Author's response to reviews

Title: Manipulation of Valve Composition to Elucidate the Role of Collagen in Aortic Valve Calcification

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Author's response to reviews: see over
The authors genuinely thank the reviewers for their helpful feedback and suggestions. We have addressed the reviewer concerns as indicated in the responses below:

**Reviewer 1:**

1. *Previous studies have used excised leaflets in bioreactors to study the effects of strain (along with various biochemical stimuli) on leaflet changes. These studies should be mentioned in the present manuscript with special emphasis placed on the difference between static and strained leaflets in these previous studies. Could the lack of strain lead to changes in the leaflet composition (i.e., the apparent drop in HA content) over time in the control samples in the current study? Adding a dynamically strained culture condition is probably beyond the scope of the current study, but the authors should at least address this limitation.*

The reviewer is correct, the lack of mechanical stimulation in this work should indeed be addressed in the manuscript. Although we were pleased to generally retain the Day 0 characteristics in the undepleted valves over 6 days, we also recognize that: 1) a dynamic environment would likely be more physiologically relevant, and 2) our outcomes may be different with mechanical stimulation. A paragraph dedicated to discussion of these issues has been added to the manuscript.

2. *Figure 4 is missing a significance marker.*

The reviewer is correct, and this error has been fixed.

3. *The authors mention that the changes in VIC phenotype were not found “to occur when ECM components other than HA or collagen are depleted.” This certainly increases the strengths of the current results, but it would be nice to mention the ECM components that have been tested and show these results in this study.*

Other treatments included: elastase (to deplete elastin), and chondroitinase (to deplete chondroitin sulfate). The analysis of these conditions was limited to histology, but neither of these conditions were associated with an increase in a-SMA, ALP, or mineralization (by von Kossa). We’ve included some representative images from our elastase trials in a Data Supplement.

**Reviewer 2:**

1. *Histological representation of collagen depletion by pentachrome staining would be informative in Figure 1.*

This is an excellent suggestion and has now been included in the manuscript (Figure 1G).

2. *It would be easier to appreciate the calcification in von Kossa stained sections if panels were included at higher magnification in Figure 7A.*

The authors agree that this better illustrates the differences in staining between conditions, and we have included higher magnification images in Figure 7A.

3. *Additional information in the figure legends would be helpful. For example, it is not completely clear that the Westerns in Figures 4 and 5 were performed on day 6 tissue. This is implied, but not explicitly stated.*

The Figure captions were indeed too sparse; this has now been fixed.
4. *The last sentence of the discussion is a bit confusing*

The last sentence has been reworded for clarity.