated the proliferation of caprine endometrial epithelial cells through activating PI3K/AKT/mTOR pathway via miR-449a/CEP55 axis [36]. Further studies demonstrated that the activation PI3K/AKT signaling could be regulated by circ_0007841/miR-338-3p/BRD4 axis in MM cells. Additionally, we found that MSCs-generated exosomes could accelerate the progression of MM via circ_0007841.

Conclusions

In summary, our studies identified a novel circ_0007841/miR-338-3p/BRD4 axis in MM. Circ_0007841 could facilitate the proliferation, cell cycle and metastasis and inhibit the apoptosis of MM cells through acting as a decoy of miR-338-3p to up-regulate BRD4. Circ_0007841/miR-338-3p/BRD4 axis might be a promising therapeutic target for MM patients.

Abbreviations

MM: multiple myeloma; qRT-PCR: Quantitative real-time polymerase chain reaction; BRD4: bromodomain containing 4; CCK8: cell counting kit-8; p-PI3K: phosphorylated-phosphatidylinositol 3-kinase; BM: bone marrow; MSCs: mesenchymal stromal cells; ncRNAs: Non-coding RNAs; circRNAs: Circular RNAs; miRNAs: microRNAs; mRNAs: messenger RNAs; siRNAs: small interfering RNAs; PVDF: polyvinylidene fluoride; MSCs: Mesenchymal stromal cells.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Fifth Affiliated Hospital of Zhengzhou University and written informed consents were collected from all patients and hospitals.

Consent for publication

Not applicable

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Yan Wang and Quande Lin performed experiments, analyzed data, and wrote the manuscript. Chung Song and Ruojin Ma designed research, performed experiments, and analyzed data. Xiaojie Li conceived and designed research.

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Figures

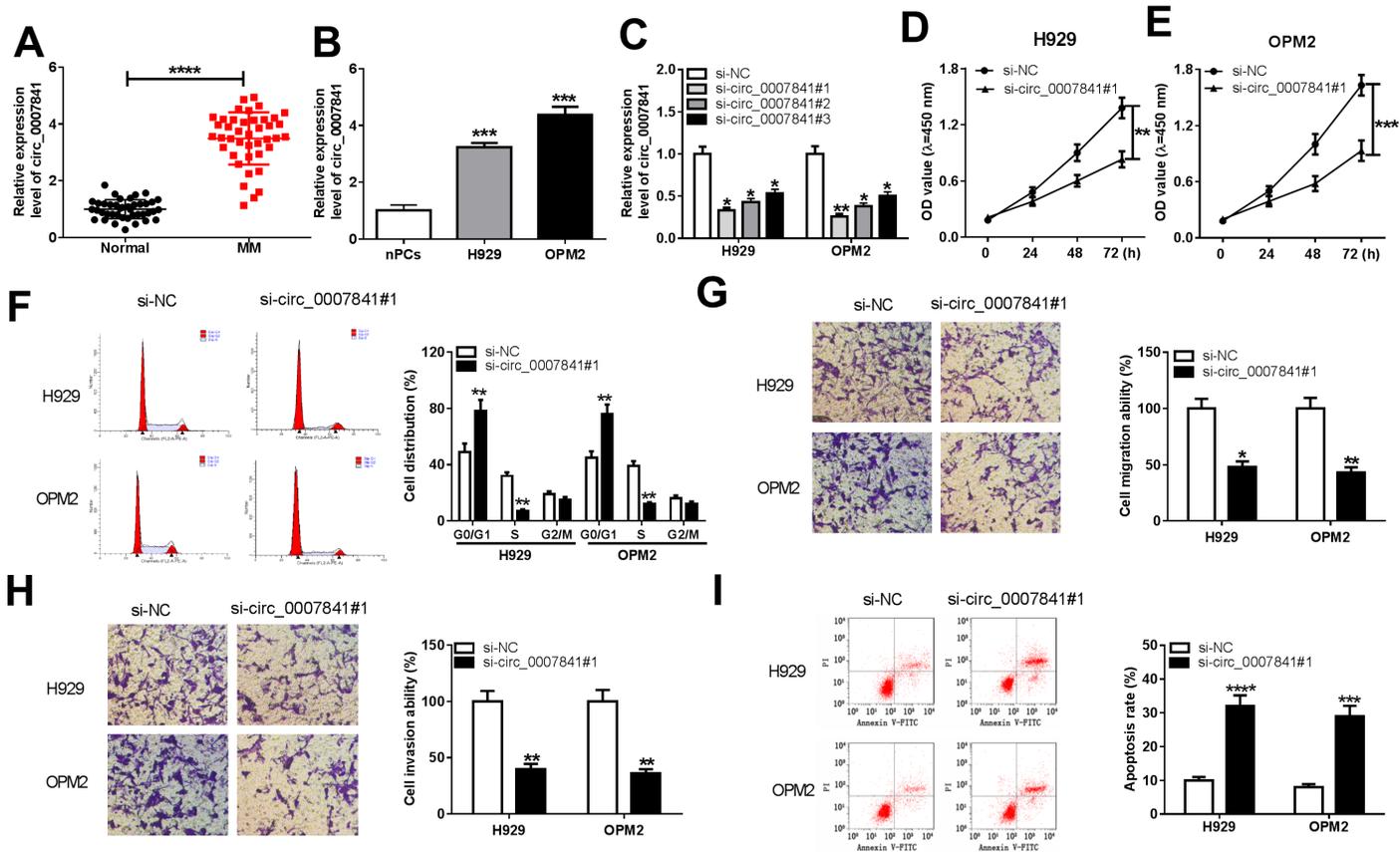


Figure 1

Circ_0007841 promotes the progression of MM. (A) The enrichment of circ_0007841 was examined in BM-derived plasma cells of MM patients and normal volunteers by qRT-PCR. (B) The expression of circ_0007841 was measured in MM cell lines and normal plasma cell line nPCs by qRT-PCR. (C) The level of circ_0007841 was detected in H929 and OPM2 cells transfected with si-NC, si-circ_0007841#1, si-circ_0007841#2 or si-circ_0007841#3 by qRT-PCR. (D-I) MM cells were transfected with si-NC or si-circ_0007841#1. (D and E) CCK8 assay was employed to assess the proliferation ability of MM cells. (F) Flow cytometry was carried out to detect the influence of circ_0007841 silencing on the cycle of MM cells. (G and H) The metastasis ability of MM cells was evaluated by transwell assays. (I) The apoptosis of MM cells was analyzed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

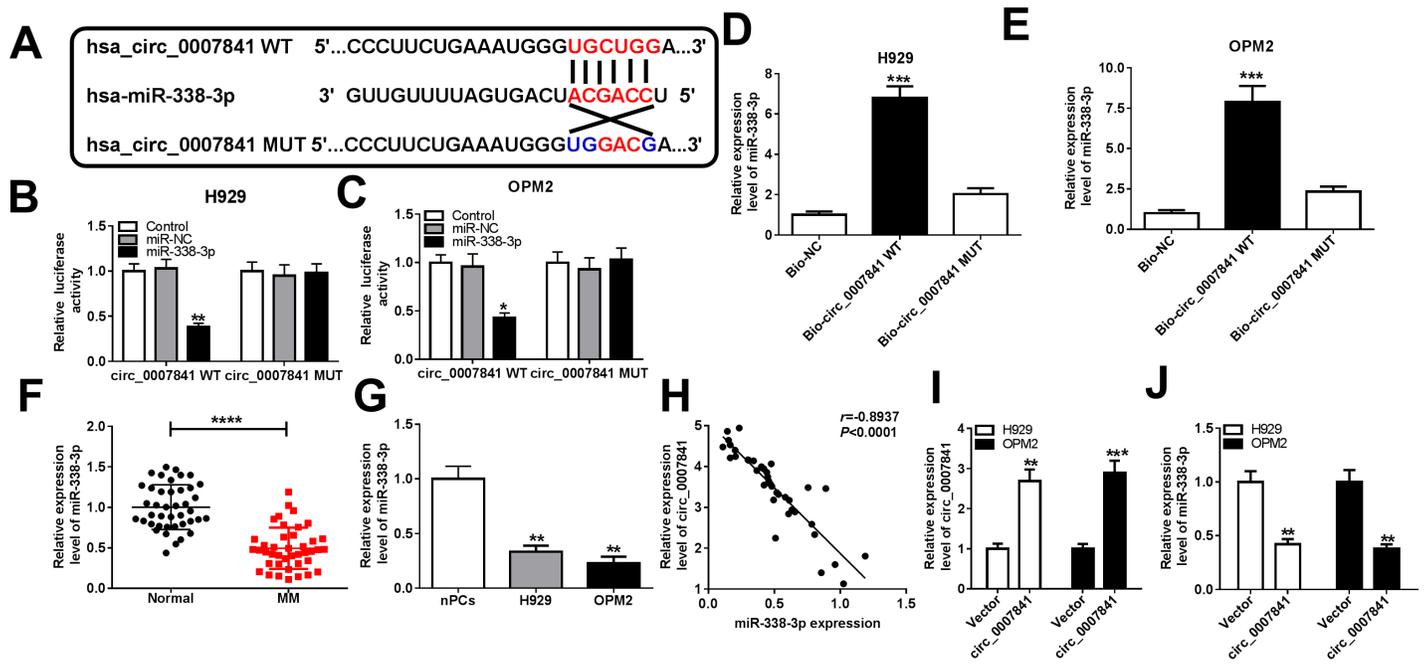


Figure 2

MiR-338-3p could directly interact with circ_0007841 in MM cells. (A) MiR-338-3p was predicted as a candidate target of circ_0007841 by circinteractome software. (B and C) Dual-luciferase reporter assay was conducted to verify whether miR-338-3p could bind to circ_0007841 in MM cells. (D and E) RNA-pull down assay was performed to confirm the target relationship between miR-338-3p and circ_0007841 in MM cells. (F and G) The expression of miR-338-3p was detected in BM-derived plasma cells of MM patients and healthy volunteers, MM cells and nPCs cells by qRT-PCR. (H) The correlation between the expression of miR-338-3p and circ_0007841 was analyzed using Spearman's coefficient. (I and J) The abundance of circ_0007841 and miR-338-3p was examined in H929 and OPM2 cells transfected with Vector or circ_0007841 by qRT-PCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

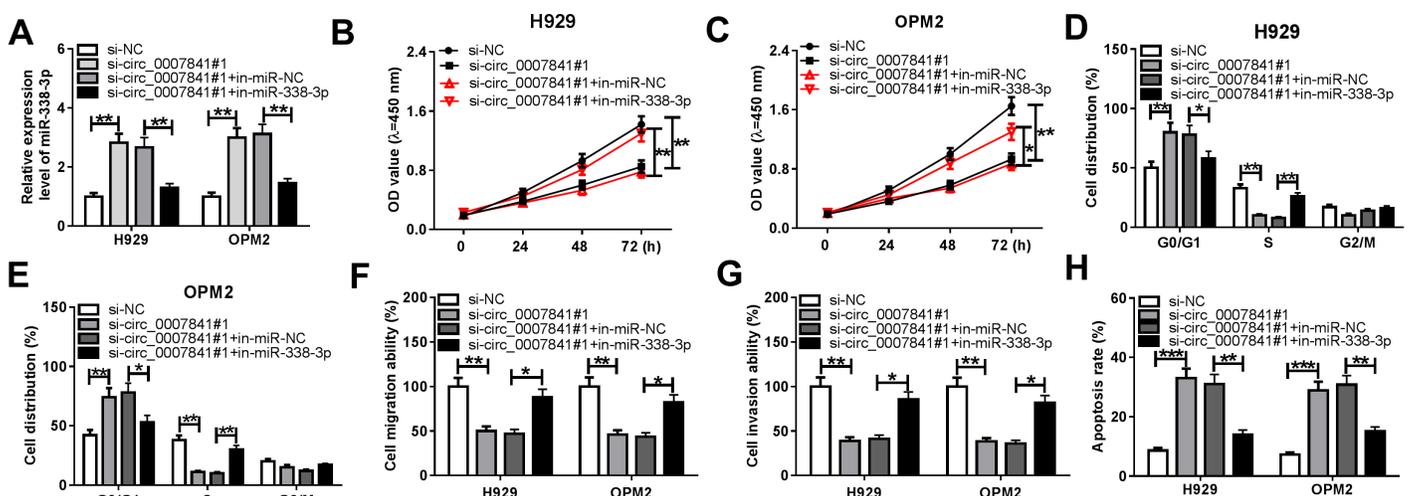


Figure 3

Circ_0007841 plays an oncogenic role through targeting miR-338-3p in MM cells. MM cells were transfected with si-NC, si-circ_0007841#1, si-circ_0007841#1 + in-miR-NC or si-circ_0007841#1 + in-miR-338-3p. (A) The level of miR-338-3p was examined in MM cells by qRT-PCR assay. (B and C) The proliferation of MM cells was measured through conducting CCK8 assay. (D and E) The percentage of MM cells in G0/G1, S or G2/M phase was analyzed using flow cytometry. (F and G) The migration and invasion abilities of MM cells were evaluated by transwell assays. (H) The apoptosis rate of MM cells in different groups was analyzed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001.

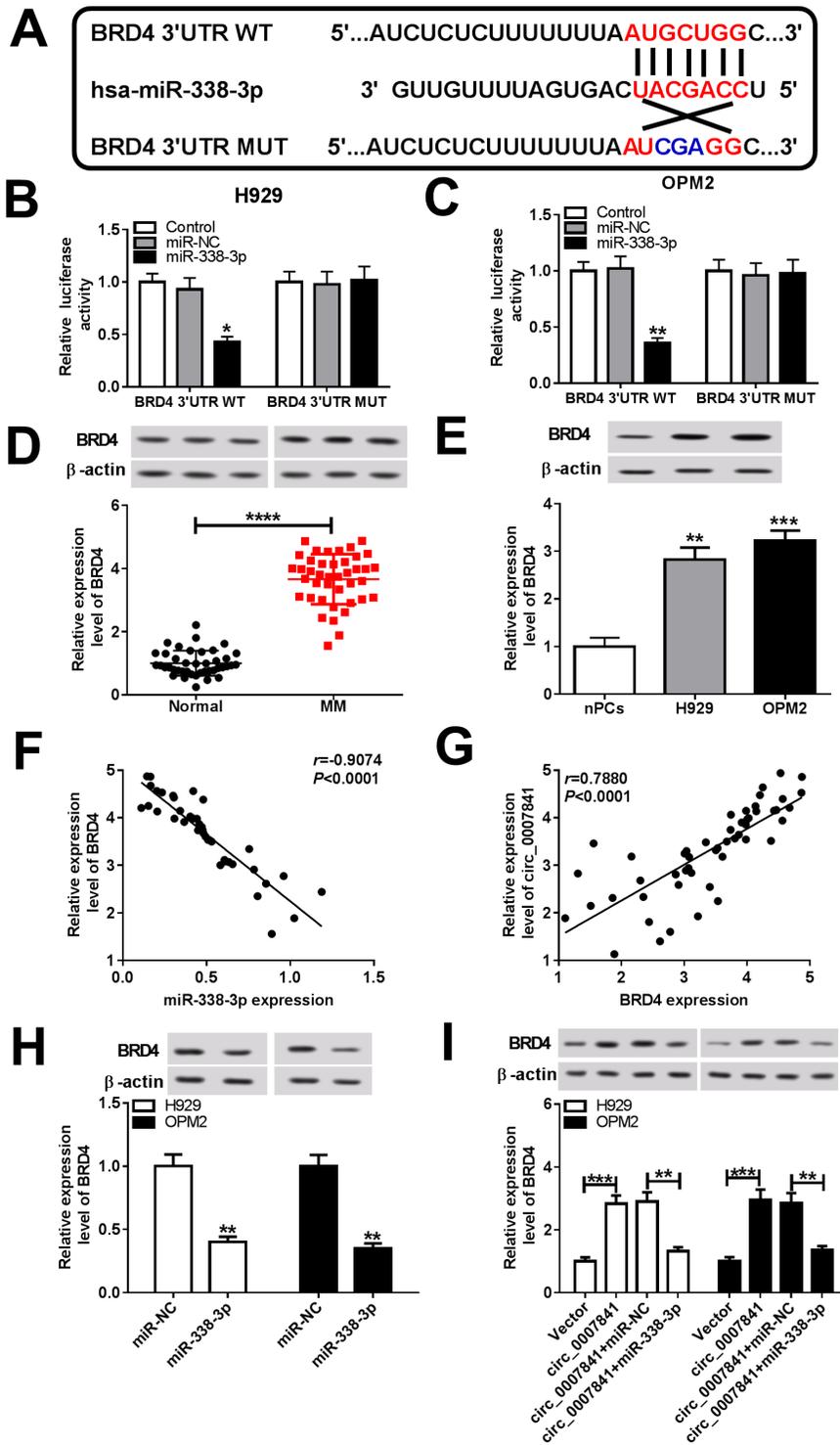


Figure 4

BRD4 is validated as a target of miR-338-3p in MM cells. (A) The complementary sites between miR-338-3p and the 3'UTR of BRD4 were predicted by targetscan software. (B and C) The luciferase activity was measured in H929 and OPM2 cells transfected with miR-NC or miR-338-3p and BRD4 3'UTR WT or BRD4 3'UTR MUT. (D) The protein level of BRD4 in BM-derived plasma cells of MM patients and healthy volunteers was detected by Western blot assay. (E) The level of BRD4 in H929, OPM2 and nPCs cells was

evaluated by Western blot assay. (F and G) The linear relationship between BRD4 and miR-338-3p or circ_0007841 was analyzed using Spearman's coefficient. (H) The expression of BRD4 was detected in MM cells transfected with miR-NC or miR-338-3p by Western blot assay. (I) The protein level of BRD4 was detected in MM cells transfected with Vector, circ_0007841, circ_0007841 + miR-NC or circ_0007841 + miR-338-3p by Western blot assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

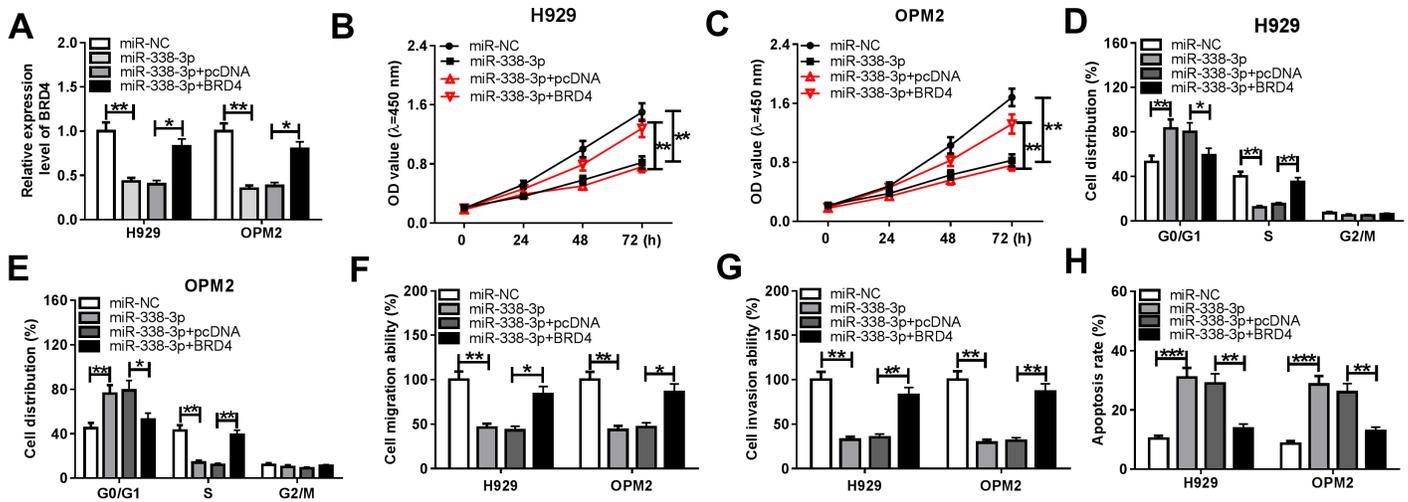


Figure 5

BRD4 overexpression attenuates the effects of miR-338-3p accumulation on MM cells. (A-H) MM cells were transfected with miR-NC, miR-338-3p, miR-338-3p + pcDNA or miR-338-3p + BRD4. (A) qRT-PCR was employed to measure the expression of BRD4 in MM cells. (B and C) CCK8 assay was applied to assess the proliferation ability of MM cells. (D and E) Flow cytometry was conducted to detect the cell cycle of MM cells. (F and G) Transwell assays were performed to detect the metastasis of MM cells. (H) The apoptosis rate of MM cells was examined by flow cytometry. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

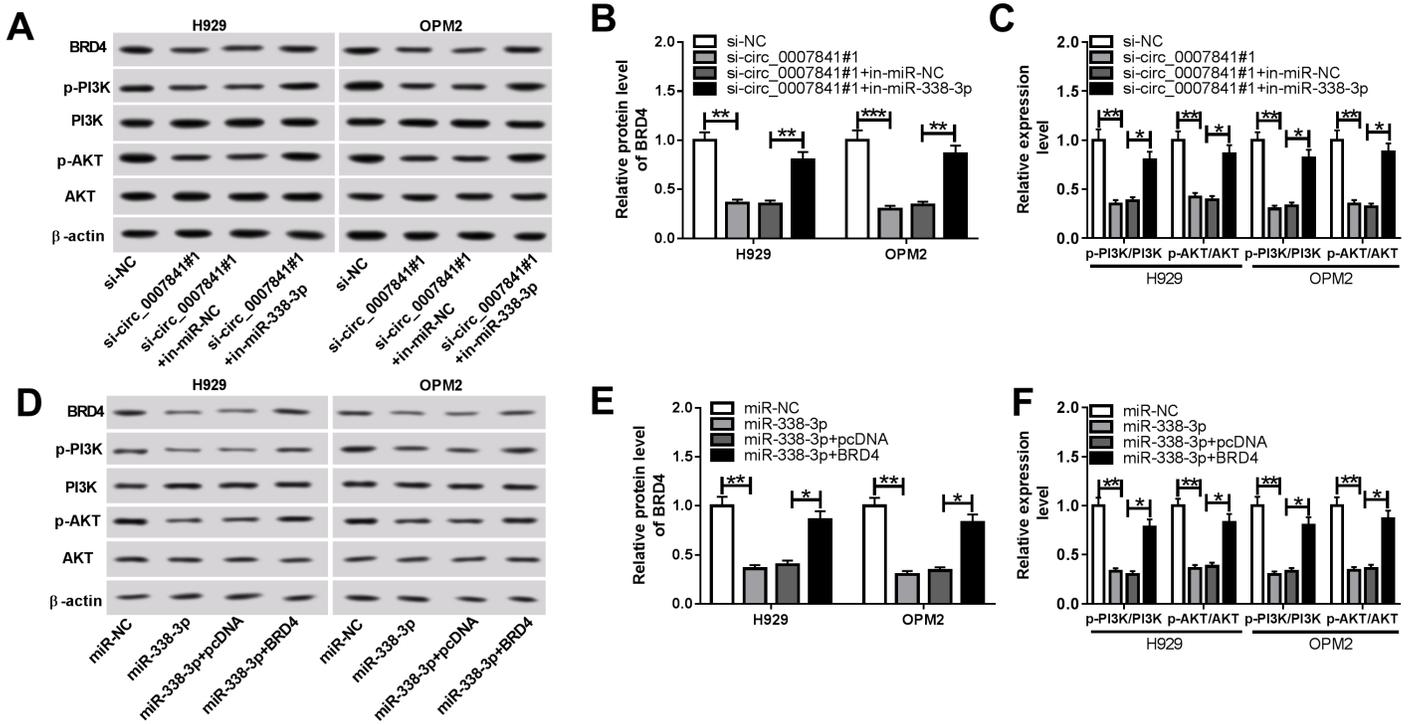


Figure 6

The activation of PI3K/AKT signal pathway is modulated by circ_0007841/miR-338-3p/BRD4 axis. (A-C) Western blot assay was performed to detect the levels of BRD4 and PI3K/AKT signaling-related proteins in MM cells transfected with si-NC, si-circ_0007841#1, si-circ_0007841#1 + in-miR-NC or si-circ_0007841#1 + in-miR-338-3p, and gray analysis was used to assess the abundance of these proteins. (D-F) The expression of BRD4 and PI3K/AKT signaling-associated proteins in MM cells transfected with miR-NC, miR-338-3p, miR-338-3p + pcDNA or miR-338-3p + BRD4 was examined by Western blot assay. *P<0.05, **P<0.01, ***P<0.001.

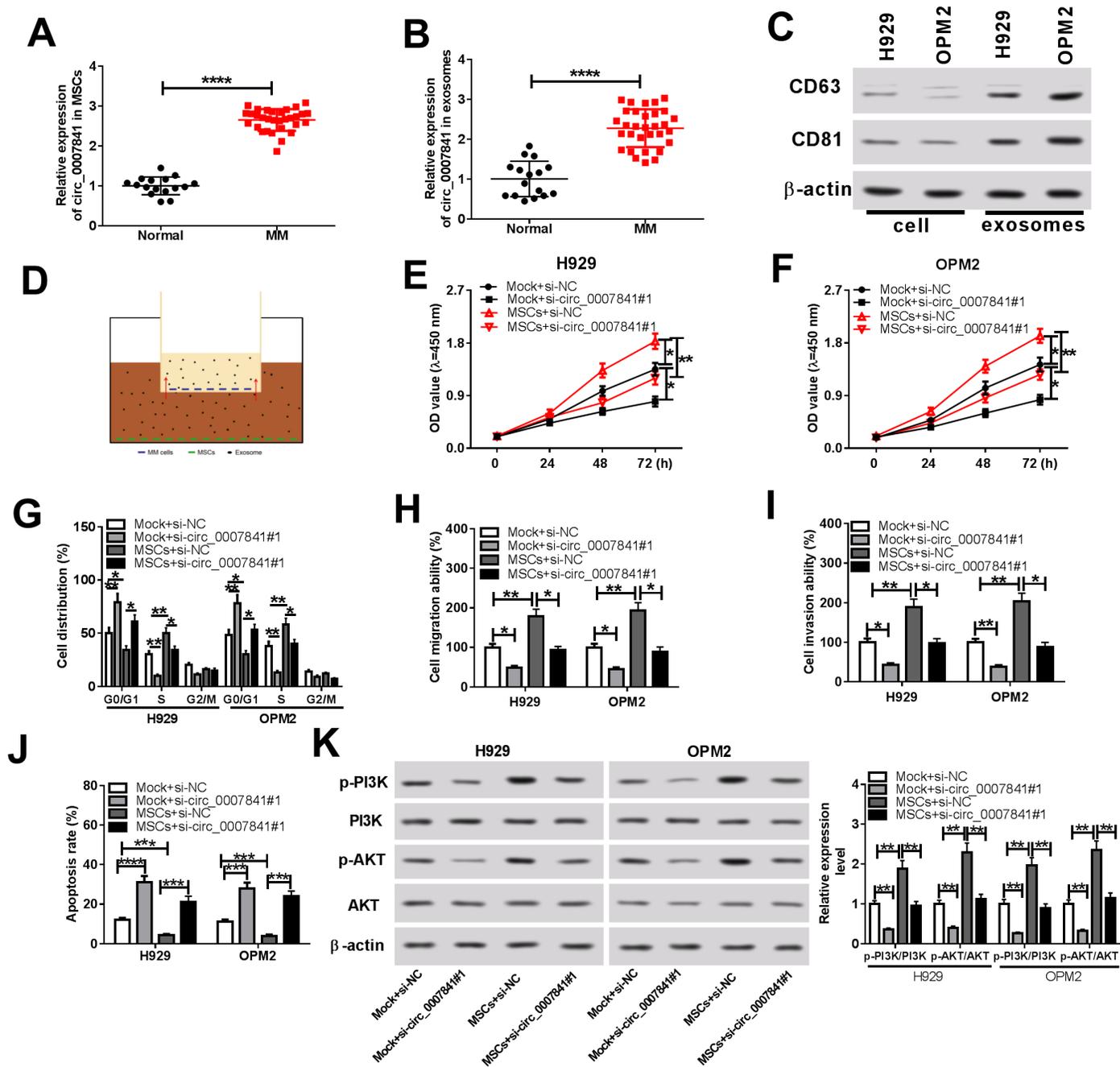


Figure 7

MSCs-generated exosomes accelerate the malignant potential of MM cells via circ_0007841. (A and B) The expression of circ_0007841 was detected in the MSCs and MSCs-generated exosomes from the adjacent tissues of MM and normal tissues by qRT-PCR. (C) Western blot assay was performed to detect the protein levels of exosome-related markers, including CD63 and CD81, in cell lysate and exosomes. (D) The model showed that MM cells were co-cultured with MSCs, and only exosomes could move from the lower chambers to the upper chambers. (E-K) MM cells transfected with si-NC or si-circ_0007841#1 were co-cultured with MSCs or not. (E and F) CCK8 assay was performed to assess the proliferation of MM cells. (G) The cell cycle of MM cells was detected through conducting flow cytometry. (H and I) The

abilities of migration and invasion of MM cells were assessed by transwell assays. (J) The apoptosis of MM cells was examined through performing flow cytometry. (K) The levels of p-PI3K, PI3K, p-AKT and AKT were detected in MM cells by Western blot assay. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.