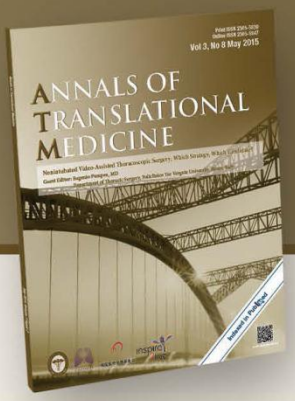
ANNALS OF TRANSLATIONAL MEDICINE



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Reviewer A

This study suggests that rosavin suppresses osteoclastogenesis via blocking the NF-kappa B and MAPK pathways and it may be a promising therapeutic candidate for osteoporosis. It is a well-designed study. However, there are many minor mistakes, the graph is small, and the characters on the graph cannot be recognized. The following points should be considered by the author for modification.

Reply: Thank you for your kind and constructive advice. We have substantially improved our manuscript based on your suggestions.

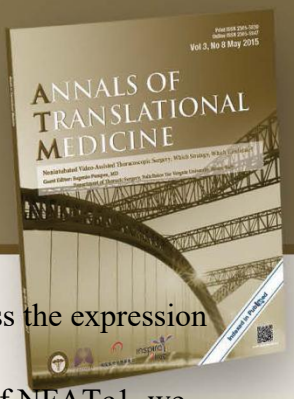
Comment 1: c-FMs in the paper should be c-fms.
Reply 1: Thank you for pointing our this and we have all corrected it as c-fms.
Changes in the text: page 4, line 2; figure 3E.

Comment 2: In the abstract, abbreviations should not be used at first. For example, MAPK should be mitogen-activated protein kinase (MAPK).
Reply 2: Thank you for your reminding. We have all rewritten the abbreviations as they first appear in the text.
Changes in the text: Page 3, line 6, line 15, line 22; Page 4, line 4, line 6, 7.

Comment 3: It is necessary to explain the abbreviation again in the body of the paper. For example, RANKL should be the receptor activator of nuclear factor -□B ligand (RANKL).
Reply 3: As is in comment 2, we have all rewritten the abbreviations as they first appear in the text.
Changes in the text: Page 3, line 7.

Comment 4: In the method, the strain of the mouse used in the study should be described in osteoclastogenesis assay in vitro.
Reply 4: Thank you for your advice. We have added the mice strain used in the in vitro study in the method.
Changes in the text: Page 7, line 9.

Comment 5: In the method, abbreviations should not be used at first. FITC, BMSCs, and OVX should be written in full at first.
Reply 5: Thank you for your advice. We have all rewritten the abbreviations in full when they first appear in the text.
Changes in the text: Page 8, line 4; Page 10, line 3; Page 3, line 13; Page 4, line 9.



Comment 6: In Figure 2D (page 14, line 6), rosavin does not appear to suppress the expression of NFATc1. The author should explain.
Reply 6: Thank you for your suggestions. In the immunofluorescent staining of NFATc1, we intended to explore the cellular location of NFATc1 before and after treatment of rosavin. In Figure 2D, we demonstrate that before the treatment, NFATc1 is mainly localized in the cytoplasm. RANKL induction translocated NFATc1 from the cytoplasm to the nucleus for the gene transcription while rosavin significantly ablated the translocation. Since it could easily lead to misunderstandings, and we have examined the expression with PCR, we removed this result.

Comment 7: In Figure 3 (page 14, line 14- line 17), the experimental procedure is unclear. For "early stage" and "late stage", describe in detail how rosavin was applied.
Reply: Thank you for your suggestions. We have added more details.
Changes in the text: To investigate the process of osteoclastogenesis influenced by the rosavin, after induction with M-CSF and RANKL, we treated BMMCs and RAW264.7 cells with rosavin on day 1, 3, or 5 after the induction. TRAP staining results showed that rosavin mainly suppressed osteoclast formation on the first day (**$p<0.01$). However, when given on day 3 for RAW 264.7 cells and day 5 for BMMCs, rosavin could not inhibit osteoclast formation (Figure 3A and 3B).

Comment 8: In Figure 4 (page 15, line 2- line 10), rosavin does not appear to suppress the nuclear translocation of p65. The graph is also small and it is difficult to determine whether it is significantly suppressed.
Reply 8: Thank you for your advice. We have adjusted the graph and make it clear to see. In Control group, we could see that the p65 is mainly localized in the cytoplasm with almost no fluorescence in the nucleus. RANKL incubation almost drives all p65 into the nucleus while rosavin significantly blocks the translocation.
Changes in the text: Figure 4A.

Comment 9: In Figure 5B (page 16, line 9- line 10), it is difficult to determine the inhibitory effect of rosavin on micro CT, so it should be described in more detail.
Reply: Thank you for your advice. We have added more detail in the micro CT results. We added the 3D reconstruction representative images and the BV/TV, BMD, Tb.N and Tb. Area analysis.
Changes in the text: Figure 5B. Page 16, line 9.

Comment 10: Overall, the graph is too small, and the effect of rosavin in immunostaining is difficult to understand. Therefore, it is necessary to improve the quality of the figure. There are many mistakes in the paper, and all of them cannot be list. All mistakes should be corrected.
Reply: Thank you for your comments. We have improved all the images and corrected the mistakes in the text.

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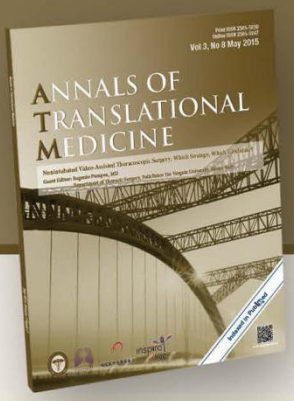


Reviewer B

Zhang et al seeks to identify mechanism of rosavin regulated bone formation. The study would benefit from increased clarity on methods used and time points when experiments were performed.
Reply: Thank you for your comments. We have made the corresponding changes based on your suggestions.

Major comments:
Comment 1: Statistics should be added to MTT assay for figure 1A.
Reply: Thank you for your advice. We have supplemented the statistics in MTT assay.

Comment 2: Graphs for figure 1, 2 are cloudy, small, and need to be higher resolution.
Reply: Thank you for your advice. We have selected clear graphs for figure 1 and 2.

Comment 3: To improve clarity, text for figure 1 B-E should identify time points for how long cells (BMSC and BMMCs) were induced for osteogenesis and osteoclast formation along with information on how long and at what point during induction treatment for rosavin was performed.
Reply: Many thanks for your comments. We have clarified the time points in studies in figure 1 B-E.
Changes in the text: Page 7, line 17, seven days later; Page 8, line 16, twenty days later.

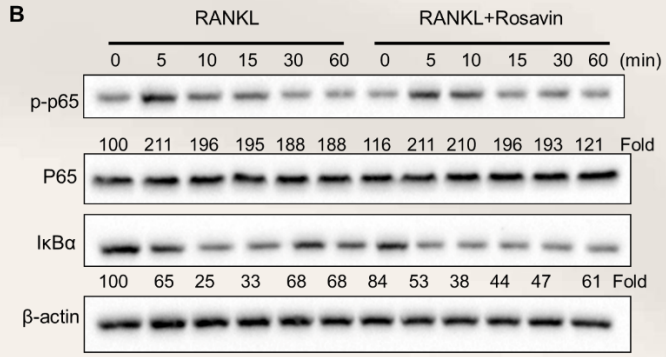
Comment 4: To improve clarity, text for figure 2 should identify time point for treated with rosavin and induction. In addition, 2C says RANKL + 1d, 3d, 5d. There is no reference to treatment with rosavin or concentration used however results mention use of rosavin.
Reply: Many thanks for your comments. We have moved precious Figure 2C to Figure 3C because it shows the effects when rosavin was given on 1, 3 and 5.
Changes in the text: Figure 3C.

Comment 5: Methods should be included on how BMMCs and BMSC were acquired in addition to identify how the cells were validated prior to use. In addition are Bone marrow monocytes (BMMCs) and BMSCs: are cells from a single mouse donor or are triplicates representative of multiple mice? What is western blot representative of-tissues, cells?
Reply: We have added the isolation methods for BMMCs and BMSC. Cells are triplicates representative of multiple mice. Western blotting results represent cells. We have clarified in the text. Many thanks.
Changes in the text: Page 7, line 15-18. BMMCs were washed from the femurs of the C57 mice. Cells were cultured in α-MEM (1% penicillin, 1% streptomycin and 10% FBS). Cells were

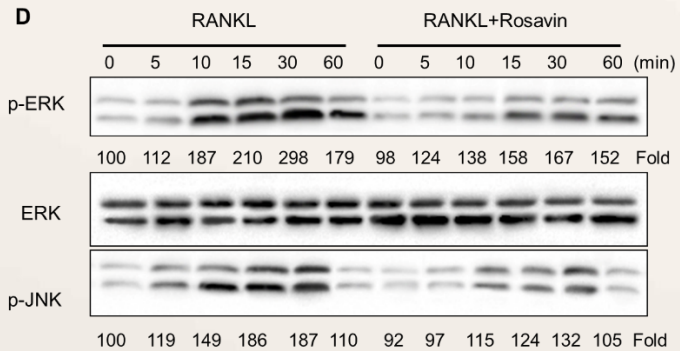
seeded into 96-well plates containing M-CSF (30 ng/mL) and RANKL (100 ng/mL) meanwhile incubated with various concentrations of rosavin (0, 1.25, 2.5, and 5 μM).

Comment 6: Concentration of rosavin should be identified for figure 3
Reply 6: The concentration is 5 μM.
Changes in the text: Page 14, line 8.

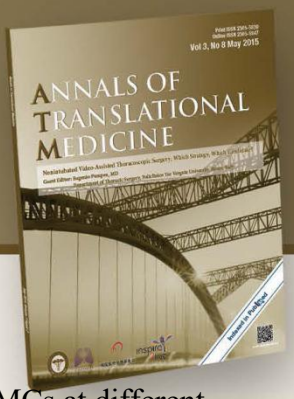
Comment 7: Figure 4 demonstrates what appears to be a loss of total proteins (ERK, p65) well as phospho-protein. Authors should describe how normalization was done and demonstrated that loss of phospho activity is not due to loss of total protein expression. If these results are referenced in graphs for figure 4 the figure should be made larger with enhanced resolution. Furthermore, the difference in phosphorylation (ERK and JNK) between RANKL and RANKL + rosavin does not appear to be great. The trends appear similar and to be a factor of time and total protein instead of treatment.
Reply: Thank you for your comments. We obtained the grey scale of each band and found that the grey scale of each band of p65 is not statistically different from each other. For pp65 expression, we set the 0 time point grey scale as 100 based on which the grey values of each band were calculated.



As we could see, the concentration of p-ERK and p-JNK gradually reached the peak at 30 min with the grey values being 298 and 187 respectively. After rosavin treatment, the peak values were 167 and 132. Thus, we could conclude that the phosphorylation of ERK and JUK was significantly inhibited with rosavin treatment.



8. In the text what is the distinction between c-Fos, and C-Fos?



Reply: Many thanks for your reminding. We confirm it is c-Fos.

9. What does the legend for figure 3C represent?
Reply: Many thanks and it represents PCR results of *rank* and *c-fms* from BMMCs at different time points after M-CSF treatment. We show that rosavin did not present rank and c-fms transcription after M-CSF induction. We have moved it to Figure 3D.

10. Figure 5 how long was rosavin treatment for?
Conclusion section is hard to interpret without increased clarity on methods
Reply: Thank you for your advice. We have clarified more details in the methods. Rosavin was used for 6 weeks until the mice were sacrificed.
Changes in text: Page 16, line 6.