

Supplementary Material

Title: MAGL blockade alleviates steroid-induced femoral head osteonecrosis by reprogramming BMSC fate in rat

Running title: MAGL reprograms BMSC fate

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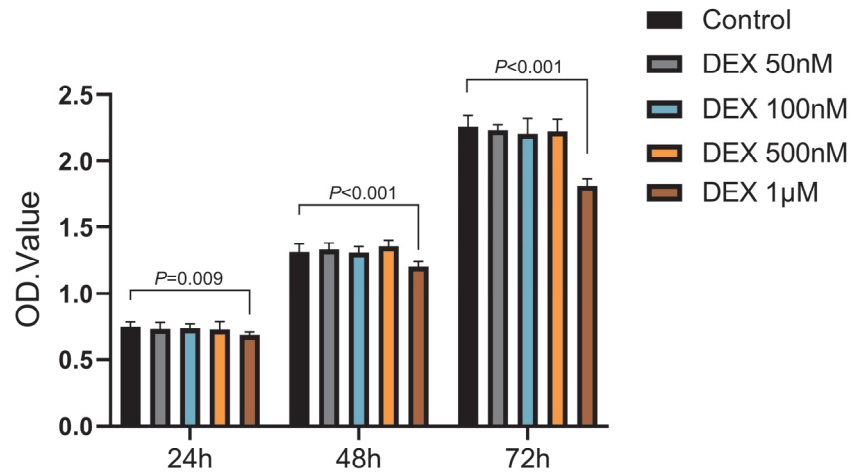
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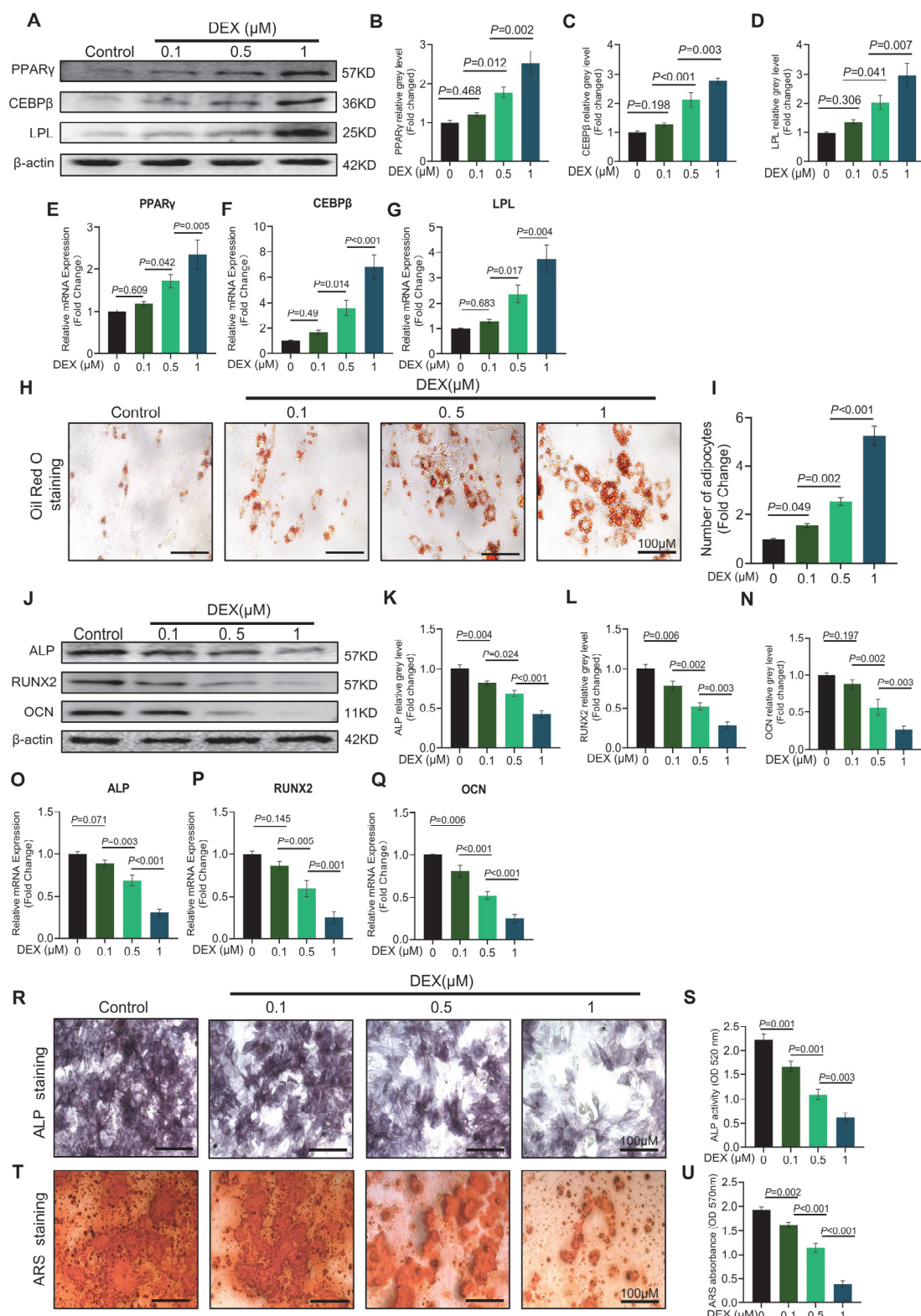
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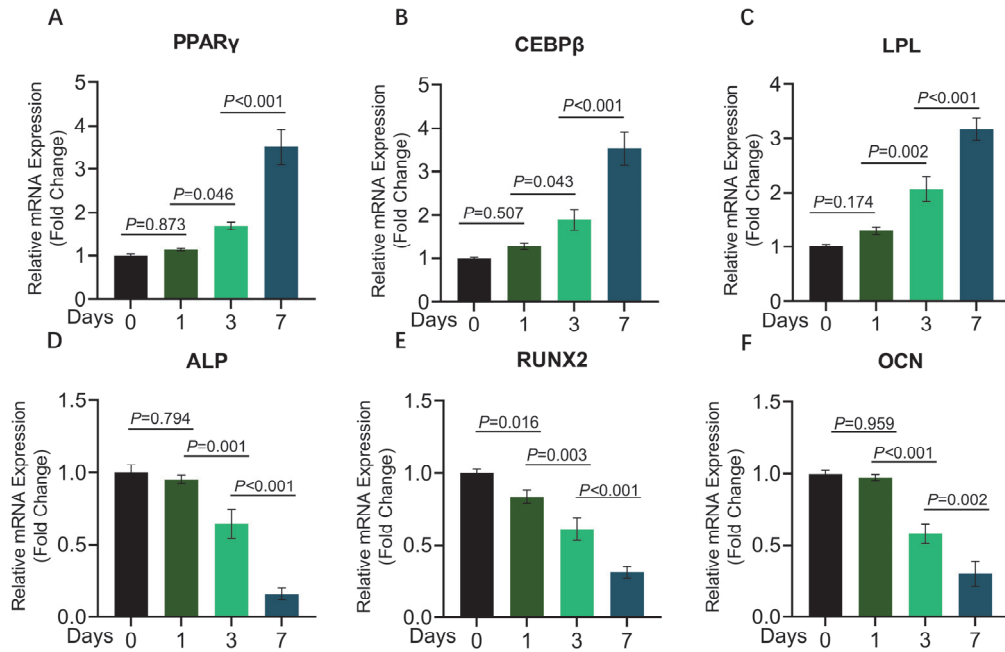


Supplemental Figure 1: DEX is toxic to BMSCs at concentrations $\geq 1 \mu\text{M}$. (A) The cytotoxicity of DEX was assessed in BMSCs using a CCK-8 assay. Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.

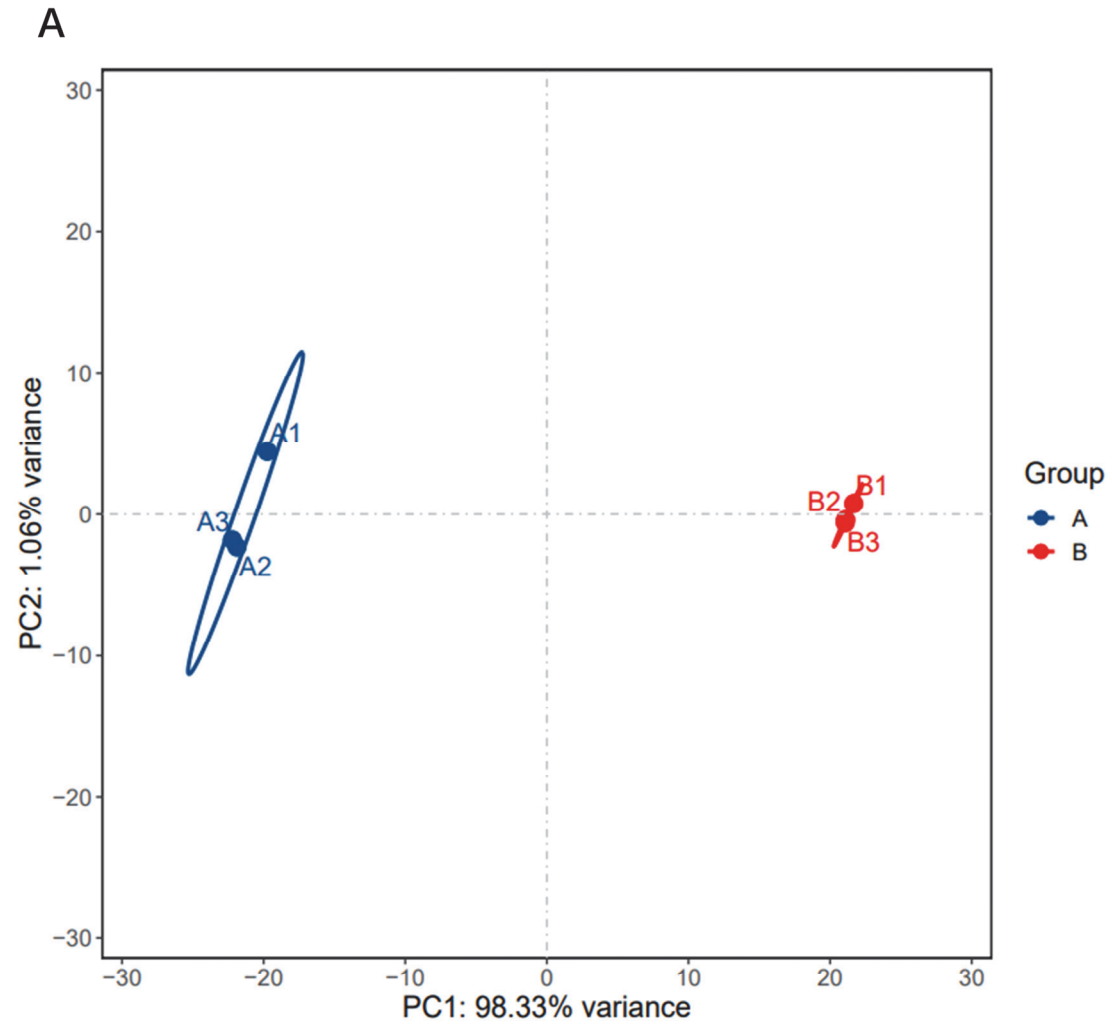


Supplemental Figure 2: DEX promotes BMSC adipogenic differentiation and inhibits BMSC osteogenic differentiation. (A-D) BMSCs were stimulated with various concentrations of DEX for 7 days, and the protein expression levels of PPAR γ , CEBP β , and LPL were analyzed by western blotting. (E-G) BMSCs were stimulated with various concentrations of DEX for 3 days, and the gene expression levels of PPAR γ , CEBP β , and

LPL were analyzed by RT-PCR. (H) Oil-red O staining was performed to test the correlation between different concentrations of DEX and the level of adipogenic differentiation. (I) Quantitative analysis of the number of adipocytes in (H). (J-N) BMSCs were stimulated with various concentrations of DEX for 7 days, and the protein expression levels of ALP and RUNX2 were analyzed by western blotting. OCN expression was evaluated on day 14. (O-Q) BMSCs were stimulated with various concentrations of DEX for 7 days, and the gene expression levels of ALP, RUNX2, and OCN were analyzed by RT-PCR. (R-S) ALP staining and ALP activity detection were performed to test the correlation between different concentrations of DEX and the level of osteogenic differentiation. (T-U) Alizarin red staining and ARS absorbance detection were performed to test the correlation between different concentrations of DEX and the level of osteogenic differentiation. Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.

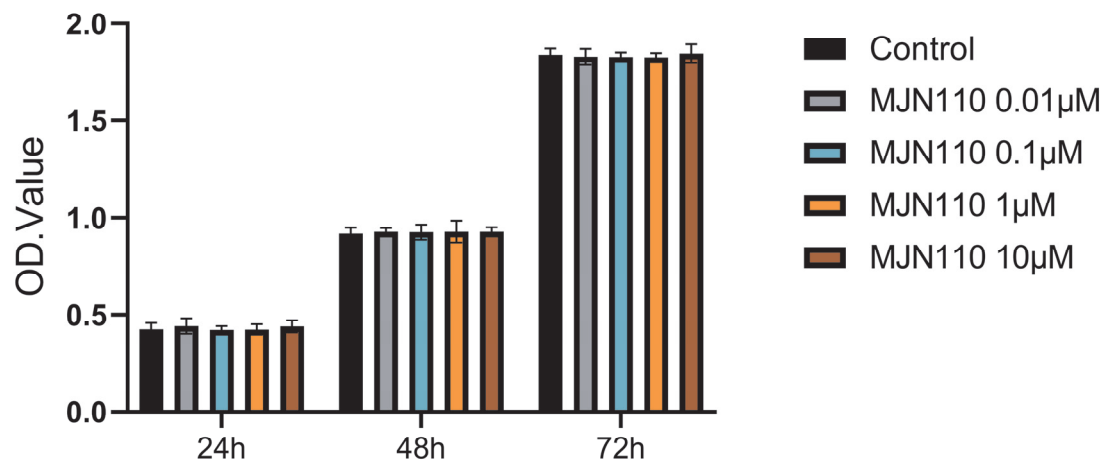


Supplemental Figure 3: DEX-induced osteogenic-related markers decrease, but adipogenic-related markers increase over time in BMSCs. (A-C) BMSCs were stimulated with DEX (1 μ M) for 0 days, 1 day, 3 days, or 7 days, and the expression levels of PPAR γ , CEBP β , and LPL were analyzed by RT-PCR. (D-F) BMSCs were stimulated with DEX (1 μ M) for 0 days, 3 days, 7 days, or 14 days, and the expression levels of ALP, RUNX2, and OCN were analyzed by RT-PCR. Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.

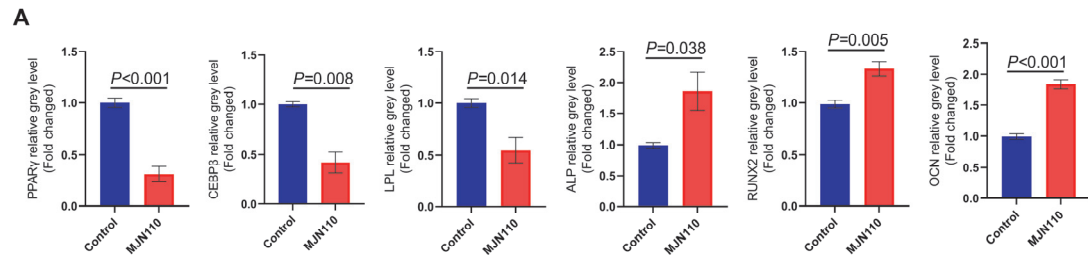


Supplemental Figure 4: Principal component analysis (PCA) of the RNA-seq data. (A) According to the PCA of the RNA-seq data, A group (shNC group) or B group (shMAGL-treated group), BMSCs were clustered and explained 98.33% of the observed variation.

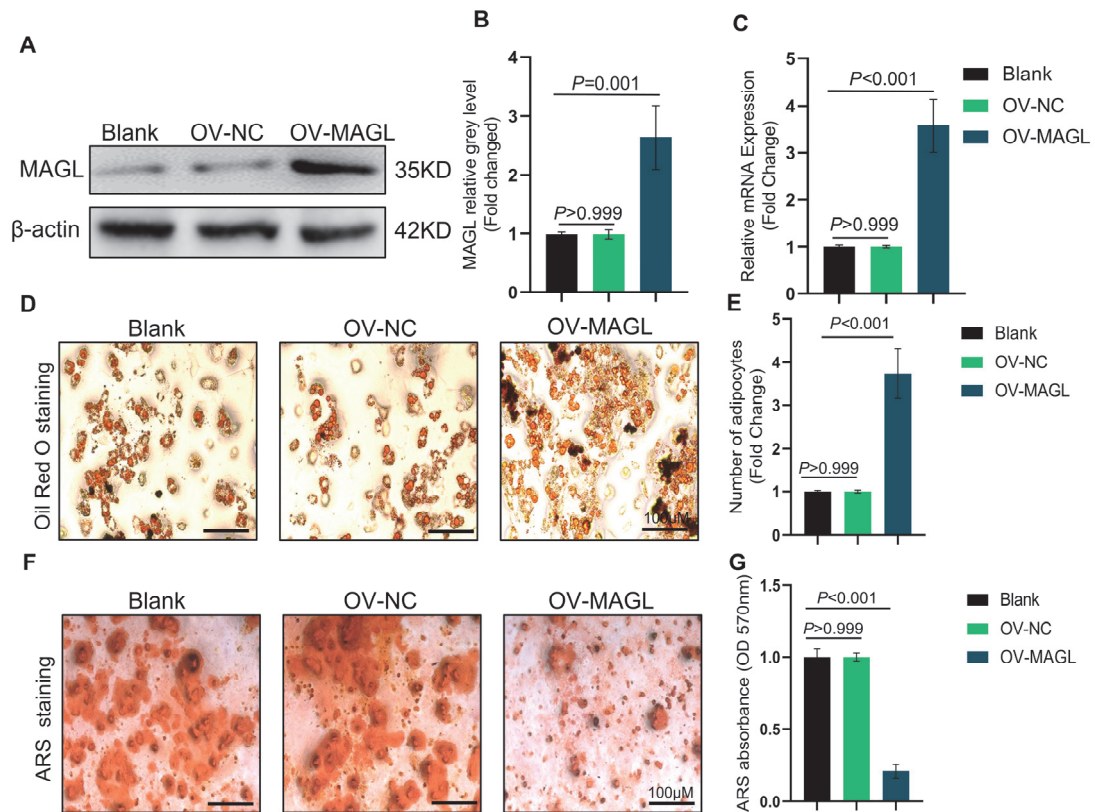
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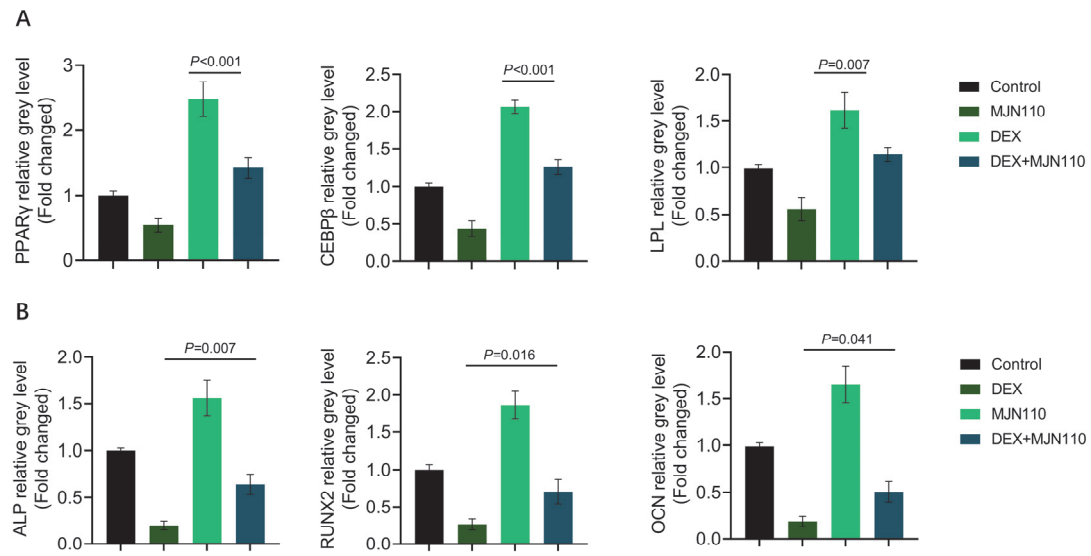
Supplemental Figure 5: MJN110 is not cytotoxic in BMSCs. (A) The cytotoxicity of MJN110 was assessed in BMSCs using a CCK-8 assay. Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.



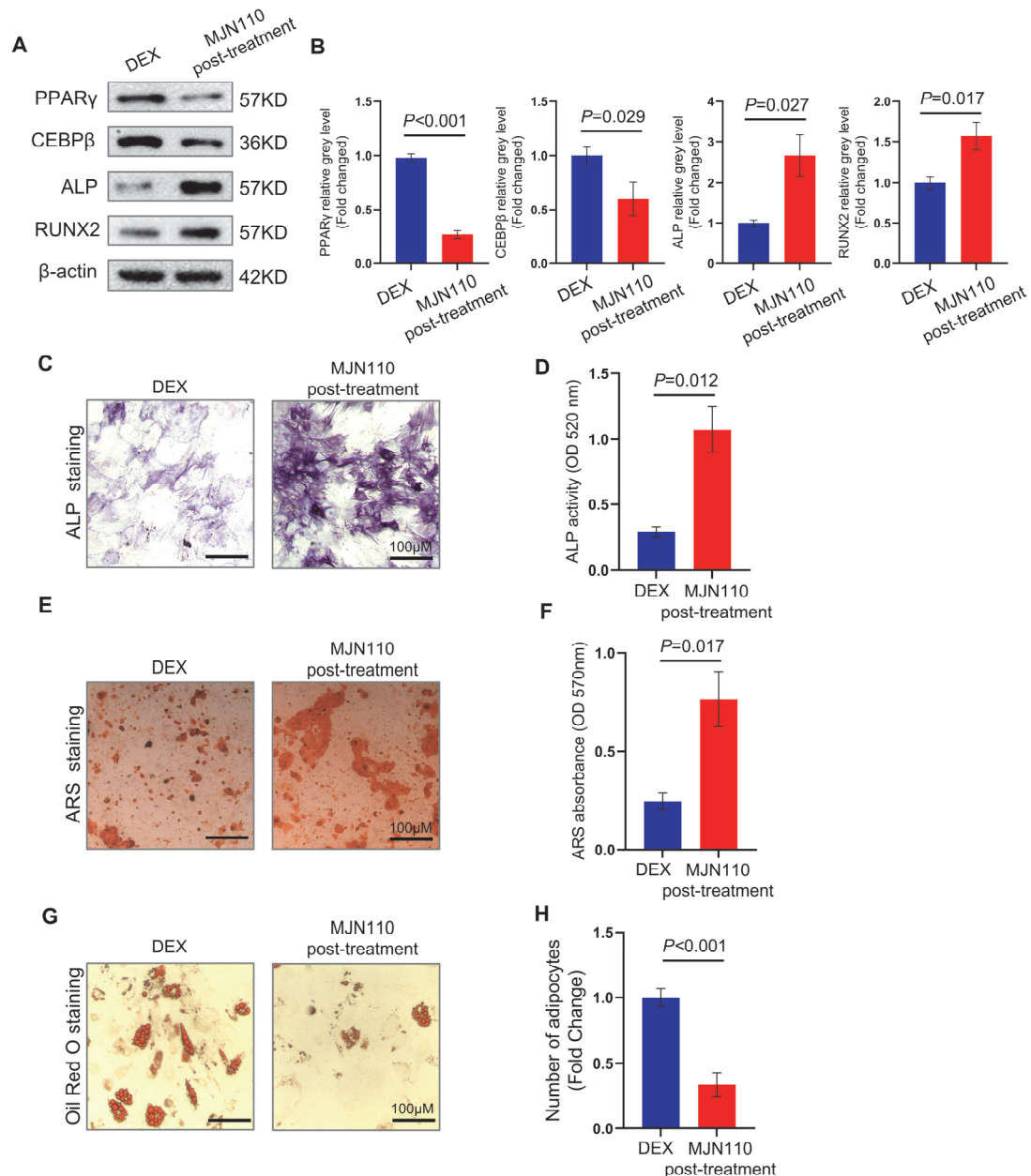
Supplemental Figure 6: (A) Quantitative analysis of the grayscale values of Western Blot bands in Figure2(M and N). Data represent the mean $\pm$ S.D. (n=3).



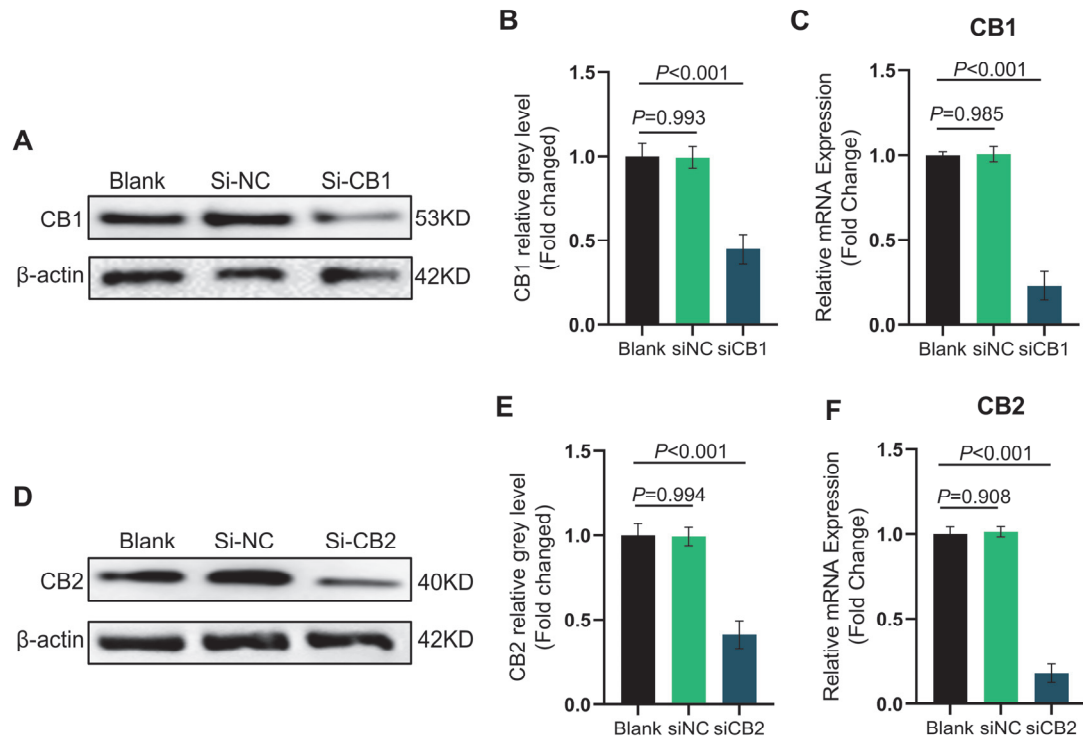
Supplemental Figure 7: MAGL overexpression promotes the adipogenic differentiation of BMSCs and inhibits their osteogenic differentiation. (A-C) The expression of MAGL was increased in the OV-MAGL group compared with the blank group and the negative control (OV-NC) group. (D-E) Osteogenic differentiation was confirmed by Alizarin red staining and ARS absorbance detection on day 14. (F) Oil-red O staining was performed on day 14 of adipogenic differentiation. (G) Quantitative analysis of the number of adipocytes in (F). Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.



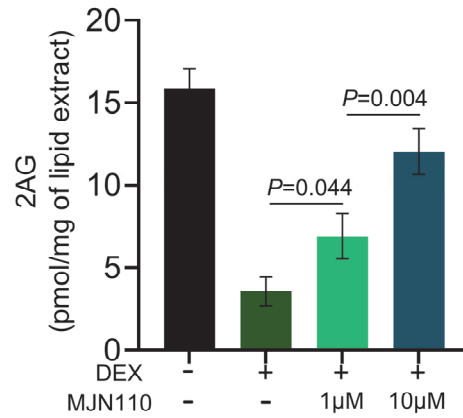
Supplemental Figure 8: (A-B) Quantitative analysis of the grayscale values of Western Blot bands in Figure3 (A and G). Data represent the mean $\pm$ S.D. (n=3).



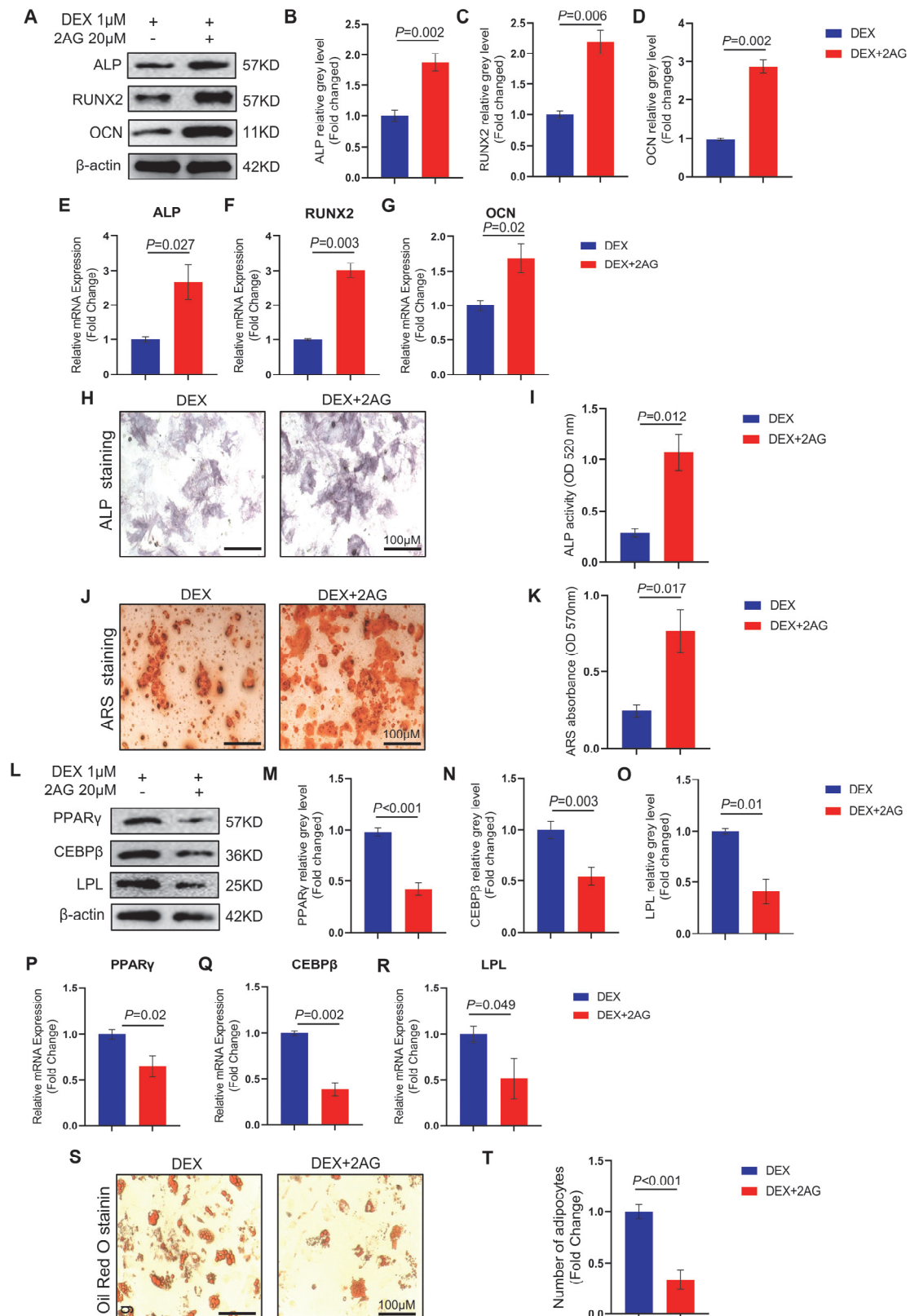
Supplemental Figure 9: Post-treatment with MJN110 still reverses the effect of DEX on the direction of BMSC differentiation. (A-B) Western blot analyses of the expression of osteogenic marker genes. In the MJN110 post-treatment group, BMSCs were pretreated with DEX (1 μ M) for 24 h. (C-D) ALP staining and ALP activity detection were performed on day 7 of osteogenic differentiation. (E-F) Osteogenic differentiation was confirmed by Alizarin red staining and ARS absorbance detection on day 14. (G) Oil-red O staining was performed on day 14 of adipogenic differentiation. (H) Quantitative analysis of the number of adipocytes in (G). Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.



Supplemental Figure 10: Knockdown of CB1 and CB2 by siRNA oligos decreased the expression of CB1 and CB2 in BMSCs. (A-C) The expression of CB1 was knocked down by siRNA (si-CB1 group) compared with the blank and negative control (si-NC) groups. (D-F) The expression of CB2 was knocked down by siRNA (si-CB2 group) compared with the blank and negative control (si-NC) groups. Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.

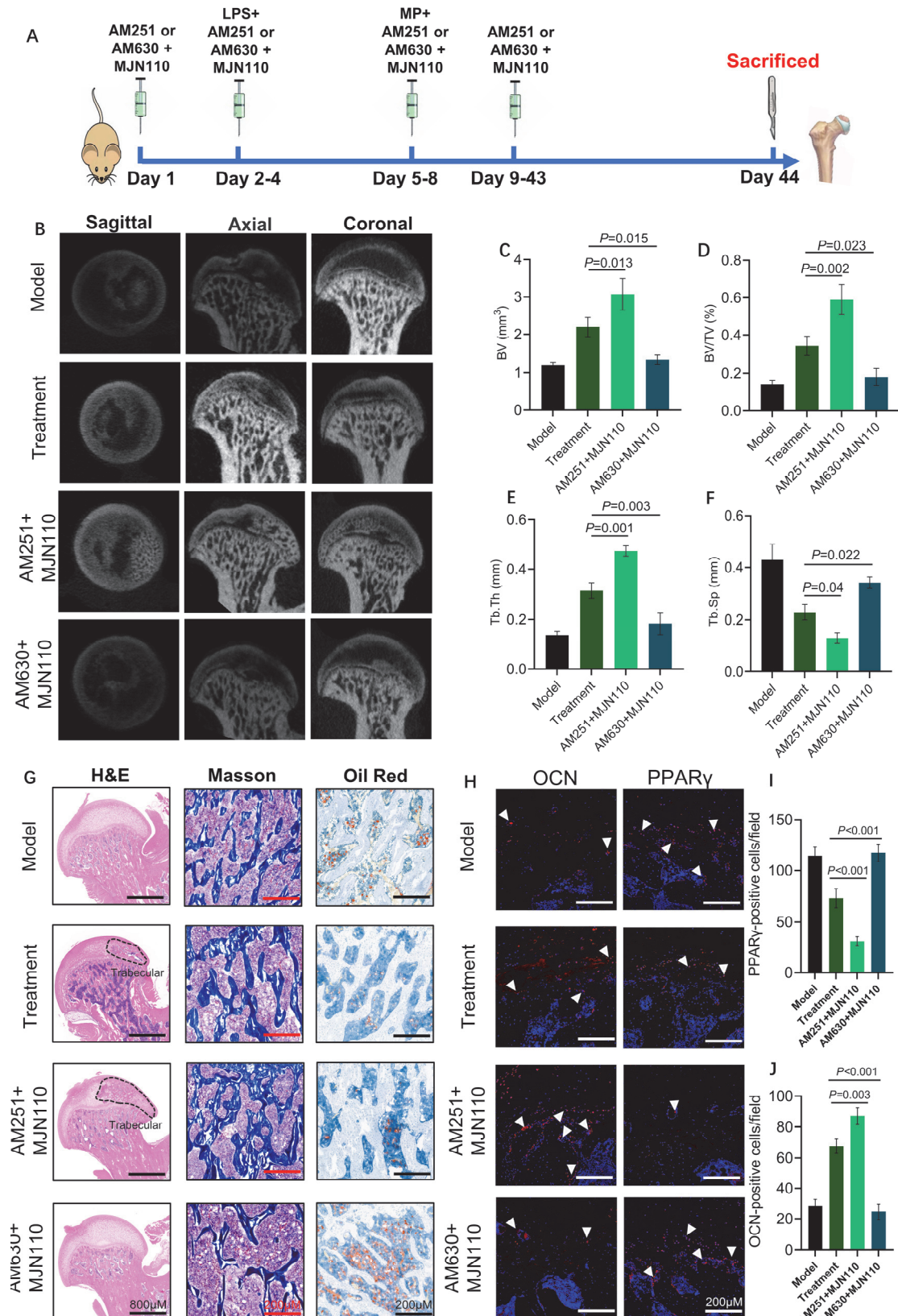


Supplemental Figure 11: The detection of the intracellular content of 2AG in the BMSCs. BMSCs were incubated with a medium containing various components; lipids were extracted from chloroform from cells; 2-AG was then quantified by LC/MS. Data represent the mean $\pm$ S.D. These studies were performed with at least 3 biological replicates.



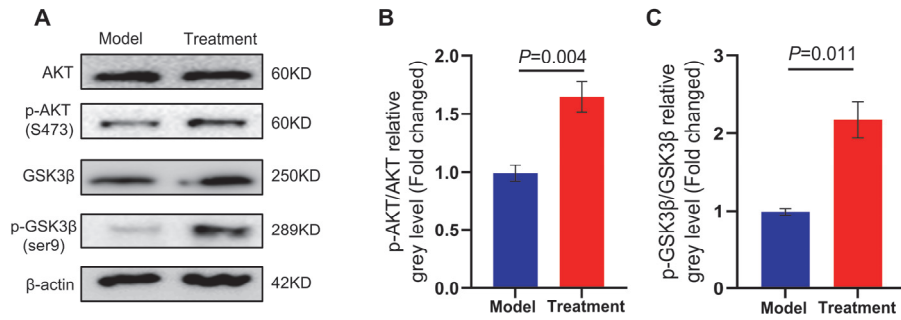
Supplemental Figure 12: 2AG can mimic the efficacy of MAGL inhibitors and partially reverse the effect of DEX on the direction of BMSC differentiation. (A-G) Western blot and RT-PCR analyses of the expression of osteogenic marker genes. In the DEX+2AG group, BMSCs were pretreated with 2AG (20 μ M) for 48 h. (H-I) ALP staining and ALP

activity detection were performed on day 7 of osteogenic differentiation. (J-K) Osteogenic differentiation was confirmed by Alizarin red staining and ARS absorbance detection on day 14. (L-R) Western blot and RT-PCR analyses of the expression of adipogenic marker genes. In the DEX+2AG group, BMSCs were pretreated with 2AG (20 μ M) for 48 h. (S) Oil-red O staining was performed on day 14 of adipogenic differentiation. (T) Quantitative analysis of the number of adipocytes in (S). Data represent the mean $\pm$ S.D. (n=3). These studies were performed with at least 3 biological replicates.

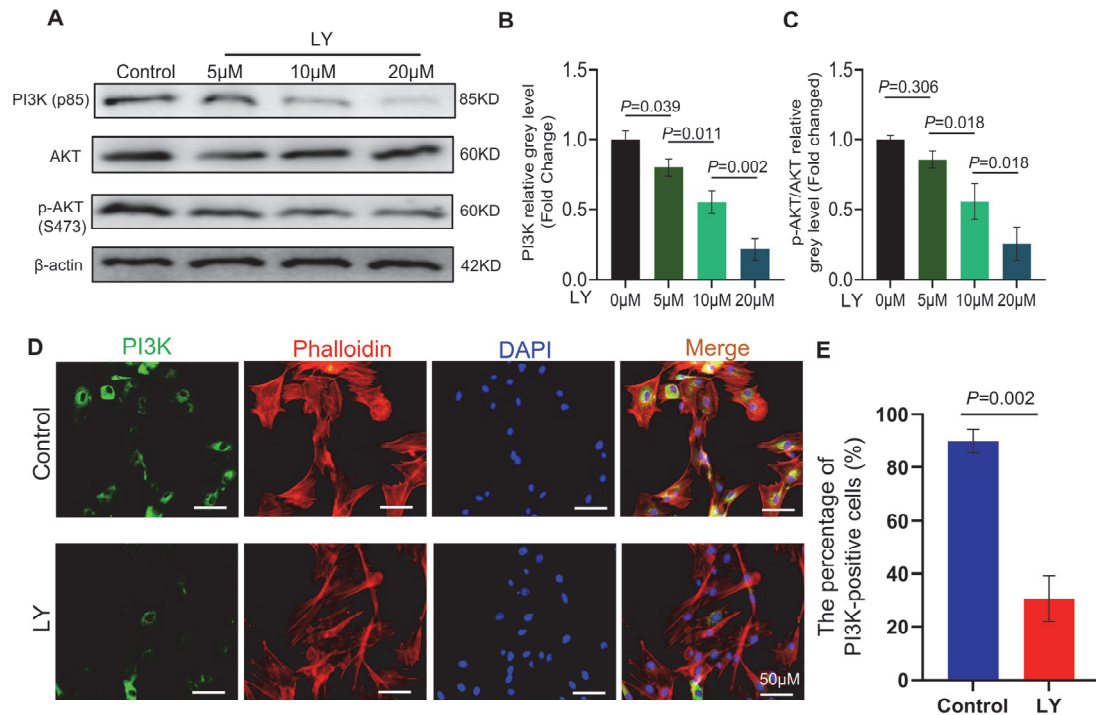


Supplemental Figure 13: The CB2 receptor, rather than the CB1 receptor, partially mediates the protective effect of MAGL blockade on GC-induced ONFH. (A) The timeline of the GC-induced ONFH model and administration of MJN110, AM251, and AM630 in vivo. (B) Images of micro-CT. (C) BV (mm³) (D) BV/TV (%). (E) Tb. Th (mm). (F)

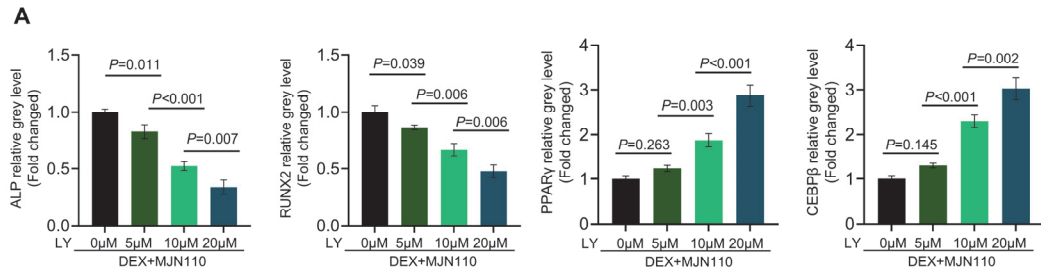
Tb. Sp (mm). (G) H&E staining, Masson staining, and Oil Red O staining of bone tissues. The lipid droplets are marked with black arrows. (H) IF staining of PPAR γ and OCN. The IF-positive cells are marked with white arrows. (I-J) Quantitative analysis of the number of IF-positive cells in (H). Data represent the mean $\pm$ S.D. (n=5). All in vitro and in vivo studies were performed with at least 3 biological replicates.



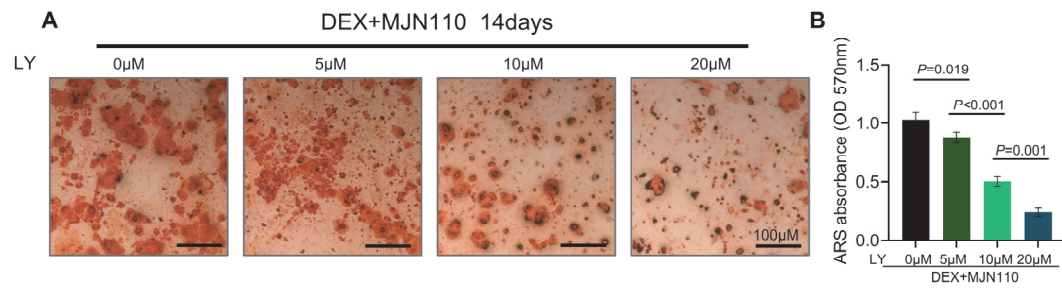
Supplemental Figure 14: MAGL inhibition enhances the protein expression of p-AKT and p-GSK3β in bone tissue. (A-C) Western blot analysis was performed to detect the expression levels of PI3K/AKT/GSK3β signaling pathway-related proteins in the bone tissue of the model group and the treatment group. Data represent mean $\pm$ S.D. (n=3).



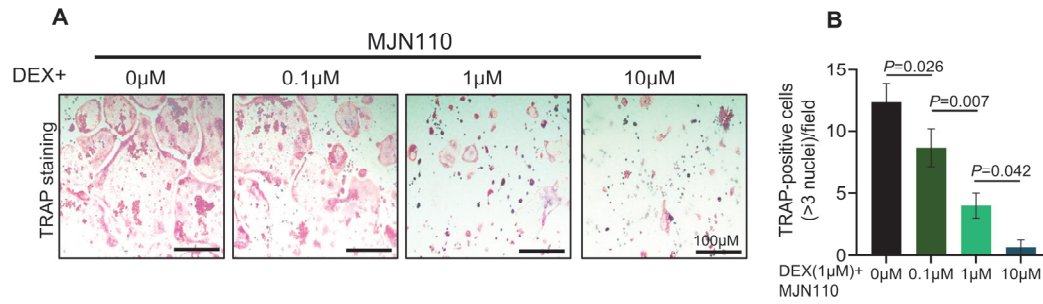
Supplemental Figure 15: The PI3K inhibitor LY294002 inhibits PI3K/AKT signaling pathway protein expression. (A-C) Western blot results for the expression of PI3K/AKT signaling pathway-related proteins. BMSCs were pretreated with various concentrations of LY294002 for 7 days. (E) Images of the immunofluorescence staining of PI3K (p85) in the control and LY groups. BMSCs were pretreated with LY294002 (20 μ M) for 7 days. (E) Quantification of the percentage of PI3Kc(p85) immunofluorescence-positively stained cells. Data represent the mean $\pm$ S.D. ($n=3$). This study included 3 biological replicates.



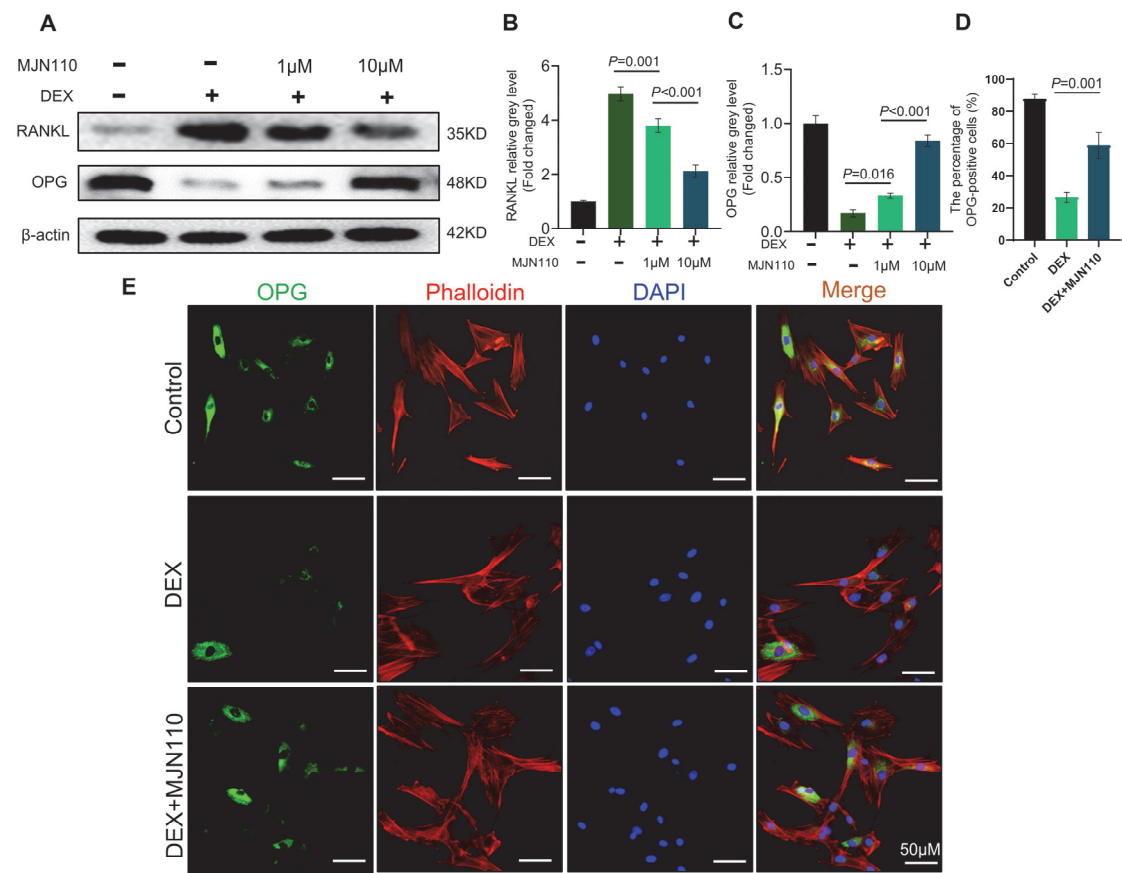
Supplemental Figure 16: (A) Quantitative analysis of the grayscale values of Western Blot bands in Figure6 (H). Data represent the mean $\pm$ S.D. (n=3).



Supplemental Figure 17: LY294002 reverses the promotive effect of MJN110 on the osteogenic differentiation of BMSCs. (A-B) Osteogenic differentiation was confirmed by Alizarin red staining and ARS absorbance detection on day 14.. Data represent mean $\pm$ S.D. (n=3).



Supplemental Figure 18: MJN110 suppresses osteoclast formation. (A) TRAP staining was performed to test the correlation between different concentrations of MJN110 and the number of osteoclasts. (B) Quantitative analysis of the number of TRAP-positive cells per field in (A). Data represent the mean $\pm$ S.D. (n=3). This study included 3 biological replicates.



Supplemental Figure 19: Blocking MAGL regulates the expressions of RANKL and OPG in BMSCs. (A-C) Western blot results for RANKL and OPG protein expressions. BMSCs were incubated with a medium containing various components for 3 days. (D-E) Images of immunofluorescence staining of OPG in BMSCs. The percentage of OPG immunofluorescence-positively stained cells was quantified. Data represent the mean $\pm$ S.D. (n=3). This study included 3 biological replicates.