nature portfolio

Peer Review File

The microbial metabolite imidazole propionate dysregulates bone homeostasis by inhibiting AMP-activated protein kinase (AMPK) signaling

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript demonstrated that the microbial metabolite imidazole propionate dysregulates bone homeostasis by inhibiting AMP-activated protein kinase (AMPK) signaling. The data suggests that the microbial metabolite ImP may be an important factor and therapeutic target for metabolic bone diseases. Although the findings are interesting but some minor comments should be addressed.

Major comments
1. Previous study has identified imidazole propionate as a microbially produced histidine-derived metabolite that is present at higher concentrations in subjects with versus without type 2 diabetes (PMID: 30401435). Whether there is a difference about imidazole propionate in osteoporosis patients versus healthy people?
2. Results-Figure 1
A, Figure 1: Why imidazole propionate was subcutaneously administrated by an osmotic pump? What is the basis for dose selection? Are there any references to support these? As far as I know, imidazole propionate was intraperitoneally administrated into mice to investigate its role on type 2 diabetes. Please explain this.
B, Figure 1: What is the injection frequency? What is the circulating concentrations of imidazole propionate after the injection of imidazole propionate with time increase?
C, Figure 1: Histological staining using osteoblast and osteoclast markers antibodies should be performed to further prove the effect of imidazole propionate on bone loss (both models).
3. Results-Figure 2/3: The osteoblast differentiation and osteoclast differentiation should be monitored using the model mice in Figure 1. Possibly, primary BMSCs from the model mice in Figure 1 should be isolated and induced in vitro. Subsequently, the biomarkers of osteoblast differentiation and osteoclast differentiation were determined, respectively.
4. Results-Figure 4: Agonist of phosphorylation of AMPK (T172) should be employed to treat primary BMSCs, then, assays about osteoblast differentiation and osteoclast differentiation should be performed for the rescue experiments. Additionally, the agonist should be applied in the in vivo assay (Figure 1).
5. Results-Figure 5
A, Figure 5: The data of Figure 5C-5E should be confirmed using the model mice or primary BMSCs from the model mice.
B, Figure 5: All the data in Figure 5 should be identified in agonist of phosphorylation of AMPK (T172)-challenged model mice.
6. Results-Figure 6: Is it possible to use an in vivo model (diabetic osteoporosis) to prove the effects of ImP on metformin-mediated the improvement?
7. How do the authorship determine if endogenous ImP transports and aggregates around BMSCs, adipocytes or osteoblast/osteoclast?

Specific comments
1. Scale bar should be provided for all radiographic images.
2. For BMP2-induced ectopic bone formation assay, please provide the source of BMP2. Please also provide detailed information on the other missed source of reagents.

Reviewer #2

(Remarks to the Author)
This manuscript demonstrated research data regarding the inhibitory effect of ImP, a microbial metabolite founded in diabetic patient with high serum level, on bone formation in vivo and in vitro. It showed its effect on inhibition of osteoblast differentiation and stimulation of adipocyte differentiation through inhibition of AMPK activation. Additionally, ImP also inhibits the metformin-induced osteoblast differentiation suggesting its importance on therapeutic target of metabolic bone diseases. Overall, this manuscript serves a valuable data for ImP effects on bone metabolism. However, I think there are some parts of this manuscript that need to be revised.

Minor Chechpoints
1. This paper discusses the inhibition of osteoblast differentiation by ImP through the suppression of AMPK activity, which is highly expressed in diabetic patients. Therefore, It would be helpful for readers to have a more detailed explanation of the relationship between diabetes and AMPK in the Introduction section.
2. In the explanation of Figure 1b, it is mentioned that Tb.Th decreased in mice treated with ImP, but there is no data in Fig 1b.
3. On page 5, lines 86-87, it is stated that the mRNA expression of Runx2, Osx, Alp, Bsp, and OC was measured, but there is no data for Osx in the results.
4. The experimental methods for cell viability measurement shown in Figures 2 and 3 are missing. Please include this assay method in the Materials and Methods section.
5. On page 6, lines 100-101, the authors conclude that ImP-induced bone loss in mice is not due to increased osteoclast activity. However, there is no data on the bone resorption activity of osteoclasts. Therefore, it would be helpful to support author's conclude to include results from a bone resorption assay.
6. ImP is reported to inhibit the phosphorylation of AMPK (T172), but other studies suggest that it inhibits the activation of MAPKs such as p38. What impact does ImP have on MAPK activity in this study?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
(1) For the comment “Results-Figure 6: Is it possible to use an in vivo model (diabetic osteoporosis) to prove the effects of ImP on metformin-mediated the improvement?”, the in vitro assay is not enough to explain the question. And I suggest to add the supplementary experimental data to the manuscript as a supplementary figure.
(2) I believe that all the other comments I provided have been adequately addressed in the revised manuscript.

Reviewer #2

(Remarks to the Author)
The manuscript by Park S and colleagues, which reports that the microbial metabolite imidazole propionate disrupts bone homeostasis by inhibiting AMPK signaling, is considered a valuable contribution to understanding new regulatory mechanisms in bone metabolism. The authors have responded to this reviewer’s comments with logical and well-founded explanations, thereby enhancing the scientific and academic significance of the study. Therefore, I believe this revised manuscript is well-suited for publication in ‘Communication Biology’.

Responses to the Reviewers' comments

We would like to thank the reviewers for their constructive comments on our manuscript. These valuable comments have helped us improve our manuscript. We have carefully addressed all issues and revised the manuscript accordingly.

Reviewer #1:

1. Previous study has identified ImP as a microbially produced histidine derived metabolite that is present at higher concentrations in subjects with versus without type 2 diabetes (PMID: 30401435). Whether there is a difference about ImP in osteoporosis patients versus healthy people?

   (Response) Thank you for your valuable comments. To clarify the involvement of ImP in the development of osteoporosis, it is important to examine changes in ImP levels in the blood of patients. However, there have been no reports on this topic to date. In this study, we attempted to measure ImP levels in blood samples from osteoporosis patients over an extended period. Unfortunately, due to limitations such as the sensitivity of the measuring equipment, we were unable to obtain valid results. Further in-depth research using more sensitive equipment is required.

2. Results-Figure 1

A, Why ImP was subcutaneously administrated by an osmotic pump? What is the basis for dose selection? Are there any references to support these? As far as I know, ImP was intraperitoneally administrated into mice to investigate its role on type 2 diabetes. Please explain this.

(Response) Thank you for your insightful question. As you mentioned, a previous study (PMID: 30401435) administered ImP intraperitoneally for a short duration to observe its effects on blood glucose regulation. However, unlike changes in blood glucose, changes in

bone tissue occur more gradually over an extended period, necessitating a longer drug administration. Administering ImP intraperitoneally for 4 weeks could cause stress to the mice, which may affect the experimental results. Additionally, slight variations in drug dosage can occur with syringe injections depending on the experimenter or environment. From a pharmacokinetic perspective, continuous drug infusion provides a more consistent blood concentration compared to intermittent injections. Considering these aspects, in this study (Figure 1), we used an osmotic pump device, which was implanted subcutaneously through a single surgery, to administer the drug. The dosage was determined based on previous studies, and as subcutaneous osmotic pumps provide a more stable blood concentration than intraperitoneal injections, we chose a lower dosage (20 μg/day, 60 μg/day).

B. What is the injection frequency? What is the circulating concentration of ImP after the injection of imidazole propionate with time increase?

(Response) To investigate the effects of ImP on bone tissue (as mentioned in Question 2), we opted to use an osmotic pump (MICRO-OSMOTIC PUMP MODEL 1004, ALZET®) implanted subcutaneously to administer ImP at a constant rate for 4 weeks, rather than intraperitoneal injections. According to the manufacturer, this pump releases drugs into the body at a rate of 0.11 μl/hr. Using the concentration of ImP we set, the dosage administered is approximately 0.83 μg/hr or 2.5 μg/hr. Unfortunately, we were unable to obtain accurate values for the circulating concentration of ImP over time due to the challenges mentioned in Question 1.

C. Histological staining using osteoblast and osteoclast markers antibodies should be performed to further prove the effect of ImP on bone loss (both models).

(Response) Thank you for your insightful comment. Following your suggestion, we performed histological staining using a ALP (osteoblast marker) antibody and a TRAP (osteoclast marker) detection kit. ALP expression in the bone tissue was decreased in the

ImP-treated group, while TRAP expression showed no significant change. These results have been included in Figure 1c–d and the main text (page 6, lines 2-5). These findings support our hypothesis that ImP inhibits osteoblast differentiation, leading to an imbalance in bone homeostasis.

3. Results-Figure 2/3: The osteoblast differentiation and osteoclast differentiation should be monitored using the model mice in Figure 1. Possibly, primary BMSCs from the model mice in Figure 1 should be isolated and induced in vitro. Subsequently, the biomarkers of osteoblast differentiation and osteoclast differentiation were determined, respectively.

(Response) In response to your request, we isolated BMSCs and BMM cells from ImP-injected animals and assessed their differentiation potential into osteoblasts and osteoclasts. The formation of Alizarin Red-positive colonies, which indicates the differentiation potential of BMSCs, was reduced in the ImP-treated group compared to the control group. However, the number of TRAP-positive cells, which represents osteoclast differentiation of BMM cells, showed no difference between the groups. These results have been included in Figure 1e–f and the main text (page 6, lines 7-9). These findings suggest that the bone loss observed with ImP administration is due to a decrease in osteoblast activity rather than an increase in osteoclast activity.

4. Results-Figure 4: Agonist of phosphorylation of AMPK (T172) should be employed to treat primary BMSCs, then, assays about osteoblast differentiation and osteoclast differentiation should be performed for the rescue experiments. Additionally, the agonist should be applied in the in vivo assay (Figure 1).

(Response) Thank you for your valuable suggestion. Following this recommendation, we treated primary BMSCs with metformin (an agonist of AMPK(T172) phosphorylation) and assessed the effects of ImP on osteoblast differentiation. ImP treatment reduced the phosphorylation of AMPK (T172) and the expression of the osteoblast differentiation transcription factor OSX, while the addition of metformin reversed these effects.

Additionally, we injected ImP and the AMPK agonist (metformin) into mice, isolated MSCs from the animals, and assessed their osteoblast differentiation potential. In the ImP-treated group, alizarin-positive CFU-osteoblast formation was decreased, and this was restored in the group receiving additional metformin treatment. These results suggest that the reduction in osteoblast differentiation caused by ImP is linked to the inhibition of AMPK (T172) phosphorylation. These findings have been included in Figure 6c–d and the main text (page 11, lines 3- 5).

5. Results-Figure 5

A, Figure 5: The data of Figure 5C-5E should be confirmed using the model mice or primary BMSCs from the model mice.

(Response) We fully agree. As requested, we additionally isolated BMSCs from ImP-injected mice to assess changes in adipocyte differentiation potential. In the ImP-treated group, lipid droplet formation in BMSCs induced by adipogenic medium increased, and phosphorylation of AMPK(T172) was reduced. On the other hand, in the group co-treated with ImP and metformin (an agonist of phosphorylation of AMPK), lipid droplet formation decreased compared to the ImP-only group, along with an increase in phosphorylation of AMPK(T172) (Figs 4b, 7C, D). These findings confirm that ImP is a contributing factor in promoting adipocyte differentiation. These data have been included in Figures 4b, 7C, D and described the main text (page 8, lines 8-9, page 12, lines 1-3).

B, Figure 5: All the data in Figure 5 should be identified in agonist of phosphorylation of AMPK (T172)-challenged model mice.

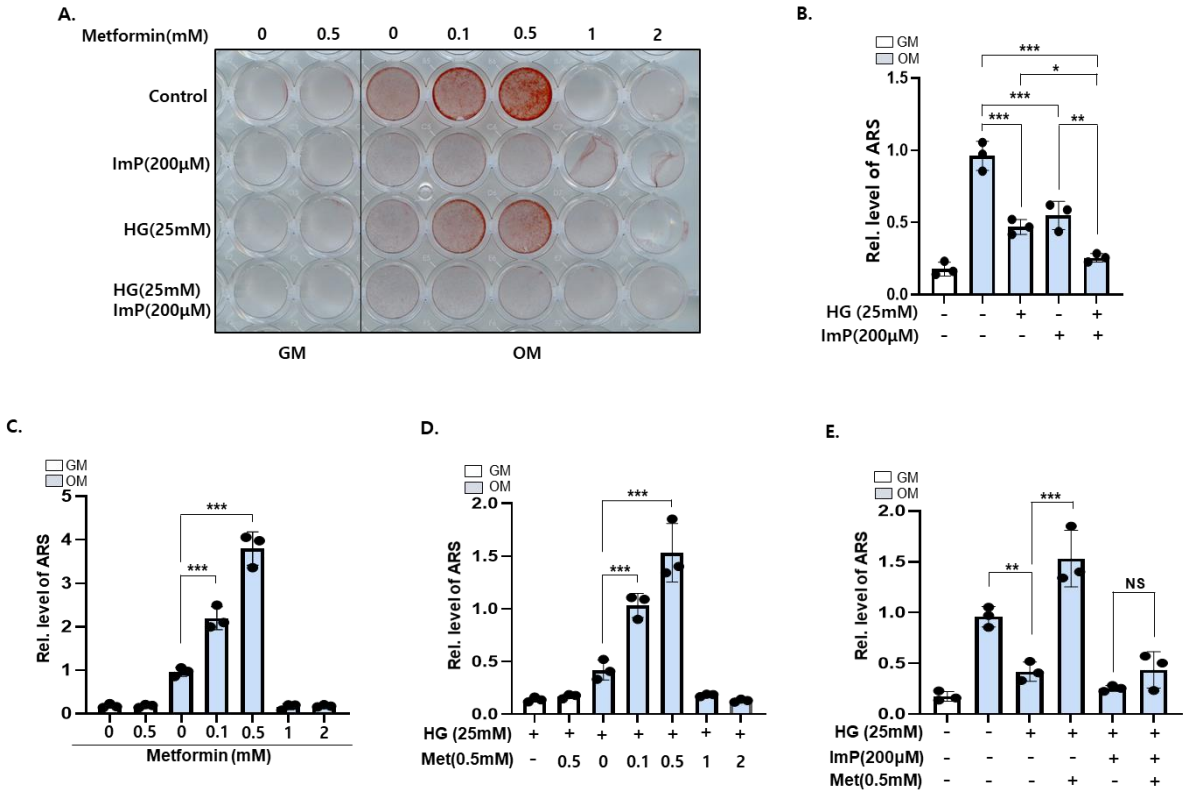
(Response) This point has been addressed in our response provided for Question 5A.

6. Results-Figure 6: Is it possible to use an in vivo model (diabetic osteoporosis) to prove the effects of ImP on metformin- mediated the improvement?

(Response) In response to this comment, we established an *in vitro* diabetes model to

evaluate the effect of ImP on metformin-mediated osteoblast differentiation. BMSCs were cultured under high glucose conditions (25 mM) with the respective drugs, and calcium deposition was assessed using Alizarin Red staining. In osteogenic medium, calcium deposition increased when BMSCs were cultured, but this deposition decreased under high glucose and ImP-treated conditions (Response Fig. A, B). Metformin treatment alone increased calcium deposition in a dose-dependent manner at concentrations below 0.5 mM (Response Fig. A, C), and this effect was consistently observed, although to a lesser extent, under high glucose conditions (Response Fig. A, D). However, ImP treatment further reduced calcium deposition in the presence of both high glucose and metformin (Response Fig. A, E, below).

Considering these results, it is expected that increased ImP production in a diabetic state would exacerbate bone loss, and this response appears to be related to modulating AMPK activity. Further confirmation using an animal model is necessary for a conclusive answer, and we plan to address this in future research.



(Response Figure legend) Changes in calcium deposition in BMSCs cultured under high glucose conditions with metformin and ImP treatments. ImP, Imidazole propionate; HG, High glucose; Met, Metformin; GM, Growth medium; OM, Osteogenic medium. NS, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; compared to the control group.

7. How do the authorship determine if endogenous ImP transports and aggregates around BMSCs, adipocytes or osteoblast/osteoclast?

(Response) According to current researches, the microbial metabolite imidazole propionate can enter the bloodstream from the gut and affect various tissues, including the liver and lung (PMID: 32783890, PMID: 34768867). While specific mechanisms detailing how ImP enters cells and exerts its effects are not fully understood, it has been reported to influence intracellular signaling pathways such as MAPK, AKT, and AMPK, leading to cellular responses (PMID: 32783890).

In our study, after administering ImP to mice for 4 weeks, we isolated BMSCs and BMM cells to assess its effects on osteoblast, adipocyte, and osteoclast differentiation (revision figures 1e, f, and 4.b). Our results showed that ImP inhibited osteoblast differentiation from BMSCs while promoting adipocyte differentiation, with associated changes in p38γ and AMPK phosphorylations. Based on these findings, we suggest that ImP, produced by gut microbiota, may influence bone tissue and potentially contribute to bone loss.

Specific comments

1. Scale bar should be provided for all radiographic images.

(Response) As requested, we have added scale bars to all radiographic images.

2. For BMP2-induced ectopic bone formation assay, please provide the source of BMP2. Please also provide detailed information on the other missed source of reagents.

(Response) As requested, we have included the source of BMP2 and the detailed information regarding other reagents in the Materials and Methods section.

Thank you for your valuable comments.

Reviewer #2:

1. This paper discusses the inhibition of osteoblast differentiation by ImP through the suppression of AMPK activity, which is highly expressed in diabetic patients. Therefore, it would be helpful for readers to have a more detailed explanation of the relationship between diabetes and AMPK in the Introduction section.

   (Response) Thank you for your advice. As suggested, we have included additional information about the relationship between diabetes and AMPK in the Introduction section to further support our claims and enhance the readers' understanding (page 4, line 18 – page 5, line 2).

2. In the explanation of Figure 1b, it is mentioned that Tb.Th decreased in mice treated with ImP, but there is no data in Fig 1b.

   (Response) Thank you for bringing this to our attention. We have now added the Tb.Th data in Figure 1b, which was mistakenly omitted.

3. On page 5, lines 86-87, it is stated that the mRNA expression of Runx2, Osx, Alp, Bsp, and OC was measured, but there is no data for Osx in the results.

   (Response) Thank you for your comment. This was a mistake, and we have now added the missing Osx PCR results in Figure 2b.

4. The experimental methods for cell viability measurement shown in Figures 2 and 3 are missing. Please include this assay method in the Materials and Methods section.

   (Response) Thank you for your suggestion. The experimental methods for cell viability measurement has been included in the Materials and Methods section (page 18, line 14 – line 19).

5. On page 6, lines 100-101, the authors conclude that ImP-induced bone loss in mice is not

due to increased osteoclast activity. However, there is no data on the bone resorption activity of osteoclasts. Therefore, it would be helpful to support author's conclude to include results from a bone resorption assay.

(Response) Thank you for your valuable advice. As requested, to further clarify the lack of osteoclast involvement in ImP-induced bone loss, we used a Bone Resorption Assay Kit 48 (CSR-BRA-48KIT, Cosmo Bio, Tokyo, Japan) to assess changes in the bone resorption activity of osteoclasts. As shown in Figure 3d, there was no significant change in pit formation by osteoclasts. These results suggest that osteoclasts may not be involved in ImP-induced bone loss. This information has been added to Figure 3f and the main text (page 7, lines 16-17).

6. ImP is reported to inhibit the phosphorylation of AMPK (T172), but other studies suggest that it inhibits the activation of MAPKs such as p38. What impact does ImP have on MAPK activity in this study?

   (Response) Thank you for your insightful comment. Previous studies (PMID: 30401435, PMID: 32783890) have reported that ImP stimulates p38γ phosphorylation in HEK-293 cells and inhibits AMPK (T172) phosphorylation in liver cells. Based on current research, there is limited direct evidence that ImP specifically regulates the MAPK pathway. In this study, we additionally observed that ImP stimulates p38γ phosphorylation in BMSCs and suppresses osteoblast differentiation by regulating the phosphorylation of AMPK (T172), a downstream signal of p38γ. Based on these findings, we hypothesize that ImP negatively regulates osteoblast differentiation by activating p38γ to modulate the phosphorylation of AMPK (T172). These results have been added to Figure 5a-d, and the main text (page 9, line 1 – line 17).

Thank you for your valuable comments.

References

1. Koh A, Molinaro A, Ståhlman M, Khan MT, Schmidt C, Mannerås-Holm L, Wu H, Carreras A, Jeong H, Olofsson LE, Bergh PO, Gerdes V, Hartstra A, de Brauw M, Perkins R, Nieuwdorp M, Bergström G, Bäckhed F. Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. Cell. 2018 Nov 1;175(4):947-961.e17. PMID: 30401435.
2. Koh A, Mannerås-Holm L, Yunn NO, Nilsson PM, Ryu SH, Molinaro A, Perkins R, Smith JG, Bäckhed F. Microbial Imidazole Propionate Affects Responses to Metformin through p38γ-Dependent Inhibitory AMPK Phosphorylation. Cell Metab. 2020 Oct 6;32(4):643-653.e4. PMID: 32783890.
3. Chen Z, Wang B, Dong J, Li Y, Zhang S, Zeng X, Xiao H, Fan S, Cui M. Gut Microbiota-Derived l-Histidine/Imidazole Propionate Axis Fights against the Radiation-Induced Cardiopulmonary Injury. Int J Mol Sci. 2021 Oct 23;22(21):11436. PMID: 34768867.

Responses to the Reviewers' Comments

We sincerely appreciate the reviewers' constructive feedback on this study. Your insights have greatly contributed to improving the quality and clarity of the manuscript.

Reviewer #1:

1. For the comment, "Results-Figure 6: Is it possible to use an in vivo model (diabetic osteoporosis) to prove the effects of ImP on metformin-mediated improvement?", the in vitro assay is not enough to explain the question. I suggest adding supplementary experimental data to the manuscript as a supplementary figure.

   (Response) We agree that demonstrating the effects of ImP on metformin-mediated improvement using a diabetic osteoporosis animal model would provide valuable insights. However, incorporating such experiments into this study would require substantial time and involve a complex experimental design. This has been acknowledged as a limitation of the current study and discussed in detail in the revised discussion section (page 16, lines 1 – lines 7). Additionally, in response to the reviewer's request, we have included the supplementary experimental data mentioned in the letter within the manuscript's supplementary materials and provided detailed discussions on these results (page 15, lines 14 – page 16, lines 7).
2. I believe that all the other comments I provided have been adequately addressed in the revised manuscript.

   (Response) Thank you for your thorough evaluation.

Reviewer #2:

1. The manuscript by Park S and colleagues, which reports that the microbial metabolite imidazole propionate disrupts bone homeostasis by inhibiting AMPK signaling, is considered a valuable contribution to understanding new regulatory mechanisms in bone metabolism. The authors have responded to this reviewer's comments with logical

and well-founded explanations, thereby enhancing the scientific and academic significance of the study. Therefore, I believe this revised manuscript is well-suited for publication in ‘Communication Biology’.

(Response) Thank you for your positive assessment and valuable feedback. We greatly appreciate your recognition of the study's scientific significance.

Thank you once again for your constructive feedback so far, which has been instrumental in refining this manuscript.