Peer Review File

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Reviewer A

Comment 1: Osteogenic medium (OM) and osteoblastic medium (OM) are mixed in your manuscript. Please check.

Reply 1: Thank you for this question. We have replaced osteoblastic medium (OM) with Osteogenic medium (OM) (see Page3, line3; Page20, line299)

Comment 2: Authors should provide the number of patients for quantification of gal-3 mRNA. Also, please provide quantitative data with statistics for western blotting.

Reply 2: Thank you for this question. We have rewritten the figure 1 legend and added quantitative data with statistics for western blotting.

Comment 3: Immunohistochemical staining is useful for identification of gal-3-expressing cell types. Please provide the high-magnified images of gal-3-positive VICs in figure 1.

Reply 3: Thank you for this question. We have provided the high-magnified images of Immunohistochemical staining in figure 1D.

Comment 4: In fig2D, please provide quantitative data of the areas stained by red.

Reply 4: Thank you for this question. We have provided the quantitative data of the areas stained by red in figure 1D and figure 4H.

Comment 5: What are the source cells of Gal-3 production during osteoblast differentiation? Knockdown experiment targeting gal-3 is one of the solutions to clear whether VIC-derived Gal-3 promotes osteoblast differentiation via autocrine pathway.

Reply 5: We thank the reviewer for the comments. The source of Gal-3 is an important question in our study. In view of the special microenvironment of the human aortic valve, we believed that the more likely source of Gal-3 is from blood. Blood-derived Gal-3 stimulates the valve interstitial cell to undergo osteogenic transformation while we cannot rule out the possibility that autocrine Gal-3 is another stimulation. Since VIC is a collective name for a type of cell group, it contains many different types of cells, so it may require more experiments to explore the source of Gal-3.

Comment 6: Although 'control, OM, and OM+BAY11-7082' are labelled in the figure4D, authors described 'after 30 minutes of Gal3 treatment (Line 250, page14)' in the main body and figure legends. Gal-3-treated VICs? or OM-treated VICs? Please check these sentences.

Reply 6: Thank you for this question. We have corrected the mistake of label in figure4D, we treated VIC with Gal-3.

Comment 7: Many misspell are found. Please correct 'contral' to 'control' in fig 2E, 'Vitmentinl' to 'Vimentin' in fig 2E. Please check entire manuscript carefully.

Reply 7: Thank you for this question. We have corrected the spelling mistake. (see Page20, Figure 2)

Reviewer B:

Comment 1: As the authors discussed in the Discussion, the biggest problem of this paper is that the control group was heart patients, and they were terminal heart patients, lacking healthy controls. I suggest that the authors MATCH blood samples from at least 20 healthy individuals to supplement this.

Reply 1: Your insightful review is greatly appreciated. We collected 20 healthy individuals blood samples. The table below showed the result that Gal-3 levels of CAVD patients were significantly higher than that in healthy group. This result was in line with the conclusions in the published articles.

	Normal（N=20）	CAVD（N=20）	*P* value
Age (years)	53.2 ± 7.42	54.15 ± 8.01	0.6994
Male (%)	16 (80%)	16 (80%)	1
Smoking (%)	11 (55%)	10 (50%)	0.6531
Gal-3(ng/mL)	0.092 ± 0.143	9.82 ± 2.58	**<0.0001**

Comment 2: As with the previous question, it can be seen in Table 1 that there is a significant gender difference and the smoking difference between the CAVD group and the control group. This is not sufficient to mention with Limitation of Discussion. It needs to be discussed in detail in the Discussion and the possible impact of these two factors on the results. This is one of the important reasons I insisted that the authors supplement the blood of at least 20 matched healthy individuals.

Reply 2: Thank you for bringing this to our attention. According to the published articles, smoking and gender are two important risk factors for the onset of CAVD. Therefore, we were affected by the corresponding risk factors when we obtained samples from the hospital within a certain period of time. The proportion of male patients undergoing surgery is much higher than that of female patients. Factor related to smoking is also in the same situation.

Comment 3: As shown in Figure 3, RELA is listed before NF-κ B. The authors need to clarify why NF-κ B was chosen instead of RELA for the in-depth study? Again, in the Introduction section, I would like to see a complete story, not just what was done. More interested in why the authors came up with this idea. After the 1st and 2nd paragraphs of the Introduction, there is a paragraph to give the rationale for this study.

Reply 3: Thank you for this question. In figure 3D, RelA is listed before NF-κB1. Both RelA and NF-κB1 are NF-κB protein family members. Gal-3, a protein from the blood, is a an exogenous stimulus to the calcification of aortic valve. Therefore, we prefer to study the changes in upstream factors of NF-κB Signaling pathways. IκB is the inhibitor of NF-κB activition. NF-κB is activited

only when IκB is phosphorylated, so we choose IκBα as the focus of our subsequent study. We have made some modifications in the introduction. (see Page6, line95-99).

Comment 4: Critical point is that the authors found in figure 4A a decrease in phosphorylation after 30 min and to almost the same level later as in the no-treatment group. We know that in the body is a chronic process and not an acute situation of 30 min. Do the authors need to explain why it was upregulated only at 0-30 minutes? How to explain the contradiction in between?

Reply 4: Thank you for this question. As you said, calcification is a chronic process in the body, However, it does not take a long time to induce calcification in cell experiments in vitro, because long-term induction will lead to apoptosis. The process of molecular phosphorylation changes rapidly when cells are stimulated by external stimuli. We found that upon stimulation of hVICs by Gal-3 for 30 min, p-IκBα expression increased two-folds compared to the control group.

Comment 5: Figure 4E and Figure 4F should preferably also have the Gal-3-/BAY11-7082+ group to be more convincing. Also, the authors need to explain why BAY11-7082, an inhibitor of IkBa phosphorylation, its treatment alone does not affect phosphorylated IkBa levels.

Reply 5: Your suggestion is greatly appreciated. In Figure 4E, 4F also 4G and 4H, we tried to simulate a treatment process at the cellular level, wheather the cellular osteogenic transfomation could be blocked or not is teated, so we didn't set the group Gal-3-/BAY11-7082+. In Figure 4B and 4C we can see that in Gal-3-/BAY11-7082+ group the expression of P-IκBα. We thinck under physiological conditions, when inhibitors are used alone, there must be other alternative activation methods to maintain this important biochemical activity.

Comment 6: What do #1, #2, #3, #4, #5 in Figure 1C stand for? I need to explain in the figure legends.

Reply 6: Thank you for this question. We are sorry for the confusion due to our negligence of elucidations for Figure 1C. The aortic valves used for immunoblotting (Figure C) were came from two parts: 5 normal aortic valve donors and 5 calcified aortic valve donors. The #1, #2, #3, #4, #5 respectively represent the label of the patient. We have added an explanation at the corresponding position in the figure legend. (see Page18, figure1).

Comment 7: Each figure in Figure 1 and figure 2 needs to state what the sample size is.

Reply 7: Thank you for your valuable suggestion. We have rewritten the figure legend of figure 1 and 2. (see Page18, Figure 1; Page20, Figure 2)

Comment 8: The OM in Figure 4D should be Gal-3?

Reply 8: Thank you for this question. We are very sorry that we mistakenly marked Gal-3 as OM in

Figure 4D. We have replaced OM with Gal-3. (see Page23, Figure 4D)

Second round of Peer review comments

Comment 1: In upper panels of figure1E, what does the black square indicate? Is it an enlarged view of the area of upper panel? If so, I think that the area does not match enlarged area. Please check.

Reply 1: Thank you for this question. We have redrawn the figure1E (see Page18). The black squares represent the enlarged areas.

Comment 2: Author should also add the method regarding quantification of stained area by Alizarin Red S solution in the material & method or figure legends.

Reply 2: Thank you for this question. We have added the method regarding quantification of stained area by Alizarin Red S solution in the material & method (see Page9, line155-156).

Comment 3: It is important to know whether Gal-3 expression in VICs is induced during osteoblast differentiation. Given that protein and mRNA levels of Gal-3 are upregulated in the VICs of CAVD patients (Figure 1), I think that VICs are a major source of Gal-3. If so, the efficiency of osteoblast differentiation by OM treatment will be inhibited by Gal-3 siRNA. If Gal-3 knockdown will not affect osteoblast differentiation, authors will be able to conclude that blood-derived Gal-3 (via unknown mechanism) is critical for valve calcification. To clear whether Gal-3-positive VICs (fig1E) is a good therapeutic target for valve calcification, knockdown experiments and quantifications of gal-3 mRNA levels in control and OM-treated VICs or inclusion of these points in the discussion section will be helpful in the future study.

Reply 3: Thank you for this question. We have knockdown the Gal-3 in VICs, the efficiency of osteoblast differentiation by OM treatment shows any significant changes, just as we thought. In view of the special microenvironment of the human aortic valve, we believed that the more likely source of Gal-3 is from blood. Blood-derived Gal-3 stimulates the valve interstitial cell to undergo osteogenic transformation.