The miR4261/PAMM axis in multiple myeloma promotes bone resorption

Contents

Supplementary Table 1.

Table 1. The clinical characteristics of multiple myeloma patients recruited in this study.

Characteristic		MBD (n=27)	N-MBD (n=11)
Median age (range) - years		50-76（62）	53-75（65）
Male sex - number		20	6
Female sex - number		7	5
Types of Multiple Myeloma	IgG-λ	9	3
	IgG-κ	6	1
	IgA-λ	4	
	IgA-κ	2	
	IgD-λ	1	1
	IgD-κ		
	λ	1	2
	κ	2	3
	Non-secretory	2	1
D-S system	I	2	1
	II	4	2
	III	21	8
ISS system	I	2	1
	II	7	2
	III	18	8
Median C-BM-PCs (range) - %		15-92（42）	10-45（24）
Median Hb (range) - g/L		63-165（103）	86-143（120）
Median ALB (range) - g/L		17-46（33）	18-44（36.2）
Median sβ-2MG (range) - mg/L		1.2-24.8（9.09）	1.4-21.4（6.4）
Median sCa (range) – mmol/L		1.86-3.64（2.54）	1.91-2.5（2.25）

MMBD:myeloma bone disease ,N-MMBD:on-myeloma bone disease ,C-BM-PCs: Clonal Bone Marrow Plasma Cells, Hb: Hemoglobin, ALB: Albumin, sCa: Serum Calcium, sβ-2MG: Serum β-2 Microglobulin, D-S system: Durie-Salmon System, ISS system: International Staging System.

Supplementary Table 2.

Table 2. The gene-specific primers used in this study.		
Name	**sequence（5’-3’）**	
For RT-qPCR		
Human Actin	F	CACCATTGGCAATGAGCGGTTCC
	R	GTAGTTTCGTGGATGCCACAGG
Human PAMM	F	ATAGACCTGAAAACACTGGA
	R	GCAGCTTCCTCTCGACAGAG
hsa-miR-4261	F	TGCTGGACGAGGAAACAGGG
	R	TATGGTTGTTCACGAGTCCTTGTC
u6 snRNA	F	CGCTTCGGCAGCACATATAC
	R	TTCACGAATTTGCGTGTCATC
For 3’-UTR of PAMM		
PAMM(WT)	F	TCGAGTTATTGTTTCCAAGGGCATGC
	R	CAATAACAAAGGTTCCCGTACGCCGG
PAMM（MT）	F	TCGAGTTATCTCAGATAAGGGCATGC
	R	CAATAGAGTCTATTCCCGTACGCCGG
The mutant sequences are shown in the boxes. MT, mutant.		

Supplementary Table 3.

Table 3. The sequences of shRNA, mimics in this study.		
name		sequence（5’-3’）
scrambled		GCTTCGCGCCGTAGTCTTA
shPAMM#1		GGAGACAAAGTAAACCTACTT
shPAMM#2		GGAGTGAGAAACCCATTTATA
For has-miR-61 mimics overexpression		
has-miR-61 mimics	sense	AGGAAACAGGGACCCA
	antisense	GGUCCCUGUUUCCUUU
Negative control	sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT

Supplementary Figure 1.

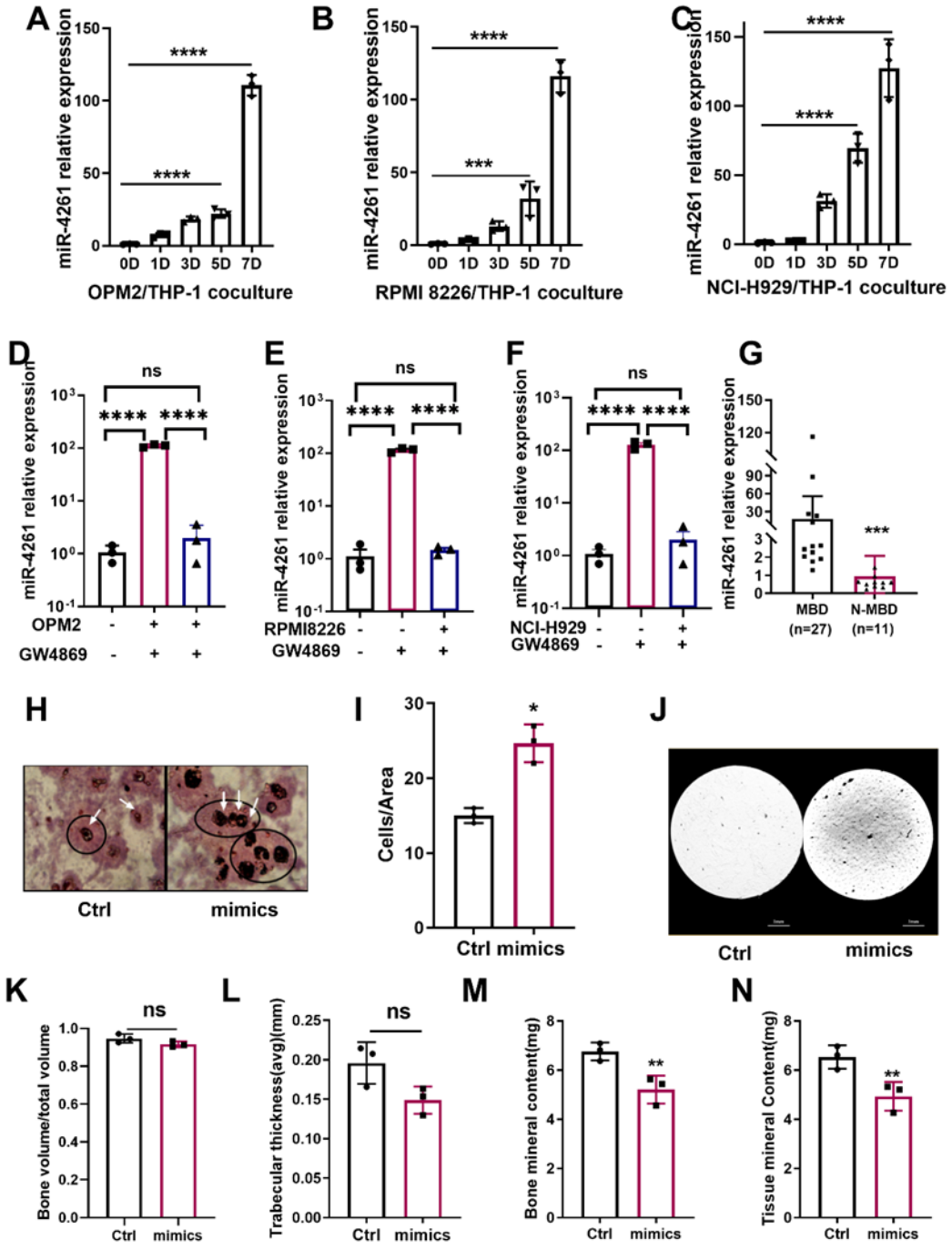


Figure 1. miR-4261 delivered by exsomes from MM cells promotes osteoclasts differentiation and causes bone resorption.

(A-C) In co-culture systems of OPM2/THP-1, RPMI 8226/THP-1, and NCI-H929/THP-1, THP-1 cells were subjected to RNA extraction, and miR-4261 expression over the duration of co-culture (up to 7 days) was quantified by RT–qPCR.(D-F) Relative expression of miR-4261 in THP-1 cells under various culture conditions, compared with untreated THP-1 cells. Conditions include: THP-1 cells cultured alone, co-culture of myeloma cells with THP-1 cells, and co-culture of myeloma cells with THP-1 cells in the presence of 4 μM exosome inhibitor GW4869. (G)The

expression of miR-4261 in myeloma bone disease（MBD）roup (n = 27) compared with the non-myeloma bone disease（N-MBD）group (n =11) was measured by RT–qPCR. (H) miR-4261 mimics and control vectors were delivered to THP-1 cells in the presence of both M-CSF and RANKL (100 ng/ml), seven days later, TRAP staining was performed. The typical photos are displayed. Multinucleated cells (≥3 nuclei per cell) with wine-red cytoplasm (TRAP-positive) are visible in the images. Cells are indicated by black circles, and nuclei are marked by white arrows (40× objective magnification).(I)The number of osteoclasts in the miR-4261 mimics-overexpressed and control groups was quantified and compared.(J) Representative Micro-CT reconstruction images of the miR-4261 mimics-overexpressing group and the control group. (K-N)The miR-4261 mimics-overexpressed and control groups were subjected to Micro-CT analysis. Original micro-CT images;BV/TV, bone volume/total volume; Tb.Th, trabecular thickness; BMC (g/cm^3), bone mineral content; TMC (g/cm^3), tissue mineral Content. Data are presented as the mean ± SD from more than 3 biological replicates. Student's t test was used to estimate the statistical significance. miR-4261 expression in MBD (n=27) vs non-MBD (n=11) groups. Significant difference was detected by Mann-Whitney U test ($P < 0.001$) despite unbalanced sample sizes.*$p<0.05$, **$p<0.01$, ***$p<0.001$,****$p<0.0001$.

Supplementary Figure 2.

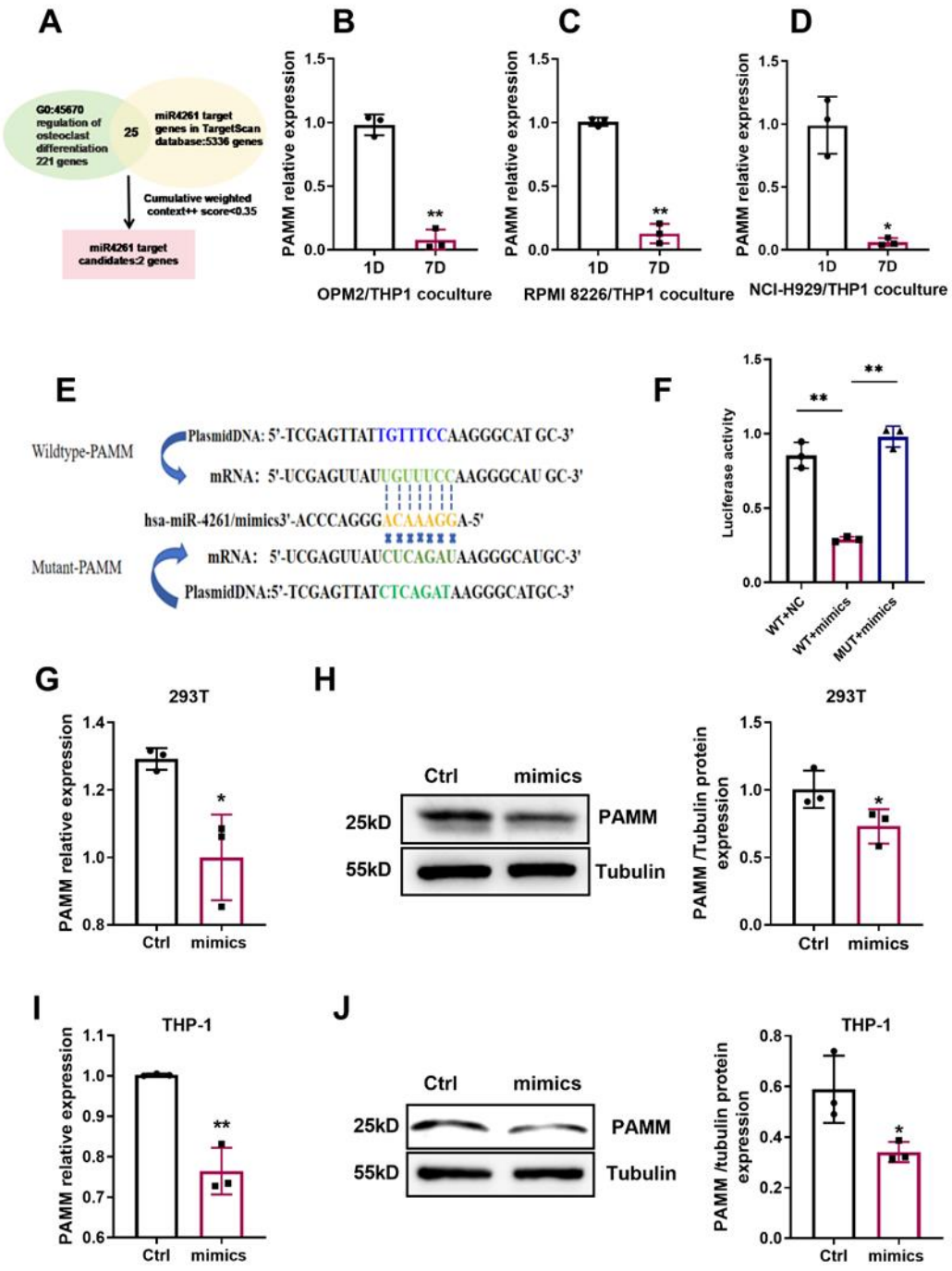
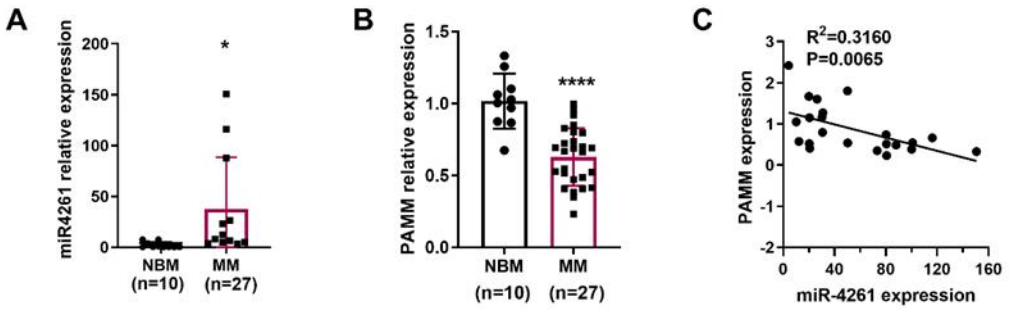


Figure 2. miR-4261 directly regulates PAMM expression in THP-1 cells

(A)A cartoon illustrates the screening process for miR-4261 targets in this study. The TargetScan database predicted 5,336 genes as putative targets of miR-4261. The Gene Ontology database identified 221 genes associated with regulated osteoclast differentiation (GO:45670). The intersection of these two datasets yielded 25 candidate genes, with 2 candidates selected under more stringent criteria. (B-D). In co-culture systems of OPM2/THP-1, RPMI 8226/THP-1, and NCI-H929/THP-1, THP-1 cells were subjected to RNA extraction, and PAMM expression was quantified by RT–qPCR. (E) Schematic diagram

depicting the predicted miR-4261 binding site in the 3'UTR of PAMM mRNA. (F). Dual luciferase reporter assay. The 3'UTR fragments (wild-type and mutant sequences) of PAMM were cloned into the psiCHECK2 vector and co-transfected with miR-4261 mimics or negative control (NC). Relative firefly/Renilla luciferase activity was compared among the wild-type (WT), mutant (MUT) and negative control (NC) groups. (G-H)PAMM expression in miR-4261-overexpressed and control 293T cells was analyzed by RT–qPCR and Western blot. (I-J)PAMM expression in miR-4261-overexpressed and control THP-1 cells was analyzed by RT–qPCR and Western blot.

Supplementary Figure 3.



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The correlation analysis of PAMM expression levels with clinical characteristics in MBD

	PAMM high expression (n=11)	PAMM low expression (n=16)	P value
Age	63.1	64.6	
Gender (Male: Female)	9:2	11:5	
Bone marrow plasma cells (%)	25.6	40.3	NS
Hb (g/L)	106	104	NS
ALB (g/L)	34.1	33.6	NS
Ca (mmol/L)	2.38	2.67	0.0032

Note: ALB: Serum albumin; Hb: hemoglobin;MBD:myeloma bone disease

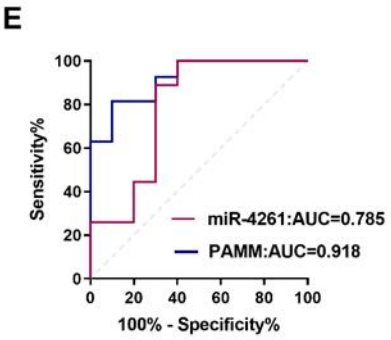

Figure 3. PAMM is negatively correlated with miR-4261 and PAMM low expression is associated with Hypercalcemia in MM patients at diagnosis.

(A–B) The bone marrow mononuclear cells from healthy donors (NBM, n=10) and multiple myeloma patients (MM, n=27) were collected and subjected to RT–qPCR analyses of miR-4261 and PAMM expression. (C) The correlation between miR-4261 and PAMM was also analyzed. (D) Clinical characteristics of MM patients with high PAMM expression (n=11) and low PAMM expression (n=16) were analyzed. Calcium ion levels in PAMM-high and PAMM-low groups were compared. (E) Receiver operating characteristic (ROC) curve analysis was performed on the miR-4261 and PAMM expression data from 27 MM patients and 10 healthy controls.

Supplementary Figure 4.

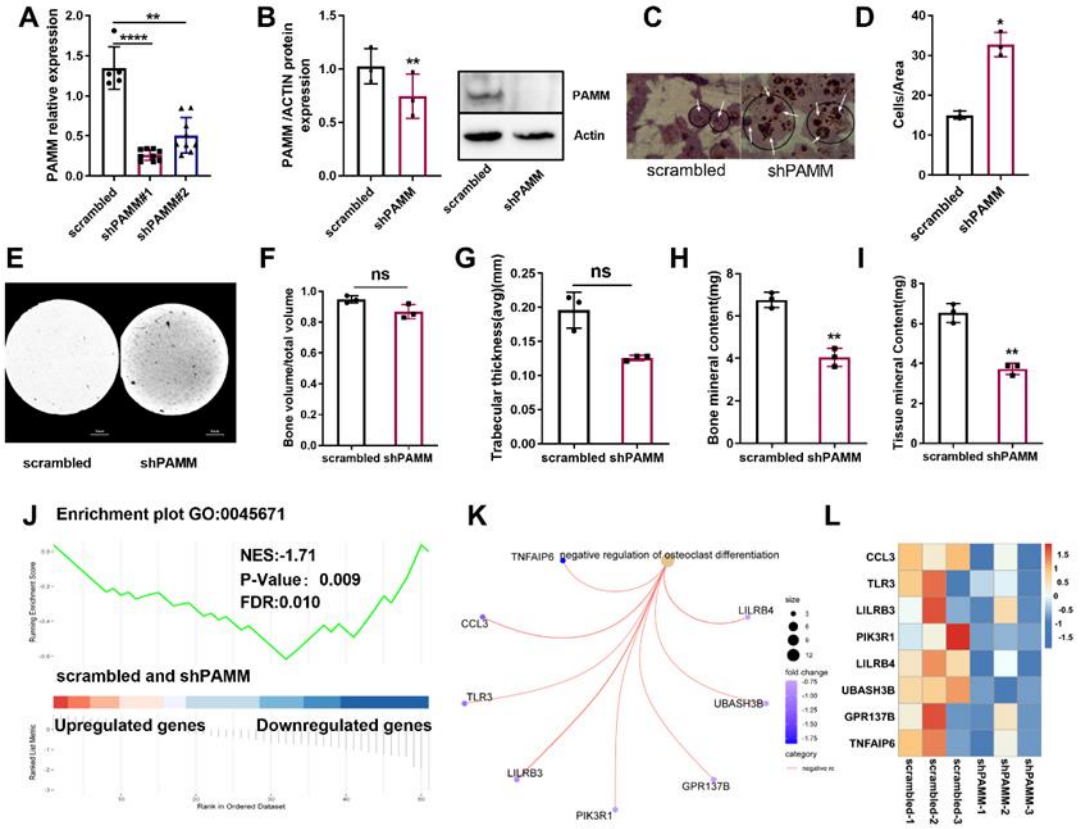

Figure4. PAMM silencing enhances THP-1 Cell differentiation to osteoclast and Enhances Osteoclast Activity.

(A) THP-1 cells were transduced by shPAMM#1, shPAMM#2, and control (scrambled) vectors, and PAMM expression was evaluated by RT–qPCR. (B) PAMM protein expression in shPAMM and scrambled THP-1 cells was assessed by Western blot. (C) Both shPAMM and scrambled cells were analyzed by TRAP staining after 1 week of differentiation, and typical photos are displayed. (D) The osteoclasts were quantified in shPAMM and control THP-1 cells. (E) Representative examples of Micro-CT reconstructed images from the shPAMM group and scrambled group. (F-I) Micro-CT analysis demonstrated that the shPAMM group exhibited significantly lower BMC, and TMC compared to the control group, with enhanced bone resorption. (J-K) Eukaryotic reference-based transcriptome analysis and GO-GSEA revealed significant downregulation of genes associated with negative regulation of osteoclast differentiation (e.g., CCL3, TLR3, LILRB3, PIK3R1, LILRB4, UBASH3B, GPR137B, and TNFAIP6) in shPAMM-treated THP-1 cells, with TNFAIP6 exhibiting the most pronounced downregulation (NES = -1.71, P = 0.009, FDR = 0.010). (L) Heatmap of differentially expressed genes in ShPAMM THP-1 cells. RNA-seq data comparing shPAMM with scrambled controls in THP-1 cells (n=3). Color scale represents expression changes ranging from downregulation (blue) to upregulation (red).

Supplementary Figure 5.

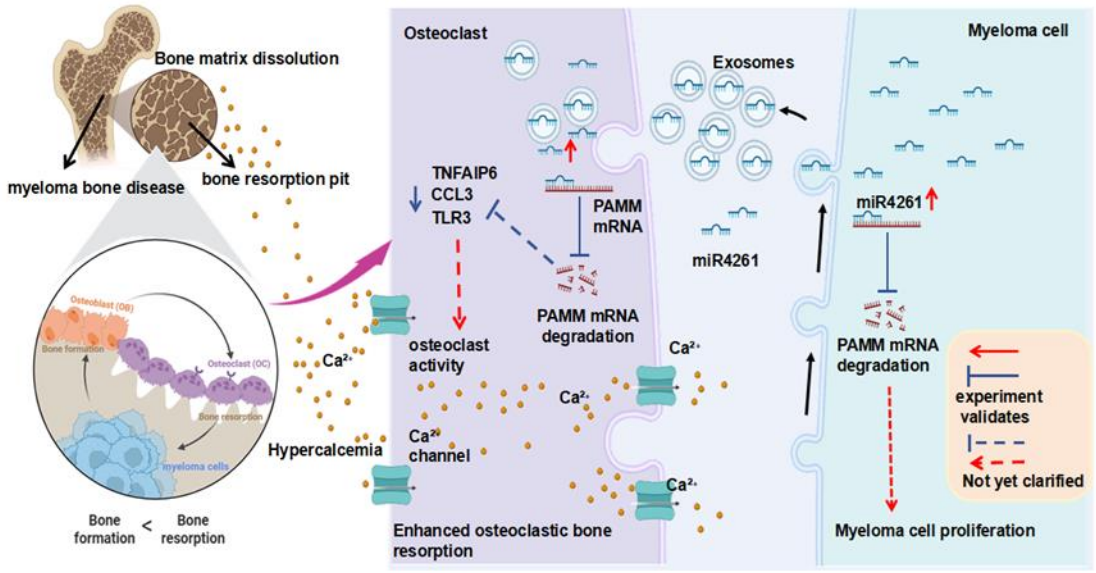

Figure 5. Targeting miR-4261 or PAMM represents an optional strategy to alleviate clinical symptoms in MBD

PAMM has been identified as a bona fide target of miR-4261. Downregulation of PAMM protein expression relieves its negative regulation on osteoclast differentiation-related genes (e.g., TNFAIP6, CCL3, TLR3), thereby enhancing osteoclast differentiation and activity. This leads to increased bone resorption, manifesting as bone matrix dissolution and resorption pit formation. The imbalance where bone resorption exceeds bone formation ultimately promotes the development of MBD and hypercalcemia.