nature portfolio

Peer Review File

EZH2-mediated macrophage-to-myofibroblast transition contributes to calcium oxalate crystal-induced kidney fibrosis

Corresponding Author: Professor Fan Cheng

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)
Manuscript Title: EZH2-mediated macrophage-to-myofibroblast transition contributes to calcium oxalate crystal-induced kidney fibrosis

In this study authors investigated the role of EZH2 in the macrophage-to-myofibroblast transition (MMT) and its contribution to kidney fibrosis induced by calcium oxalate (CaOx) crystals, a common cause of nephrocalcinosis. The researchers identified MMT cells in kidney tissues of patients with CaOx-related chronic kidney disease (CKD) and in a mouse model of CaOx nephrocalcinosis, using immunofluorescence and flow cytometry. They found that MMT cell infiltration correlated with a decline in glomerular filtration rate in patients.
To explore the role of macrophages in this process, the researchers used clodronate liposomes to deplete macrophages in mice, which attenuated CaOx crystal-induced fibrosis. Transcriptomic and single-cell sequencing revealed that EZH2 was highly expressed in kidneys with CaOx deposition, particularly in macrophages. The study demonstrated that EZH2 inducible knock-out or pharmacological inhibition by GSK-126 reduced MMT and renal fibrosis in vivo and in vitro. Mechanistically, the researchers found that EZH2 inhibition reduced the enrichment of H3K27me3 on the DUSP23 gene promoter, leading to increased DUSP23 expression. DUSP23 was shown to mediate the dephosphorylation of pSMAD3 (S423/425), a key regulator of MMT. Overexpression of DUSP23 could alleviate SMAD3-mediated MMT.
In summary, the study identifies EZH2 as a critical regulator that promotes kidney fibrosis by mediating MMT via the DUSP23/SMAD3 pathway in nephrocalcinosis. The findings suggest that targeting EZH2 could be a potential therapeutic strategy for managing MMT-mediated kidney fibrosis in patients with CaOx crystal-induced nephrocalcinosis.
The study was well designed and executed, however, a few concerns were identified as detailed below. I encourage the authors to revise the manuscript with changes included as suggested.
Review Comments:
1. The biochemical quantification of collagen content by Hydroxyproline assays is not performed in the study. This parameter is really required to quantitatively evaluate the MMT induced renal fibrosis.
2. Inconsistent capitalization: The capitalization of words are inconsistent throughout the text.
3. Spelling errors: A few words are misspelled, such as "privious" (393 line) instead of "previous" and "whereafter" (196, 376 line) instead of "where after".
4. Abbreviation inconsistencies: The abbreviations "COM" at line 384 and 629 are different.
5. In Figure 7, author mentioned n=6 (line-360), n=3 (line-127), n=5 (line-651), n=3 (line-652) why this description of experimental groups and results varies between sections. Author mentioned animals n=6, each group, but why n=3 at line 127.?
6. In Figure 9G, Were the Collagen I and fibronectin blots cropped/originate from same blot.. ? Kindly provide all original blots in triplicate and mention which was chose for the figures in manuscript.
7. Why there is inconsistency in # Cat. No. for anti-α-SMA antibody.
8. Please provide “software used & version and supplier” under “Statistical analysis” section
9. There are lot of formatting issues all over the document (extra space, missing space, capital letter that should not be, missing punctuation). Please carefully revise the manuscript.

Reviewer #2

(Remarks to the Author)
This manuscript described EZH2 via DUSP23 and SMAD3 mediating Macrophage Myofibroblast transition (MMT) in contribution to kidney fibrosis CaOx crystal-induced kidney fibrosis model.

The experiment design and results are strong and supporting. The manuscript is of high standard in experimental approaches and data presenting, and is logical in drawing conclusions.

While the MMT contribution to kidney fibrosis has been demonstrated by other labs, the EZH2 mediated via DUSP23 mediated SMAD3 signaling is probably the novel finding of this study regardless of CaOx crystal-induced or other factor induced kidney fibrosis via MMT, although large efforts has been focused on MMT of macrophages treated with CaOx.

To demonstrate the CaOx induced MMT contributes to kidney fibrosis, a quantitative study of ECM production by the MMT macrophages via non-macrophage myofibroblasts would probably more convicting.

Reviewer #3

(Remarks to the Author)
EZH2-mediated macrophage-to-myofibroblast transition contributes to calcium oxalate crystal-induced kidney fibrosis is and interesting manuscript, trying to elucidate the underlying mechanisms of nephrocalcinosis. A possible role for EZH2 is investigated/ elucidated.

I have an important question though. If I correctly understand the order of events that are supposed to occur during nephrocalcinosis is as follows: CaOx crystal deposition, macrophage attraction (in order to clear the crystals), MMT cell differentiation (in these cells EZH2 is inducing collagen/fibronectin production as a start of renal fibrosis process).
If this is correct I should not expect that in a model of EZH2 depletion, by gene KO (figure 5) or inhibitor (figure 9), the amount of CaOx crystals is lower in case of EZE2 depletion. In contrast, I should expect that an identical number of crystals (induced by glyoxylic acid administration) should induce a lower degree of fibrosis. (One could even conclude it is completely normal that a lower number of crystals induce a milder degree fibrosis).
Please can the authors explain this ?

Reviewer #4

(Remarks to the Author)
I wanted to thank the authors for this well-written and well-presented manuscript. This manuscript establishes mechanistic details around macrophage to myofibroblast fibrosis transition in kidney using a CaOX injury model in mice and includes relevant human disease biology. It builds on a growing field of work around macrophage/myofibroblast and its role in kidney fibrosis. The model is well characterized and described and the experiments to establish causality with macrophages were well-thought out.

Major and minor concerns are outlined below.

Major concerns:

The animal RNAseq profiles must be deposited into a publicly accessible database –NCBI GEO or EMBL Expression Atlas are the two most common. This is a non-negotiable point. There is simply no way to verify RNAseq findings reported without the information being available to the scientific community. No methods are provided for how differential expression analyses was performed. Did the authors use DESeq2, Voom-Limma, or something else?

While EZH2 was clearly a reasonable target to go after given the data presented in Figure 3, what is the rationale for preferentially focus on this given the number of DEGs among methyltransferase genes? Without the full DEG profile available (see first point), there's no way to know if this was a reasonable gene target to pursue.

There are no methods for any of the single-cell RNAseq analysis from the Kidney Precision Medicine Project – not for cell annotations and not for differential expression analyses. Please provide sufficient details on these analyses.
• I believe the bar plots are incorrect. Median expression of EZH2 across these cell types would be 0 as the vast majority of cells do not express EZH2 (see attached violin plots and dot plots for EZH2 expression in monocytes from this dataset). It is true that some monocytes in patients with AKI do have higher EZH2 expression, but again, the bulk of cells do not express EZH2 which makes any differential expression calculation suspect.
• Given the prominent up-regulation shown for monocytes and phagocytic macrophages in Figure 4E, I tried to replicate the upregulation in each cell type (reference vs AKI). Using a Wilcoxon rank sum approach, the difference between patients with AKI and control samples was not significant for monocytes ($p=0.27$, p-adj=0.57) nor for dendritic macrophages ($p=0.54$, p-adj=0.74).

Line 183/4 – the statement on the collagen 1 western blot seems reasonable but the blot has a number of bubbles which make quantitation suspect. Please re-run the collagen 1 western blot.

Minor concerns:
For all heatmaps, please indicate whether these are absolute, normalized, or median centered levels of expression.

Re: Line 117 – A high level of MMT cell infiltration was associated with glomerular filtration rate decline (Fig 1F) . As far as I can tell this is measuring GFR at a single point in time for each sample and not a GFR decline (which is a rate over time per sample). “MMT cell infiltration was associated with lower glomerular filtration rates.” is more appropriate wording here.

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Line 237 should read …EZH2 suppressed pSMAD3(Ser423/425) instead of simply pSMAD3 as pSMAD3 (Thr179) is not affected.

Figure 2 and figure 3 legend – insert space between nephorcalcinosis and mice (lines 606, 607)and nephrocalcinosis and group (line 617)

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I have no more concerns

Reviewer #3

(Remarks to the Author)
The authors answered in a satisfying way to my concern.

Reviewer #4

(Remarks to the Author)
I have no additional comments. The authors have addressed all concerns I had with the original submission.

Response to reviewers

Dear reviewers:

On behalf of my co-authors, we thank you very much for your letter and comments on our manuscript entitled ***" EZH2-mediated macrophage-to-myofibroblast transition contributes to calcium oxalate crystal-induced kidney fibrosis ".***

We appreciate the constructive and valuable comments. We have revised our manuscript considerably according to the comments, questions, and suggestions. In the event that we missed any one of the comments please let us know. The revised portion are marked in YELLOW in revised manuscript.

Overview of revision: (1) the raw data of RNA-seq was uploaded through GEO; (2) the content of scRNA-seq was deleted and snATAC-seq was added; (3) Quantification of collagen deposition was evaluated by measuring hydroxyproline concentration; (4) All the original western blot images were uploaded as supplementary materials; (5) we detected endothelial-to-mesenchymal transition (EndMT); (7) We added that the relationship between collagen deposition and stone formation in discussion; (8) We have reproofed the manuscript and corrected the spelling errors.

Here are my responses to your questions:

Reviewer 1

Comment 1: The biochemical quantification of collagen content by Hydroxyproline assays is not performed in the study. This parameter is really required to quantitatively evaluate the MMT induced renal fibrosis.

Reply 1: Thank you for your meticulous review and positive and encouraging comments on our manuscript. Hydroxyproline assays are an important method for the quantitative analysis of renal tissue fibrosis **(Krones E, Eller K, Pollheimer MJ, et al. NorUrsodeoxycholic acid ameliorates cholemic nephropathy in bile duct ligated mice. J Hepatol. 2017;67(1):110-119. doi: 10.1016/j.jhep.2017.02.019.)**. Based on your kind reminder, quantification of collagen

deposition in kidney was evaluated by measuring hydroxyproline concentration. Consistent with the Masson staining, hydroxyproline assay also indicated that the EZH2 deletion attenuated CaOx crystal-induced kidney fibrosis (Fig. 2C; Fig. 3D; Supplementary Fig. 2B-C).

Changes in revised manuscript:

(1) Line 124~126, 150~152, 177~180, 250~253, and 406~412 in yellow;

(2) Fig. 2C; Fig. 3D; Supplementary Fig. 2B-C.

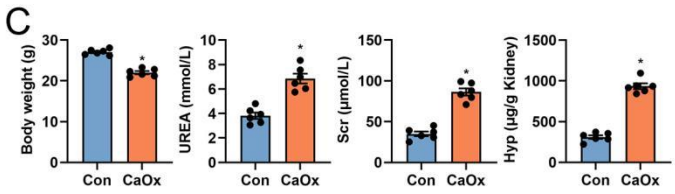


Fig. 2C

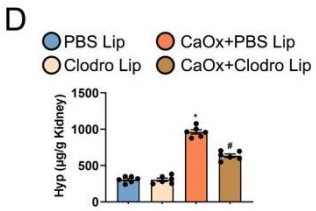
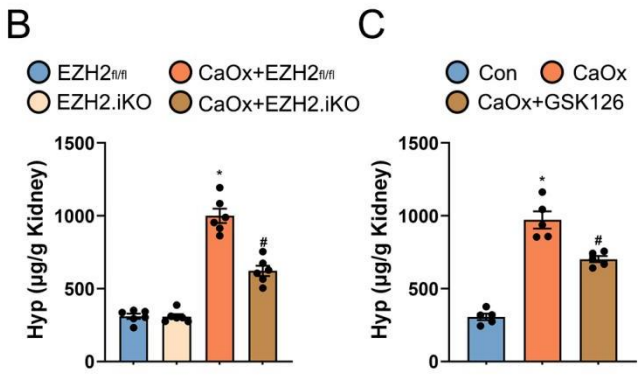


Fig. 3D



Supplementary Fig. 2B-C

Comment 2: Inconsistent capitalization: The capitalization of words are inconsistent throughout the text.

Reply 2: Thanks for your good suggestion. I apologize for our oversight. We have reproofed the manuscript and corrected the errors.

Comment 3: Spelling errors: A few words are misspelled, such as "privious" (393 line) instead of "previous" and "whereafter" (196, 376 line) instead of "where after".

Reply 3: Thank you for your meticulous review. We have corrected the errors.

Changes in revised manuscript:

(1) Line 201, 404, and 386 in yellow.

Comment 4: Abbreviation inconsistencies: The abbreviations "COM" at line 384 and 629 are different.

Reply 4: Thank you for your meticulous review. We have corrected the spelling errors.

Changes in revised manuscript:

(1) Line 168, 394, 763 and in yellow.

Comment 5: In Figure 7, author mentioned n=6 (line-360), n=3 (line-127), n=5 (line-651), n=3 (line-652) why this description of experimental groups and results varies between sections. Author mentioned animals n=6, each group, but why n=3 at line 127?

Reply 5: Thanks for your good suggestion. We apologize for the confusion caused by our unclear description. In this study, 6 mice were included in each group for histological analysis. However, only 3 mice were randomly selected for transcriptomic analysis. We have revised the annotations in the manuscript to minimize misunderstanding.

Changes in revised manuscript:

(1) Line 445~447 in yellow.

Comment 6: In Figure 9G, Were the Collagen I and fibronectin blots cropped/originate from

same blot.. ? Kindly provide all original blots in triplicate and mention which was chose for the figures in manuscript.

Reply 6: Thank you for your meticulous review. Thank you for the reminder. We acknowledge that the Collagen I and fibronectin blots in Figure 9G do appear very similar. However, we have located the original bands for both blots and confirmed that they are indeed different images. Additionally, we have prepared all the original Western blot images and uploaded them as supplementary materials for your review.

Changes in revised manuscript:

(1) Unedited blot and gel images.pdf in SUPPLEMENTAL MATERIAL

Comment 7: Why there is inconsistency in # Cat. No. for anti-α-SMA antibody.

Reply 7: Thank you for your meticulous review. Our laboratory has two flow cytometers: one dedicated to cell samples and the other to tissue samples. Due to differences in channel settings, we used the anti-F4/80-BV421 antibody for cell samples and the anti-F4/80-APC antibody for tissue samples. We have also updated the antibody information in the Methods section accordingly.

Changes in revised manuscript:

(2) Line 383~389 in yellow.

Comment 8: Please provide "software used & version and supplier" under "Statistical analysis" section

Reply 8: Thank you for your meticulous review. We have updated the Statistical Analysis section with details on the software used, including the version and supplier information.

Changes in revised manuscript:

(1) Line 470~473 in yellow.

Comment 9: There are lot of formatting issues all over the document (extra space, missing space, capital letter that should not be, missing punctuation). Please carefully revise the manuscript.

Reply 9: Thank you for your meticulous review. I apologize for our oversight. We have reproofed the manuscript and corrected the errors.

Reviewer 2

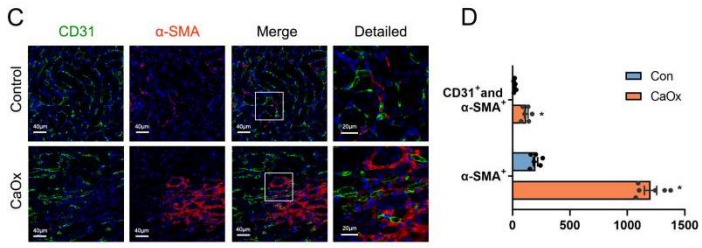
Comment 1: To demonstrate the CaOx induced MMT contributes to kidney fibrosis, a quantitative study of ECM production via non-macrophage myofibroblasts would probably more convicting.

Reply 1: We appreciate your constructive and valuable comments. We have revised our manuscript considerably according to your comments, questions, and suggestions. Given that endothelial cells are another important source of renal myofibroblasts **(Lovisa S, Fletcher-Sananikone E, Sugimoto H, et al. Endothelial-to-mesenchymal transition compromises vascular integrity to induce Myc-mediated metabolic reprogramming in kidney fibrosis. Sci Signal. 2020;13(635):eaaz2597. doi: 10.1126/scisignal.aaz2597.)**, we also utilized IF to detect endothelial-to-mesenchymal transition (EndMT). The results showed that, although EndMT was activated in the kidneys of nephrocalcinosis mice, $CD31^+$ α-SMA^+ accounted for only 9.96% of the total α-SMA^+ cells **(Supplementary Fig. 1C-D)**. These results indicated that in nephrocalcinosis patients and nephrocalcinosis mice, macrophages are one of the major sources of myofibroblasts.

Changes in revised manuscript:

(1) Line 133~139 in yellow;

(2) Supplementary Fig. 1C-D.



Supplementary Fig. 1C-D

Reviewer 3

Comment 1: I have an important question though. If I correctly understand the order of events that are supposed to occur during nephrocalcinosis is as follows: CaOx crystal deposition, macrophage attraction (in order to clear the crystals), MMT cell differentiation (in these cells EZH2 is inducing collagen/fibronectin production as a start of renal fibrosis process). If this is correct I should not expect that in a model of EZH2 depletion, by gene KO (figure 5) or inhibitor (figure 9), the amount of CaOx crystals is lower in case of EZE2 depletion. In contrast, I should expect that an identical number of crystals (induced by glyoxylic acid administration) should induce a lower degree of fibrosis. (One could even conclude it is completely normal that a lower number of crystals induce a milder degree fibrosis). Please can the authors explain this ?

Reply 1: We appreciate your constructive and valuable comments. This is a very interesting question. We have added additional explanation to this issue in the Discussion section. Crystal deposition in kidney is a complex process often accompanied by renal interstitial fibrosis **(Evan A, Lingeman J, Coe FL, et al. Randall's plaque: pathogenesis and role in calcium oxalate nephrolithiasis. Kidney Int. 2006 Apr;69(8):1313-8. doi: 10.1038/sj.ki.5000238.)**. Recent studies have shown that collagen deposition can further promotes kidney stone formation through collagen mineralization and the expansion of crystal plaques **(Khan SR, Canales BK,**

Dominguez-Gutierrez PR. Randall's plaque and calcium oxalate stone formation: role for immunity and inflammation. Nat Rev Nephrol. 2021 Jun;17(6):417-433. doi: 10.1038/s41581-020-00392-1.). Consequently, EZH2 deletion or inhibition not only reduced collagen deposition but also prevented further crystal deposition from the early stages.

Changes in revised manuscript:

(1) Line 316~320 in yellow.

Reviewer 4

Comment 1:The animal RNAseq profiles must be deposited into a publicly accessible database –NCBI GEO or EMBL Expression Atlas are the two most common. This is a non-negotiable point. There is simply no way to verify RNAseq findings reported without the information being available to the scientific community. No methods are provided for how differential expression analyses was performed. Did the authors use DESeq2, Voom-Limma, or something else?

Reply 1: We appreciate your constructive and valuable comments. (1) We have supplemented the methods of RNA-seq and bioinformatics analysis in detail in the Methods section. "Differential expression genes were determined by using the "limma" package in the R software with the criteria of $|\log_2 FC|>0.75$ and adjust. The "ggplot2" package and normalized data was used to draw heatmaps and volcano plots for visualization." (2) We uploaded the raw data of transcriptome sequencing through Gene Expression Omnibus (GEO). The datasets analyzed for this study can be found in the Gene GEO of NCBI (https://www.ncbi.nlm.nih.gov/geo/) (Accession: GSE280007). Supporting data related to this work are available upon request. It should be noted that due to unknown reasons, the raw data for the EZH2 KO group failed to upload. As a substitute, we have uploaded the corresponding processed data in the supplementary materials. We hope for your

understanding. Once the technical issue is resolved, we will promptly submit the EZH2 KO group data to GEO.

Changes in revised manuscript:

(1) Line 449~456 in yellow;

(2) Line 481~483 in yellow.

Comment 2: While EZH2 was clearly a reasonable target to go after given the data presented in Figure 3, what is the rationale for preferentially focus on this given the number of DEGs among methyltransferase genes? Without the full DEG profile available (see first point), there's no way to know if this was a reasonable gene target to pursue.

Reply 2: We uploaded the raw data of RNA-Seq through Gene Expression Omnibus (GEO). The datasets analyzed for this study can be found in the Gene GEO of NCBI (https://www.ncbi.nlm.nih.gov/geo/) (Accession: GSE280007). EZH2 is one of the most significantly altered genes among the differentially expressed genes, which is the primary reason for our subsequent research focus on it.

Changes in revised manuscript:

(1) Line 481~483 in yellow.

Comment 3: There are no methods for any of the single-cell RNAseq analysis from the Kidney Precision Medicine Project – not for cell annotations and not for differential expression analyses. Please provide sufficient details on these analyses.

• I believe the bar plots are incorrect. Median expression of EZH2 across these cell types would be 0 as the vast majority of cells do not express EZH2 (see attached violin plots and dot plots for EZH2 expression in monocytes from this dataset). It is true that some monocytes in patients

with AKI do have higher EZH2 expression, but again, the bulk of cells do not express EZH2 which makes any differential expression calculation suspect.

• Given the prominent up-regulation shown for monocytes and phagocytic macrophages in Figure 4E, I tried to replicate the upregulation in each cell type (reference vs AKI). Using a Wilcoxon rank sum approach, the difference between patients with AKI and control samples was not significant for monocytes (p=0.27, p-adj=0.57) nor for dendritic macrophages (p=0.54, p-adj=0.74).

Reply 3: Thank you for your meticulous review and kind reminder. We reanalyzed the data using the KMAP database. As you pointed out, the KMAP data does not support the conclusion that EZH2 is significantly upregulated in renal macrophages during AKI. Therefore, we have removed Figure 4D, 4E, Supplementary Figure 2, and the related descriptions. As a replacement, we used snATAC-Seq data from the Kidney Interactive Transcriptomics database, which indicated that EZH2 was activated in immune cells during the chronic progression of the injury (Fig. 4D). The relevant methodology has been detailed in the Methods section.

Changes in revised manuscript:

(1) Line 163~165. 452~456 in yellow;

(2) Fig. 4D.

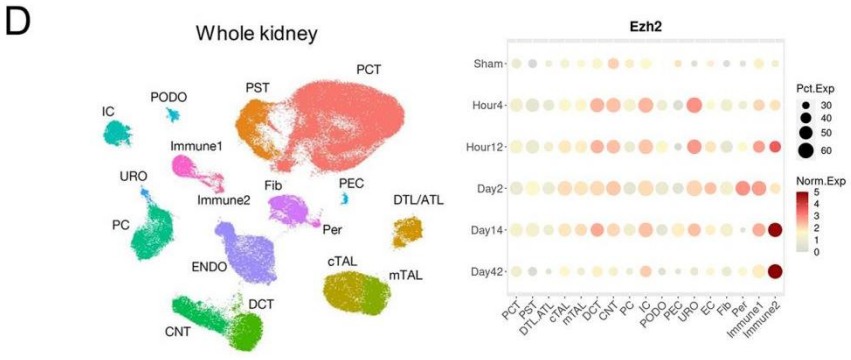


Fig. 4D

Comment 4: the statement on the collagen 1 western blot seems reasonable but the blot has a number of bubbles which make quantitation suspect. Please re-run the collagen 1 western blot.

Reply 4: Thank you for your meticulous review and kind reminder. We have repeated the Western blot experiment and replaced the images with clearer bands. Additionally, we have prepared all the original Western blot images and uploaded them as supplementary materials for your review.

Changes in revised manuscript:

(1) Fig. 6D.

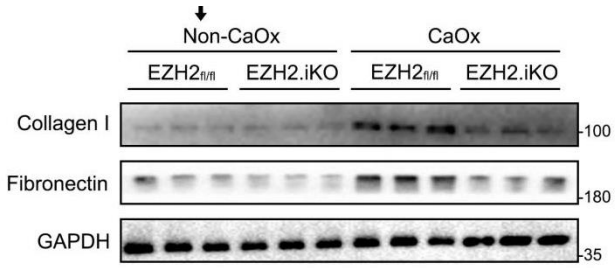


Fig. 6D

Comment 5: For all heatmaps, please indicate whether these are absolute, normalized, or median centered levels of expression.

Reply 5: Thanks for your good suggestion. Normalized data was used to draw heatmaps. We have provided additional details in the Methods section.

Changes in revised manuscript:

(1) Line 449~451 in yellow.

Comment 6: Line 117 – A high level of MMT cell infiltration was associated with glomerular filtration rate decline (Fig 1F) . As far as I can tell this is measuring GFR at a single point in time for each sample and not a GFR decline (which is a rate over time per sample). "MMT cell

infiltration was associated with lower glomerular filtration rates." is more appropriate wording here.

Reply 6: Thanks for your good suggestion. We have revised the descriptions according to your suggestions to ensure clearer expression.

Changes in revised manuscript:

(1) Line 31~32, 117~118, 297~298 in yellow.

Comment 7: Line 139, add s to macrophage.

Reply 7: Thanks for your good suggestion. We apologize for our spelling errors. We have revised according to your suggestions.

Changes in revised manuscript:

(1) Line 144 in yellow.

Comment 8: Line 154 - Why was literature review performed to summarize histone methyltransferase enzymes (Supplemental Table 3) rather than leveraging available Histone Methyltransferase gene sets Gene Ontology?

Reply 8: Thanks for your good suggestion. We apologize for the inconsistent terminology. We intended to refer to the "Histone methyltransferase gene" and have made the corrections.

Changes in revised manuscript:

(1) Line 159 in yellow;

(2) Supplementary Table 3.

Comment 9: Line 157 – Unless the authors specific excluded patient samples from tumor nephrectomies, healthy specimens is not correct. The reference tissue from KPMP is comprised

of both living transplant donors and tumor nephrectomies.

Reply 9: Thanks for your good suggestion. We have removed the KMAP generated Figure 4D, 4E, Supplementary Figure 2, and the related descriptions. As a replacement, we used snATAC-Seq data from the Kidney Interactive Transcriptomics database, which indicated that EZH2 was activated in immune cells during the chronic progression of the injury (Fig. 4D). The relevant methodology has been detailed in the Methods section.

Changes in revised manuscript:

(1) Line 163~165. 452~456 in yellow;

(2) Fig. 4D.

Comment 10: Line 217 – "knockdown of EZH2 restored the expression of H3K27me3 and DUSP23 (Figure 8D) "? While the restoration of DUSP23 is clear, knockdown of EZH2 does not restore H3K27me3, it reduces it.

Reply 10: Thank you for your meticulous review. We apologize for our errors. We have revised.

Changes in revised manuscript:

(1) Line 221~222 in yellow.

Comment 11: Line 237 should read …EZH2 suppressed pSMAD3(Ser423/425) instead of simply pSMAD3 as pSMAD3 (Thr179) is not affected.

Reply 11: Thank you for your meticulous review. We apologize for the mistake. We have revised.

Changes in revised manuscript:

(1) Line 242~243 in yellow.

Comment 12: Figure 2 and figure 3 legend – insert space between nephorcalcinosis and mice (lines 606, 607)and nephrocalcinosis and group (line 617)

Reply 12: Thank you for your meticulous review. We apologize for our errors. We have revised according to your suggestions.

Changes in revised manuscript:

(1) Line 649~650, 662 in yellow.

We really appreciate the editor and reviewers' positive and insightful suggestions on our manuscript. And we have revised our manuscript considerably according to their comments point by point. Under your kind help and professional guidance, we believe that our manuscript has been improved substantially. We are looking forward to your further suggestions.

Sincerely yours,

Fan Cheng
E-mail: urology1969@aliyun.com

12/05/2024

Response to reviewers

Dear reviewers:

On behalf of my co-authors, we thank you very much for your letter and comments on our manuscript entitled ***" EZH2-mediated macrophage-to-myofibroblast transition contributes to calcium oxalate crystal-induced kidney fibrosis ".***

We appreciate the constructive and valuable comments. We have revised our manuscript considerably according to the comments, questions, and suggestions. In the event that we missed any one of the comments please let us know. The revised portion are marked in YELLOW in revised manuscript.

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deposition in kidney was evaluated by measuring hydroxyproline concentration. Consistent with the Masson staining, hydroxyproline assay also indicated that the EZH2 deletion attenuated CaOx crystal-induced kidney fibrosis (Fig. 2C; Fig. 3D; Supplementary Fig. 2B-C).

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(2) Fig. 2C; Fig. 3D; Supplementary Fig. 2B-C.

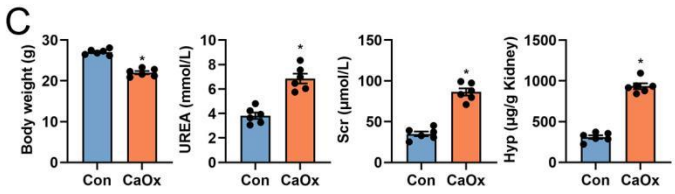


Fig. 2C

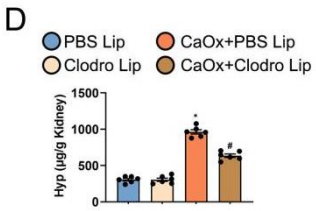
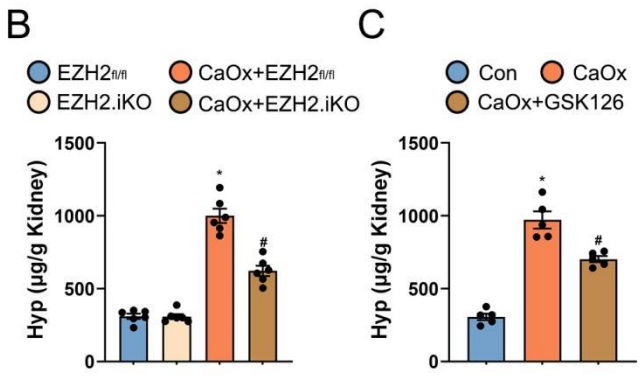


Fig. 3D



Supplementary Fig. 2B-C

Comment 2: Inconsistent capitalization: The capitalization of words are inconsistent throughout the text.

Reply 2: Thanks for your good suggestion. I apologize for our oversight. We have reproofed the manuscript and corrected the errors.

Comment 3: Spelling errors: A few words are misspelled, such as "privious" (393 line) instead of "previous" and "whereafter" (196, 376 line) instead of "where after".

Reply 3: Thank you for your meticulous review. We have corrected the errors.

Changes in revised manuscript:

(1) Line 201, 404, and 386 in yellow.

Comment 4: Abbreviation inconsistencies: The abbreviations "COM" at line 384 and 629 are different.

Reply 4: Thank you for your meticulous review. We have corrected the spelling errors.

Changes in revised manuscript:

(1) Line 168, 394, 763 and in yellow.

Comment 5: In Figure 7, author mentioned n=6 (line-360), n=3 (line-127), n=5 (line-651), n=3 (line-652) why this description of experimental groups and results varies between sections. Author mentioned animals n=6, each group, but why n=3 at line 127?

Reply 5: Thanks for your good suggestion. We apologize for the confusion caused by our unclear description. In this study, 6 mice were included in each group for histological analysis. However, only 3 mice were randomly selected for transcriptomic analysis. We have revised the annotations in the manuscript to minimize misunderstanding.

Changes in revised manuscript:

(1) Line 445~447 in yellow.

Comment 6: In Figure 9G, Were the Collagen I and fibronectin blots cropped/originate from

same blot.. ? Kindly provide all original blots in triplicate and mention which was chose for the figures in manuscript.

Reply 6: Thank you for your meticulous review. Thank you for the reminder. We acknowledge that the Collagen I and fibronectin blots in Figure 9G do appear very similar. However, we have located the original bands for both blots and confirmed that they are indeed different images. Additionally, we have prepared all the original Western blot images and uploaded them as supplementary materials for your review.

Changes in revised manuscript:

(1) Unedited blot and gel images.pdf in SUPPLEMENTAL MATERIAL

Comment 7: Why there is inconsistency in # Cat. No. for anti-α-SMA antibody.

Reply 7: Thank you for your meticulous review. Our laboratory has two flow cytometers: one dedicated to cell samples and the other to tissue samples. Due to differences in channel settings, we used the anti-F4/80-BV421 antibody for cell samples and the anti-F4/80-APC antibody for tissue samples. We have also updated the antibody information in the Methods section accordingly.

Changes in revised manuscript:

(2) Line 383~389 in yellow.

Comment 8: Please provide "software used & version and supplier" under "Statistical analysis" section

Reply 8: Thank you for your meticulous review. We have updated the Statistical Analysis section with details on the software used, including the version and supplier information.

Changes in revised manuscript:

(1) Line 470~473 in yellow.

Comment 9: There are lot of formatting issues all over the document (extra space, missing space, capital letter that should not be, missing punctuation). Please carefully revise the manuscript.

Reply 9: Thank you for your meticulous review. I apologize for our oversight. We have reproofed the manuscript and corrected the errors.

Reviewer 2

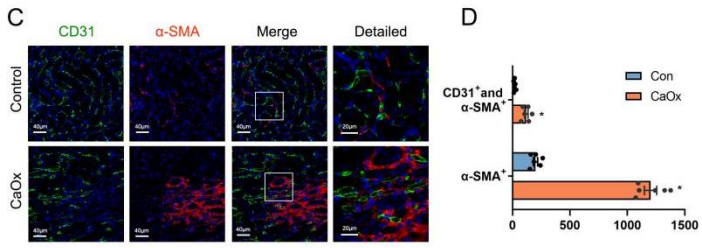
Comment 1: To demonstrate the CaOx induced MMT contributes to kidney fibrosis, a quantitative study of ECM production via non-macrophage myofibroblasts would probably more convicting.

Reply 1: We appreciate your constructive and valuable comments. We have revised our manuscript considerably according to your comments, questions, and suggestions. Given that endothelial cells are another important source of renal myofibroblasts **(Lovisa S, Fletcher-Sananikone E, Sugimoto H, et al. Endothelial-to-mesenchymal transition compromises vascular integrity to induce Myc-mediated metabolic reprogramming in kidney fibrosis. Sci Signal. 2020;13(635):eaaz2597. doi: 10.1126/scisignal.aaz2597.)**, we also utilized IF to detect endothelial-to-mesenchymal transition (EndMT). The results showed that, although EndMT was activated in the kidneys of nephrocalcinosis mice, $CD31^+$ α-SMA^+ accounted for only 9.96% of the total α-SMA^+ cells **(Supplementary Fig. 1C-D)**. These results indicated that in nephrocalcinosis patients and nephrocalcinosis mice, macrophages are one of the major sources of myofibroblasts.

Changes in revised manuscript:

(1) Line 133~139 in yellow;

(2) Supplementary Fig. 1C-D.



Supplementary Fig. 1C-D

Reviewer 3

Comment 1: I have an important question though. If I correctly understand the order of events that are supposed to occur during nephrocalcinosis is as follows: CaOx crystal deposition, macrophage attraction (in order to clear the crystals), MMT cell differentiation (in these cells EZH2 is inducing collagen/fibronectin production as a start of renal fibrosis process). If this is correct I should not expect that in a model of EZH2 depletion, by gene KO (figure 5) or inhibitor (figure 9), the amount of CaOx crystals is lower in case of EZE2 depletion. In contrast, I should expect that an identical number of crystals (induced by glyoxylic acid administration) should induce a lower degree of fibrosis. (One could even conclude it is completely normal that a lower number of crystals induce a milder degree fibrosis). Please can the authors explain this ?

Reply 1: We appreciate your constructive and valuable comments. This is a very interesting question. We have added additional explanation to this issue in the Discussion section. Crystal deposition in kidney is a complex process often accompanied by renal interstitial fibrosis **(Evan A, Lingeman J, Coe FL, et al. Randall's plaque: pathogenesis and role in calcium oxalate nephrolithiasis. Kidney Int. 2006 Apr;69(8):1313-8. doi: 10.1038/sj.ki.5000238.)**. Recent studies have shown that collagen deposition can further promotes kidney stone formation through collagen mineralization and the expansion of crystal plaques **(Khan SR, Canales BK,**

Dominguez-Gutierrez PR. Randall's plaque and calcium oxalate stone formation: role for immunity and inflammation. Nat Rev Nephrol. 2021 Jun;17(6):417-433. doi: 10.1038/s41581-020-00392-1.). Consequently, EZH2 deletion or inhibition not only reduced collagen deposition but also prevented further crystal deposition from the early stages.

Changes in revised manuscript:

(1) Line 316~320 in yellow.

Reviewer 4

Comment 1:The animal RNAseq profiles must be deposited into a publicly accessible database –NCBI GEO or EMBL Expression Atlas are the two most common. This is a non-negotiable point. There is simply no way to verify RNAseq findings reported without the information being available to the scientific community. No methods are provided for how differential expression analyses was performed. Did the authors use DESeq2, Voom-Limma, or something else?

Reply 1: We appreciate your constructive and valuable comments. (1) We have supplemented the methods of RNA-seq and bioinformatics analysis in detail in the Methods section. "Differential expression genes were determined by using the "limma" package in the R software with the criteria of $|\log_2 FC|>0.75$ and adjust. The "ggplot2" package and normalized data was used to draw heatmaps and volcano plots for visualization." (2) We uploaded the raw data of transcriptome sequencing through Gene Expression Omnibus (GEO). The datasets analyzed for this study can be found in the Gene GEO of NCBI (https://www.ncbi.nlm.nih.gov/geo/) (Accession: GSE280007). Supporting data related to this work are available upon request. It should be noted that due to unknown reasons, the raw data for the EZH2 KO group failed to upload. As a substitute, we have uploaded the corresponding processed data in the supplementary materials. We hope for your

understanding. Once the technical issue is resolved, we will promptly submit the EZH2 KO group data to GEO.

Changes in revised manuscript:

(1) Line 449~456 in yellow;

(2) Line 481~483 in yellow.

Comment 2: While EZH2 was clearly a reasonable target to go after given the data presented in Figure 3, what is the rationale for preferentially focus on this given the number of DEGs among methyltransferase genes? Without the full DEG profile available (see first point), there's no way to know if this was a reasonable gene target to pursue.

Reply 2: We uploaded the raw data of RNA-Seq through Gene Expression Omnibus (GEO). The datasets analyzed for this study can be found in the Gene GEO of NCBI (https://www.ncbi.nlm.nih.gov/geo/) (Accession: GSE280007). EZH2 is one of the most significantly altered genes among the differentially expressed genes, which is the primary reason for our subsequent research focus on it.

Changes in revised manuscript:

(1) Line 481~483 in yellow.

Comment 3: There are no methods for any of the single-cell RNAseq analysis from the Kidney Precision Medicine Project – not for cell annotations and not for differential expression analyses. Please provide sufficient details on these analyses.

• I believe the bar plots are incorrect. Median expression of EZH2 across these cell types would be 0 as the vast majority of cells do not express EZH2 (see attached violin plots and dot plots for EZH2 expression in monocytes from this dataset). It is true that some monocytes in patients

with AKI do have higher EZH2 expression, but again, the bulk of cells do not express EZH2 which makes any differential expression calculation suspect.

• Given the prominent up-regulation shown for monocytes and phagocytic macrophages in Figure 4E, I tried to replicate the upregulation in each cell type (reference vs AKI). Using a Wilcoxon rank sum approach, the difference between patients with AKI and control samples was not significant for monocytes (p=0.27, p-adj=0.57) nor for dendritic macrophages (p=0.54, p-adj=0.74).

Reply 3: Thank you for your meticulous review and kind reminder. We reanalyzed the data using the KMAP database. As you pointed out, the KMAP data does not support the conclusion that EZH2 is significantly upregulated in renal macrophages during AKI. Therefore, we have removed Figure 4D, 4E, Supplementary Figure 2, and the related descriptions. As a replacement, we used snATAC-Seq data from the Kidney Interactive Transcriptomics database, which indicated that EZH2 was activated in immune cells during the chronic progression of the injury (Fig. 4D). The relevant methodology has been detailed in the Methods section.

Changes in revised manuscript:

(1) Line 163~165. 452~456 in yellow;

(2) Fig. 4D.

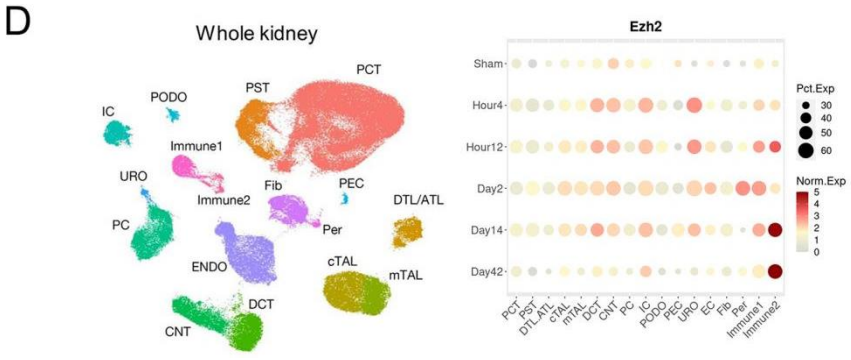


Fig. 4D

Comment 4: the statement on the collagen 1 western blot seems reasonable but the blot has a number of bubbles which make quantitation suspect. Please re-run the collagen 1 western blot.

Reply 4: Thank you for your meticulous review and kind reminder. We have repeated the Western blot experiment and replaced the images with clearer bands. Additionally, we have prepared all the original Western blot images and uploaded them as supplementary materials for your review.

Changes in revised manuscript:

(1) Fig. 6D.

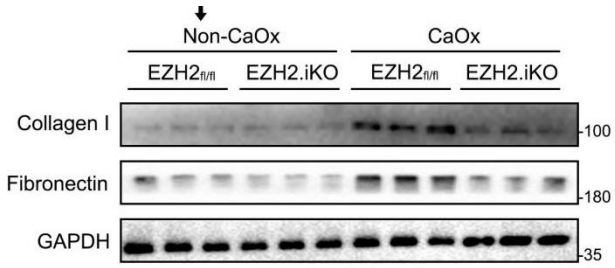


Fig. 6D

Comment 5: For all heatmaps, please indicate whether these are absolute, normalized, or median centered levels of expression.

Reply 5: Thanks for your good suggestion. Normalized data was used to draw heatmaps. We have provided additional details in the Methods section.

Changes in revised manuscript:

(1) Line 449~451 in yellow.

Comment 6: Line 117 – A high level of MMT cell infiltration was associated with glomerular filtration rate decline (Fig 1F) . As far as I can tell this is measuring GFR at a single point in time for each sample and not a GFR decline (which is a rate over time per sample). "MMT cell

infiltration was associated with lower glomerular filtration rates." is more appropriate wording here.

Reply 6: Thanks for your good suggestion. We have revised the descriptions according to your suggestions to ensure clearer expression.

Changes in revised manuscript:

(1) Line 31~32, 117~118, 297~298 in yellow.

Comment 7: Line 139, add s to macrophage.

Reply 7: Thanks for your good suggestion. We apologize for our spelling errors. We have revised according to your suggestions.

Changes in revised manuscript:

(1) Line 144 in yellow.

Comment 8: Line 154 - Why was literature review performed to summarize histone methyltransferase enzymes (Supplemental Table 3) rather than leveraging available Histone Methyltransferase gene sets Gene Ontology?

Reply 8: Thanks for your good suggestion. We apologize for the inconsistent terminology. We intended to refer to the "Histone methyltransferase gene" and have made the corrections.

Changes in revised manuscript:

(1) Line 159 in yellow;

(2) Supplementary Table 3.

Comment 9: Line 157 – Unless the authors specific excluded patient samples from tumor nephrectomies, healthy specimens is not correct. The reference tissue from KPMP is comprised

of both living transplant donors and tumor nephrectomies.

Reply 9: Thanks for your good suggestion. We have removed the KMAP generated Figure 4D, 4E, Supplementary Figure 2, and the related descriptions. As a replacement, we used snATAC-Seq data from the Kidney Interactive Transcriptomics database, which indicated that EZH2 was activated in immune cells during the chronic progression of the injury (Fig. 4D). The relevant methodology has been detailed in the Methods section.

Changes in revised manuscript:

(1) Line 163~165. 452~456 in yellow;

(2) Fig. 4D.

Comment 10: Line 217 – "knockdown of EZH2 restored the expression of H3K27me3 and DUSP23 (Figure 8D) "? While the restoration of DUSP23 is clear, knockdown of EZH2 does not restore H3K27me3, it reduces it.

Reply 10: Thank you for your meticulous review. We apologize for our errors. We have revised.

Changes in revised manuscript:

(1) Line 221~222 in yellow.

Comment 11: Line 237 should read …EZH2 suppressed pSMAD3(Ser423/425) instead of simply pSMAD3 as pSMAD3 (Thr179) is not affected.

Reply 11: Thank you for your meticulous review. We apologize for the mistake. We have revised.

Changes in revised manuscript:

(1) Line 242~243 in yellow.

Comment 12: Figure 2 and figure 3 legend – insert space between nephorcalcinosis and mice (lines 606, 607)and nephrocalcinosis and group (line 617)

Reply 12: Thank you for your meticulous review. We apologize for our errors. We have revised according to your suggestions.

Changes in revised manuscript:

(1) Line 649~650, 662 in yellow.

We really appreciate the editor and reviewers' positive and insightful suggestions on our manuscript. And we have revised our manuscript considerably according to their comments point by point. Under your kind help and professional guidance, we believe that our manuscript has been improved substantially. We are looking forward to your further suggestions.

Sincerely yours,

Fan Cheng
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