Hydrostatic mechanical stress regulates growth and maturation of the atrioventricular Valve

David M Bassen, Mingkun Wang, Duc Pham, Shuofei Sun, Rashmi Rao, Rishabh Singh and Jonathan Butcher
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AUTHORS: David M Bassen, Duc Pham, Mingkun Wang, Rashmi Rao, Rishabh Singh, and Jonathan Butcher

I sincerely apologise for the long delay before being able to come back to you. The current circumstances tend to make the review process longer than usual. I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors investigated the role of tensile and compressive stress during valve development. They used osmotic stress on atrioventricular (AV) cushions to analyze valve growth and maturation. Compressive stress is obtained with hypertonic medium, while tensile stress is obtained with hypotonic medium. Proliferation increased with compressive stress condition. The authors examined the role of BMP signaling in valve growth. Compress stress condition showed a highest pSmad1/5 activity compared to control and tensile stress conditions. BMP treatment decreased compaction while treatment with BMP receptor inhibitor induced compaction. Based on these observations the authors conclude that compressive stress regulates tissue compaction through BMP signaling. To assess contractile mechanism for osmotic and BMP growth phenotype, the authors used a myosin inhibitor mostly specific for myosin II (Blebbistatin). Thus, tissue compaction was inhibited at lower doses of blebbistatin. Blebbistatin treatment can also block tissue rounding. They authors found that Rho activity localized to endothelial cells during compaction and that RhoA-GTP staining patterns in the cytoplasm of cells under compressive. BMP treatment reduced Ser-19 NM-Myosin II light chain activation. To validate their observations the authors used left-atrial-ligation model to alter the hemodynamics on the AV valve. In this model pSmad1/5 staining is increased in the mitral valve leaflets. They found spatial localization of pSmad, pERK and pSer-19 NM Myosin II within the cushion. Using cushions from different staged embryos the authors showed that compressive/tensile stress regulates growth and compaction across stages of valve development. In conclusion, the authors propose that compression promotes growth while tension promotes cushion remodeling and leaflet thinning. These finding are important to understand how mechanical environment modify activation of signaling pathway during valve development.

Comments for the author

While the basic insight regarding the role of mechanical stress during growth and maturation of the AV valve is interesting, there are some weakness to the arguments and data reported in this study.

Major concerns:
- My main concern is related to the identification of the mechanism controling changes in contractile and proliferation behaviors. To determine the role of tensile and compressive stress the authors added or reduced the level of sucrose in the base media, which is known to increase or decrease osmolarity respectively. Indeed, studies have shown that glucose promotes cell proliferation through AMPK/mTor MAPK pathways. The authors should demonstrate that compressive/tensile stress directly regulate cell proliferation.
- Another concern is related to the type of cells which response to the mechanical stress induced by the osmolarity. AV cushions are composed of endothelial and mesenchymal cells. Thus, the authors should show which cells proliferate and if there is a difference in the activation of the BMP signaling between endothelial and mesenchymal cells.
- It is clear that contractility is important during valve growth and maturation. The authors should further examine the cellular event involved in these processes. A cellular resolution is required to better visualize cell polarity and cell-cell contact.
- The authors used LAL model to show that compression upregulates Smad1/5 activation. Looking at the figure 5C it is obvious that valve leaflets are wider in LAL compared to Sham hearts. Thus, the authors should quantify the length and the width of the leaflets to determine if changes in compression affects valve development.
- Cell volume changes upon osmotic stress regulate transcription of many genes. It would be important to compare transcriptional variations between hyper and hypotonic conditions in order to propose a better mechanistic model.
- There is no discussion about the type of mechano-transducer potentially involved in compressive/tensile stress.

Minor points:
- Many references are missing.
- Figure legends are very short and acronyms must be explained.

Reviewer 2

Advance summary and potential significance to field

Bassen et al have studied the regulation of heart valve remodeling by mechanical forces during valvulogenesis in an avian explant model. Using osmotic pressure to induce mechanical stress in valve primordia explant cultures the authors infer that compressive stress drives growth through BMP signaling and tensile stress compaction through myosin activation. They indicate that these phases are separated by a mechanical switch. Although the experimental approach used to address this important question is pioneering, the following points and additional in vivo support are needed to reinforce the authors' conclusions.

Comments for the author

1. Certain of the results sub-sections are very short and should be expanded. For example, in the in vivo section concerning Figure 5 there appears to be more information in the section title than in the text that follows. Valve structure should be detailed after LAL. Moreover, in vivo support for the authors' conclusions should be extended, potentially by directly manipulating BMP signaling as well as the LAL approach.

2. The authors take an informed candidate approach to identify downstream effectors of mechanical stress. Can they provide data evaluating other signaling pathways? For example FGF (possibly associated with ERK activation) or Notch signaling.

3. The concept of a compressive to tensile switch is an important feature of this study. Can the authors indicate whether this would be gradual or correlate with a precise stage of valve development and ideally add in vivo evidence for such a switch? For example, can the timing of LAL be varied?

4. Can the authors evaluate explant volume in addition to area?

5. Is it possible to manipulate other features of the ECM to reinforce the conclusions?

6. The relative mechanical importance of the endothelial layer and underlying mesenchyme are difficult to distinguish. Please provide more clarity on this point. Would the results change if the cushion explants were still attached to the underlying contractile myocardial wall?

7. T, U and C should be explained in the figures.

8. Please qualify the meaning of valve shape "fidelity" at the end of the discussion. Do the authors mean the robustness of the valve sculpting process?

9. The authors should at least discuss the possibility that altering osmotic pressure may have additional effects on cushion cell biology beyond inducing mechanical stress.

10. The following typos need to be corrected:
last paragraph of introduction - should read "to isolate" call out to Fig S3B error missing "conditions" (page 5) discussion end of second last page "across" or "between"; outflow "tract"; "Our" hypothesis "mitigating".

Reviewer 3

Advance summary and potential significance to field

The study by Bassen et al. investigates the role of hydrostatic mechanical stress in the development of the cardiac atrioventricular valve. Interestingly, different types of stress lead to different effects on valve development, with compressive forces driving growth and tensile stresses inducing compaction. These effects are mediated by different molecular pathways: BMP signaling induced by compressive stress and MLC2 contractility induced by tensile stress. This study uncovers a previously unappreciated difference in the roles of different mechanical pressures in valve morphogenesis. Osmotic stress also leads to condensation and elongation through the collagen matrix.

Comments for the author

Major points:
1. While the method of altering osmotic pressure is an interesting way to alter pressure, whether the osmotic gradients mimic physiological conditions is unclear. What is the evidence that left-atrial-ligation is an in vivo equivalent of increasing compressive forces, but not tensile forces? While we appreciate the difficulty in addressing the role of compressive vs. tensile forces in vivo, it is a relevant question considering the authors are studying their roles in valve morphogenesis.
2. Have the authors ensured that the changes in osmotic pressure do not induce changes in cellular contractility, growth or activation of the different pathways due to changes in cell survival or stress? In particular, could the differences in valve size be caused by differential water content within the tissue?
It would be more convincing if the authors apply a different method other than altering osmotic stress in increasing compressive/tensile stress (e.g. directed flow experiments?).
3. Have the authors tested the effects on the outflow valve and examine whether the effects are uniform in valves of different regions?
4. In the Discussion, the authors wrote “While during valve maturation mesenchymal cells became mature interstitial cells and they emerged as a more contractile driver in tension induced compaction.” What is the direct evidence for this claim? Did the authors use the distance from the valve surface as a readout of mesenchymal cells? No experiment was performed to test whether interstitial cells properly form in the tissue explant (with VIC specific markers e.g. Sox9 or Vimentin), or whether their development is impaired and they stay (partially) endothelial.
5. What is the hypothesis by which compression, and not tensile stress activates BMP-Smad1/5, and tensile stress, but not compression, upregulate pSer19?
Considering that BMP and Myosin can be mechanically activated, it would be interesting to understand the mechanisms by which different stresses are selective in their activation.
6. Have the authors tested stages in which the ECM is stratified to assess its role in withstanding the two mechanical stresses?

First revision

Author response to reviewers' comments

Revision Report
We highly appreciate the reviewers for their insightful comments and criticism, which have greatly helped us improve both the content and the presentation of our work. In particular, we thank reviewers’ suggestion that we should give a more comprehensive analysis of in-vivo data and a more solid description of in-vivo results. Following these suggestions, we add new data in Figure 5B and expand the paragraph describing in-vivo results in the Results section. We also thank reviewers for pointing out their concerns over the possible side effects of media content and osmotic pressure on cell behaviors, especially proliferation. To address this concern, we add a new paragraph to discuss the robustness of our experimental approach. Please find our detailed responses to the reviewers inlined below.

Reviewer 1 Advance summary and potential significance to field...

In this study, the authors investigated the role of tensile and compressive stress during valve development. They used osmotic stress on atrioventricular (AV) cushions to analyze valve growth and maturation. Compressive stress is obtained with hypertonic medium, while tensile stress is obtained with hypotonic medium. Proliferation increased with compressive stress condition. The authors examined the role of BMP signaling in valve growth. Compress stress condition showed a highest pSmad1/5 activity compared to control and tensile stress conditions. BMP treatment decreased compaction while treatment with BMP receptor inhibitor induced compaction. Based on these observations the authors conclude that compressive stress regulates tissue compaction through BMP signaling. To assess contractile mechanism for osmotic and BMP growth phenotype, the authors used a myosin inhibitor mostly specific for myosin II (Blebbistatin). Thus, tissue compaction was inhibited at lower doses of blebbistatin. Blebbistatin treatment can also block tissue rounding. They authors found that Rho activity localized to endothelial cells during compaction and that RhoA-GTP staining patterns in the cytoplasm of cells under compressive. BMP treatment reduced Ser-19 NM-Myosin II light chain activation. To validate their observations the authors used left-atrial-ligation model to alter the hemodynamics on the AV valve. In this model pSmad1/5 staining is increased in the mitral valve leaflets. They found spatial localization of pSmad, pERK and pSer-19 NM Myosin II within the cushion. Using cushions from different staged embryos the authors showed that compressive/tensile stress regulates growth and compaction across stages of valve development. In conclusion, the authors propose that compression promotes growth while tension promotes cushion remodeling and leaflet thinning. These finding are important to understand how mechanical environment modify activation of signaling pathway during valve development.

We thank the reviewer for the accurate summary of our work, and especially for the appreciation of the importance of our findings.

Reviewer 1 Comments for the author...

While the basic insight regarding the role of mechanical stress during growth and maturation of the AV valve is interesting, there are some weakness to the arguments and data reported in this study.

Major concerns:
- My main concern is related to the identification of the mechanism controling changes in contractile and proliferation behaviors. To determine the role of tensile and compressive stress the authors added or reduced the level of sucrose in the base media, which is known to increase or decrease osmolarity respectively. Indeed, studies have shown that glucose promotes cell proliferation through AMPK/mTor MAPK pathways. The authors should demonstrate that compressive/tensile stress directly regulate cell proliferation.

We thank the reviewer for pointing out this important question. Sucrose has been widely used in studies of how osmotic and mechanical stress regulate cartilage biology. Several studies discussed whether cell proliferation and ECM production is a result of sucrose or osmolarity (Takeno et al., J Neurosurg Spine. 2007 Dec; 7(6):637-44). They found the addition of sucrose, NaCl, or use of PEG to change media osmolarity produced similar results and did not change cell phenotype, suggesting cell proliferation and ECM synthesis is related to mechanics rather than sucrose or Na+, Cl- ions. Furthermore, one study showed that animal cells cannot effectively utilize sucrose as an energy source for growth in absence of glucose (Leong, D., et al. Sci Rep 7, 45216 (2017)). These studies support that compressive/tensile stress leads to the significant difference among cell proliferation in different media.

We add this discussion as a new paragraph in the Discussion section:
"To induce the compressive/tensile stress, we used sucrose to mediate the osmolality of culture media. Sucrose has been widely used in studies of how osmotic and mechanical stress regulate cartilage biology. Several studies discussed whether cell proliferation and ECM production is a result of sucrose or osmolarity [32]. They found the addition of sucrose, NaCl, or use of PEG to change media osmolarity produced similar results and did not change cell phenotype, suggesting cell proliferation and ECM synthesis is related to mechanics rather than sucrose or Na+, Cl- ions. Furthermore, one study showed that animal cells cannot effectively utilize sucrose as an energy source for growth in absence of glucose [33]. These studies support that the addition of sucrose

does not influence the development of cushion explants, cell behaviors or their phenotype though unintended effects."

- Another concern is related to the type of cells which response to the mechanical stress induced by the osmolarity. AV cushions are composed of endothelial and mesenchymal cells. Thus, the authors should show which cells proliferate and if there is a difference in the activation of the BMP signaling between endothelial and mesenchymal cells.
We acknowledge that it is important to distinguish endothelial and mesenchymal cells. However, chicken endothelial markers, such as PECAM, are notoriously difficult to label. We will continue searching for reliable antibodies, but in the meantime, we use the spatial distance as the readout of cell type. We assume endothelial cells stay on the surface while mesenchymal cells are inside tissues, which is supported by the morphologies on the cells observed on the outermost cushion layer. This assumption allows us to overcome the technical limitation of available antibodies and instead focus on providing data to propose this previously undiscovered mechanical switch model.

We add a short discussion in the main text to address this question:
"Our results also show the valve morphogenesis requires precise spatial coordination between pSMAD1/5 and pERK, their colocalization in the same cell or neighboring cells may be required for the inhibition of NM-myosin activity and thus cell contractility. To distinguish endothelial and mesenchymal cells, we used the spatial distance to cushion the surface as the readout, due to the lack of labeling tools for chicken endothelial markers such as PECAM. We assume endothelial cells stay on the surface while mesenchymal cells are inside tissues, which is supported by the morphologies on the cells observed on the outermost cushion layer. Most studies considered endothelial cells as the "driver" in tissue shaping while mesenchymal cells as the "passengers" [24,25]. However, our data shows stress-dependent sensory roles of different cell types. Moreover, our data shows a signaling crosstalk between endocardial and mesenchymal cells during cushion compaction. We found colocalization of pERK and pSmad1/5 in the subendocardial region may synergize to regulate the endocardial cell contractility, suggesting mesenchymal BMP-SMAD1/5 could affect endocardial cell contractility. Given the studies proposing that endocardial NOTCH1 limits mesenchymal BMP signaling and subsequent mesenchymal proliferation by activating heparin-binding EGF-like growth factor (HBEGF) [26], a closed loop of interactions between endocardial and interstitial mesenchymal cells may exist."

- It is clear that contractility is important during valve growth and maturation. The authors should further examine the cellular event involved in these processes. A cellular resolution is required to better visualize cell polarity and cell-cell contact.
In this study, we identified that tensile stress drives cushion compaction (the process of valve maturation) via pSer-19 regulated MLC2 contractility, while compressive stress inhibits cell contractility. This supports our proposed mechanical switch model. Cell polarity and cell-cell contact are important factors involved in cell contraction, they would be a potential direction in the future work motivated by our findings in this work.

- The authors used LAL model to show that compression upregulates Smad1/5 activation. Looking at the figure 5C it is obvious that valve leaflets are wider in LAL compared to Sham hearts. Thus, the authors should quantify the length and the width of the leaflets to determine if changes in compression affects valve development.
We thank the reviewer for this suggestion. We measured the leaflet length and width of LAL and sham control hearts and calculated the aspect ratios. We have added this data in Figure 5B, this quantification can help the interpretation of cushion elongation. We also expand the paragraph describing in-vivo results in the Results section with subsection title of "Increased compression upregulates BMP signaling and impairs valve thinning in vivo":
"We performed partial atrial ligation experiments on ex-ovo cultured chick embryos to determine the morphogenetic impact of altered compressive/tensile stress environments on valvulogenesis in vivo. Left-atrial-ligation (LAL) is a method to surgically reduce the hemodynamics placed on the atrioventricular valve [17,18] (Figure 5A). Under LAL, the mitral septal leaflets were significantly less elongated at day 7, as compared to sham control (Sham), indicated by a smaller aspect ratio (Figure 5B). As anticipated from our results, delayed tensile stress environment enhanced the expression of pSmad1/5 in LAL leaflets. The mural leaflets generally showed high pSmad1/5 activity with a statistically insignificant increase in the LAL condition."

- Cell volume changes upon osmotic stress regulate transcription of many genes. It would be important to compare transcriptional variations between hyper and hypotonic conditions in order to propose a better mechanistic model.
We appreciate the importance of transcriptional activity in understanding mechanistic understanding. However, for this first ever study of how compression/tension mediates valve growth/compaction, we focus on bridging our knowledge gap between mechanical stress and molecular activity. Identifying the involved signaling pathways is essential for mechanistic understanding. We analyzed several candidates, including TGF-b (Figure S2) and RhoA/Rac1 GTPases (Figure S3), and determined that BMP-pSmad1/5 and SER-19 play the most important roles. These roles were further confirmed by loss of function assays (inhibition of ROCK, Alk2/3, Alk5/7, MEK and NM-Myosin II). We are also interested in transcriptional variations and would further investigate this question in the future work.

- There is no discussion about the type of mechano-transducer potentially involved in compressive/tensile stress.
There might be several transducers involved in compressive/tensile stress regulated valve growth/maturation. Candidates include mechanotransductive YAP/TAZ, primary cilia, and mechanically gated ion channels such as Piezo-1. These are three primary branches for transduction of mechanical signals into biomolecular signals.
We added this discussion as a new paragraph in the Discussion section:
"The transition from growth program to maturation program is associated with a transition from compression dominated environments to tension dominated environments. Cushions were subjected to compression dominated environments when growing thicker. While at later stages, with elongation and thinning of cushions, tensile stress became dominated. Our study suggests this mechanical transition orchestrates cushion growth and maturation via signaling coordination between BMP-SMAD1/5 and MLC2 cross endothelial and mesenchymal cells. Several mechanosensing mechanisms may be involved in the transduction of compressive/tensile stress, such as YAP/TAZ and mechanically gated ion channels. These different mechanisms may activate different signaling pathways in response to mechanical environments. The impact of mechanical stimuli may also depend on whether the stresses are transduced at the cell membrane or the cytoskeleton. Disrupted compressive/tensile stress sensing could result in defective growth and/or maturation and eventually leading to the malformation of heart valves. This concept provides a possible answer to a long-standing question why only a small minority CHDs have an identifiable genetic cause. Many clinically serious CHDs, such as Tetralogy of Fallot and Persistent Truncus Arteriosus, and prevalent but variably mild form, such as ventricular septal defect, are the result of defective cushion growth in outflow tract [28]. Our hypothesis suggests such defective growth could be the result of insufficient compression on cushions or abnormal stretch occurred in cushions during growth stages. To rectify the defects, surgical accessories may be used to apply additional compression on cushions while mitigating unnecessary tension."

Minor points:
- Many references are missing.

Some references are in the Method section.

- Figure legends are very short and acronyms must be explained.
We thank the reviewer for bringing these issues to our attention. We have added condition acronyms to the figure legend.

Reviewer 2 Advance summary and potential significance to field...

Bassen et al have studied the regulation of heart valve remodeling by mechanical forces during valvulogenesis in an avian explant model. Using osmotic pressure to induce mechanical stress in valve primordia explant cultures the authors infer that compressive stress drives growth through BMP signaling and tensile stress compaction through myosin activation. They indicate that these phases are separated by a mechanical switch. Although the experimental approach used to address this important question is pioneering, the following points and additional in vivo support are needed to reinforce the authors' conclusions.
We thank the reviewer for the accurate summary of our mechanical switch concept and the positive comments on the originality of our experimental approach.

Reviewer 2 Comments for the author...

1. Certain of the results sub-sections are very short and should be expanded. For example, in the in vivo section concerning Figure 5 there appears to be more information in the section title than in the text that follows. Valve structure should be detailed after LAL. Moreover, in vivo support for the authors' conclusions should be extended, potentially by directly manipulating BMP signaling as well as the LAL approach.
We thank the reviewer for these suggestions. We add new data in Figure 5B: the cushion area and aspect ratios of LAL and sham control hearts. We also expand the paragraph describing in-vivo results in the Results section with subsection title of "Increased compression upregulates BMP signaling and impairs valve thinning in vivo":
"We performed partial atrial ligation experiments on ex-ovo cultured chick embryos to determine the morphogenetic impact of altered compressive/tensile stress environments on valvulogenesis in vivo. Left-atrial-ligation (LAL) is a method to surgically reduce the hemodynamics placed on the atrioventricular valve [17,18] (Figure 5A). Under LAL, the mitral septal leaflets were significantly less elongated at day 7, as compared to sham control (Sham), indicated by a smaller aspect ratio (Figure 5B). As anticipated from our results, delayed tensile stress environment enhanced the expression of pSmad1/5 in LAL leaflets. The mural leaflets generally showed high pSmad1/5 activity with a statistically insignificant increase in the LAL condition."

In terms of combining BMP manipulation and LAL approach, we found it extremely difficult to employ. First, BMP signaling plays an important role in the development of many organs. Supplying BMP directly to an embryo would create artificial defects that are hard to be decoupled from mechanical influence. Secondly, it is extremely difficult to supply BMP only to the cushions without creating injury, which could also change the mechanical environments.

2. The authors take an informed candidate approach to identify downstream effectors of mechanical stress. Can they provide data evaluating other signaling pathways? For example FGF (possibly associated with ERK activation) or Notch signaling.
FGF and Notch signaling are crucial signaling pathways during valve development. We agree it would be interesting to evaluate whether they are downstream effectors of mechanical stress as a consequence of this study. However, such study is motivated by this work and would require multiple additional studies to examine those target signaling pathways.

3. The concept of a compressive to tensile switch is an important feature of this study. Can the authors indicate whether this would be gradual or correlate with a precise stage of valve development and ideally add in vivo evidence for such a switch? For example, can the timing of LAL be varied?
This work demonstrated there are phases correlated to compressive/tensile stress environments. A compressive stress environment is related to growth phase while tensile stress is related to maturation phase. Transition between these stress environments ensure the proper valvulogenesis. We also want to study whether this transition is a smooth process or a step stimulation in the next study following this work.
The time of LAL is somewhat flexible but determined by a variety of factors that can be specific to each embryo. On one hand, it should be earlier to limit the influence of surgery injury on results. On the other hand, embryos must have grown large enough for access and proper suture placement.

4. Can the authors evaluate explant volume in addition to area?
We did evaluate the explant volume and compared the volume change with area change. We found the volume compaction matches area compaction. To address this question, we add a new section "Evaluation of 2D and 3D compaction" in the supplementary information with texts and figures showing that the relationship between conditions for 2D area compaction and 3D volume compaction is unchanged.

5. Is it possible to manipulate other features of the ECM to reinforce the conclusions?
Yes, it is possible to manipulate other features of the ECM. GAG is another important component and can be digested by chondroitinase. GAG manipulation would be a future direction, but in this work, we focus on collagen as aligned collagen meshwork is one of the characteristics of mature valves.

6. The relative mechanical importance of the endothelial layer and underlying mesenchyme are difficult to distinguish. Please provide more clarity on this point. Would the results change if the cushion explants were still attached to the underlying contractile myocardial wall?
We found more positive pSMAD1/5 and pSer-19 in regions on or near the surface, where endothelial cells and underlying mesenchymal cells reside. In addition to our data, endothelial layers are anatomically directly contacted with blood flow and a well recognized sensor to a variety of stress. The crosstalk between endothelial layers and underlying mesenchymal cells are also well recognized. Therefore, we believe that the endothelial layer and underlying mesenchymal cells are more important in the transduction of mechanics into cushion morphogenic phenotype.
The results would be different if the cushion explants were still attached to the underlying myocardium. First, the contracting myocardium makes compressions and tensions cyclic. The cyclic feature is another stimulus, for example cyclic stretch regulates valve remodeling through RhoA/Rac1 GTPases (Gould et al., 2016, Current Biology 26, 27-37). While in this work, we found GTPases are not required in compression/tension mediated valve remodeling (Figure S3). Second, myocardium could provide additional signaling to mesenchymal or endothelial cells, affecting the results. The crosstalk between mesenchymal and other cell types are common throughout valve development (MacGrogan et al., 2018, Nat Rev Cardiol 15, 685-704).

7. T, U and C should be explained in the figures.

We thank the reviewer for pointing out this issue. We have added this legend to the relevant figures.

8. Please qualify the meaning of valve shape "fidelity" at the end of the discussion. Do the authors mean the robustness of the valve sculpting process?
Yes, fidelity means robustness in contrast to malformation.

9. The authors should at least discuss the possibility that altering osmotic pressure may have additional effects on cushion cell biology beyond inducing mechanical stress.
We thank the reviewer for this suggestion. The major effect of altering osmotic pressure is inducing mechanical stress, which has been discussed in the studies of cartilage biology. In addition, the osmotic pressure may also affect the positively or negatively charged ions on ECM components. Those ions play important roles in biological function. For example, the repulsion of negatively charged ions on proteoglycans due to osmotic pressure participates in cartilage load bearing. Here in this study we show that the axis of osmotic pressure driven compaction varies with the increasing collagen content at later stages. More studies are needed to determine whether osmotic pressure mediated ion influx causes such effect.

10. The following typos need to be corrected: last paragraph of introduction - should read "to isolate" call out to Fig S3B error missing "conditions" (page 5) discussion end of second last page "across" or "between"; outflow "tract"; "Our" hypothesis "Mitigating".

We thank the reviewer for finding these typos and have corrected them.

Reviewer 3 Advance summary and potential significance to field...

The study by Bassen et al. investigates the role of hydrostatic mechanical stress in the development of the cardiac atrioventricular valve. Interestingly, different types of stress lead to different effects on valve development, with compressive forces driving growth and tensile stresses inducing compaction. These effects are mediated by different molecular pathways: BMP signaling induced by compressive stress and MLC2 contractility induced by tensile stress. This study uncovers a previously unappreciated difference in the roles of different mechanical pressures in valve morphogenesis. Osmotic stress also leads to condensation and elongation through the collagen matrix.
We thank the reviewer for the accurate summary of our work, and especially for the positive comments on the originality of this work.

Reviewer 3 Comments for the author...

Major points:

1. While the method of altering osmotic pressure is an interesting way to alter pressure, whether the osmotic gradients mimic physiological conditions is unclear. What is the evidence that left-atrial-ligation is an in vivo equivalent of increasing compressive forces, but not tensile forces? While we appreciate the difficulty in addressing the role of compressive vs. tensile forces in vivo, it is a relevant question considering the authors are studying their roles in valve Morphogenesis.

*We thank the reviewer for pointing out this important question. According to the Law of Laplace, Ventricle Wall Tension (as well as tension in the cushions) = Ventricle Pressure * Ventricle Radius / (2 * Ventricle Wall Thickness). LAL diverted flow from the constricted left ventricle toward the untreated right ventricle, decreasing hemodynamic pressure on the left ventricle wall and cushions, which results in a diminished tension in the cushion.*

2. Have the authors ensured that the changes in osmotic pressure do not induce changes in cellular contractility, growth or activation of the different pathways due to changes in cell survival or stress? In particular, could the differences in valve size be caused by differential water content within the Tissue?

If the differences in valve size were caused by water content, the hyper-osmotic (with sucrose added) media should give the smallest cushion area because of water loss. However, in contrast, the area of hyper-osmotic cushion is largest. Although it is almost impossible to measure the water content in those hundreds micrometer-scale cushions, our expectation, based on what we observe when moving cushions between conditioned media, is that water content has little impact cushion size during our ex-vivo culture.

To address this question, we add a paragraph discussing the potential influence of the altered osmotic environment on cell behaviors in the Discussion section:

"To induce the compressive/tensile stress, we used sucrose to mediate the osmolality of culture media. Sucrose has been widely used in studies of how osmotic and mechanical stress regulate cartilage biology. Several studies discussed whether cell proliferation and ECM production is a result of sucrose or osmolarity [32]. They found the addition of sucrose, NaCl, or use of PEG to change media osmolarity produced similar results and did not change cell phenotype, suggesting cell proliferation and ECM synthesis is related to mechanics rather than sucrose or Na+, Cl- ions. Furthermore, one study showed that animal cells cannot effectively utilize sucrose as an energy source for growth in absence of glucose [33]. These studies support that the addition of sucrose does not interrupt the development of cushion explants, or cell behaviors and phenotype though unintended effects."

It would be more convincing if the authors apply a different method other than altering osmotic stress in increasing compressive/tensile stress (e.g. directed flow experiments?).

We thank the reviewer for this suggestion. However, directed flow experiments produce shear stress in addition to hydrostatic stress such as compressive and tensile stress. Since shear stress also plays a critical role in valve remodeling, it would be difficult to decouple the influence of compressive/tensile stress from the results.

3. Have the authors tested the effects on the outflow valve and examine whether the effects are uniform in valves of different regions?

We are also interested in outflow valves remodeling but have not tested the effects of compressive/tensile stress yet. Our hypothesis is that the effects are different, even between proximal and distal cushions. This study provides a paradigm for the future explorations of the development of these valves.

4. In the Discussion, the authors wrote "While during valve maturation, mesenchymal cells became mature interstitial cells and they emerged as a more contractile driver in tension induced compaction." What is the direct evidence for this claim? Did the authors use the distance from the valve surface as a readout of mesenchymal cells? No experiment was performed to test whether interstitial cells properly form in the tissue explant (with VIC specific markers e.g. Sox9 or Vimentin), or whether their development is impaired and they stay (partially) Endothelial.

We thank the reviewer for pointing out this unrigorous statement. We did use the distance from the surface as a readout of mesenchymal cells. Our data of Rac1 and RhoA GTPases (Fig. S3) shows a clear distinction between the surface and interior cells. On the other hand, chicken endothelial markers, such as PECAM, are notoriously difficult to label. As for the test of VIC, although we did not stain VIC markers, we think the contractile cells are more mature as they are able to compact cushions. This is also indirectly supported by their association with reduced BMP signaling and proliferation.

We rewrote this sentence in the Discussion section:
"Our results also show the valve morphogenesis requires precise spatial coordination between pSMAD1/5 and pERK, their colocalization in the same cell or neighboring cells may be required for the inhibition of NM-myosin activity and thus cell contractility. To distinguish endothelial and mesenchymal cells, we used the spatial distance to cushion the surface as the readout, due to the lack of labeling tools for chicken endothelial markers such as PECAM. We assume endothelial cells stay on the surface while mesenchymal cells are inside tissues, which is supported by the morphologies on the cells observed on the outermost cushion layer. Most studies considered endothelial cells as the "driver" in tissue shaping while mesenchymal cells as the "passengers" [24,25]. However, our data shows stress-dependent sensory roles of different cell types. Moreover, our data shows a signaling crosstalk between endocardial and mesenchymal cells during cushion compaction. We found colocalization of pERK and pSmad1/5 in the subendocardial region may synergize to regulate the endocardial cell contractility, suggesting mesenchymal BMP-SMAD1/5 could affect endocardial cell contractility. Given the studies proposing that endocardial NOTCH1 limits mesenchymal BMP signaling and subsequent mesenchymal proliferation by activating heparin-binding EGF-like growth factor (HBEGF) [26], a closed loop of interactions between endocardial and interstitial mesenchymal cells may exist."

5. What is the hypothesis by which compression, and not tensile stress, activates BMP-Smad1/5, and tensile stress, but not compression, upregulate pSer19? Considering that BMP and Myosin can be mechanically activated, it would be interesting to understand the mechanisms by which different stresses are selective in their activation.
There might be several mechanosensing mechanisms involved in activation or inactivation of BMP or Myosin, including YAP/TAZ, primary cilia, mechanically gated ion channels such as Piezo-1. They can activate different signaling pathways in response to mechanical environments. Another possibility is that BMP is activated via membrane receptors while Myosin is activated by cytoskeleton reorganization.Their activation depends on where the stress applies. Compressive stress mainly works on cell membrane, thus activating BMP signaling, while tensile stress stretches cytoskeleton and initiates Myosin activation.

6. Have the authors tested stages in which the ECM is stratified to assess its role in withstanding the two mechanical stresses?
Yes, we have tested the influence of osmotic pressure on HH40 cushions/leaflets, HH40 is the stage when ECM is mostly stratified. The results showed cushion compaction is attenuated compared to HH25 or HH34. However, the compaction became similar to earlier stages when collagen is digested via collagenase. The results are shown in Figure 7 A and B and suggest collagen matrices could have negative effects on compressive/tensile stress mediated growth/maturation.

Second decision letter

MS ID#: DEVELOP/2020/196519

MS TITLE: Hydrostatic Mechanical Stress Regulates Growth and Maturation of The Atrioventricular Valve

AUTHORS: David M Bassen, Duc Pham, Mingkun Wang, Rashmi Rao, Rishabh Singh, and Jonathan Butcher

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the remaining request from Rev 3 is addressed. This concerns issues that were brought up in the first review. I concur with the referee that these issues ought to be adressed experimentally, unless there is a fundamental reason why this should not be possible. Please attend to these issues your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors investigated the role of tensile and compressive stress during valve development. They used osmotic stress on atrioventricular (AV) cushions to analyze valve growth and maturation. The authors propose that compression promotes growth while tension promotes cushion remodeling and leaflet thinning. These finding are important to understand how mechanical environment modify activation of signaling pathway during valve development.

Comments for the author

The authors have replied to my main concern regarding the regulation of cell proliferation by the sucrose. I do understand the difficulty to address which type of cells responds to the mechanical stress induced by the osmolarity. The authors argue that chicken endothelial markers are notoriously difficult to label. Several points raised by my comments are now discussed.

Reviewer 2

Advance summary and potential significance to field

This study indicates how a switch between forces impacting on the developing AV valve drives sequential morphogenetic steps.

Comments for the author

The authors have made some changes to their manuscript, extending the results section and justifying the use of sucrose to modulate osmolarity induce compressive stress. These changes address my previous concerns.

Reviewer 3

Advance summary and potential significance to field

See previous review

Comments for the author

I thank the authors for their revised manuscript. Overall, the authors made significant changes to the Discussion section and provided more literature evidence that can support their claims. However, they failed to address my first question as to whether LAL is an in vivo equivalent of increasing compressive, and NOT tensile forces, as they answered with "LAL diverted flow from the constricted left ventricle toward the untreated right ventricle, decreasing hemodynamic pressure on the left ventricle wall and cushions, which results in a diminished tension in the cushion." Clarification on this point is needed.
The experimental revisions do not appear to be as thorough as the literature revisions. One particular experiment to note, all 3 reviewers brought up concerns regarding the fates of the endothelial vs. mesenchymal cells, which the authors stated that it is due to antibody staining problems. However, the authors only mentioned PECAM staining, and I asked whether they have also tested other antibodies, e.g. Sox9 or Vimentin in VICs or morphology membrane/cytoplasmic staining. Similarly, all 3 reviewers mention potential effects of altering osmolarity on non-mechanical aspects of development; while addressed through literature review, the authors should carry out additional experiments, e.g. qPCR on AMPK pathway or AMPK inhibitor treatment as Reviewer 1 suggested.

Second revision

Author response to reviewers' comments

Revision Report

Reviewer 1 Advance Summary and Potential Significance to Field:
In this study, the authors investigated the role of tensile and compressive stress during valve development. They used osmotic stress on atrioventricular (AV) cushions to analyze valve growth and maturation. The authors propose that compression promotes growth while tension promotes cushion remodeling and leaflet thinning. These finding are important to understand how mechanical environment modify activation of signaling pathway during valve development.

Reviewer 1 Comments for the Author:
The authors have replied to my main concern regarding the regulation of cell proliferation by the sucrose. I do understand the difficulty to address which type of cells responds to the mechanical stress induced by the osmolarity. The authors argue that chicken endothelial markers are notoriously difficult to label. Several points raised by my comments are now discussed.

We appreciate Reviewer 1's understanding and acceptance of our revision.

Reviewer 2 Advance Summary and Potential Significance to Field:
This study indicates how a switch between forces impacting on the developing AV valve drives sequential morphogenetic steps.

Reviewer 2 Comments for the Author:
The authors have made some changes to their manuscript, extending the results section and justifying the use of sucrose to modulate osmolarity induce compressive stress. These changes address my previous concerns.
We thank Reviewer 2 for accepting our revision.

Reviewer 3 Advance Summary and Potential Significance to Field:
See previous review.

Reviewer 3 Comments for the Author:
I thank the authors for their revised manuscript. Overall, the authors made significant changes to the Discussion section and provided more literature evidence that can support their claims. However, they failed to address my first question as to whether LAL is an in vivo equivalent of increasing compressive, and NOT tensile forces, as they answered with "LAL diverted flow from the constricted left ventricle toward the untreated right ventricle, decreasing hemodynamic pressure

on the left ventricle wall and cushions, which results in a diminished tension in the cushion." Clarification on this point is needed.

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We thank Reviewer 3's further comments. We conducted new experiments and provided extended data to address the reviewer's concerns.

1. Regarding the first question "whether LAL is an in vivo equivalent of increasing compressive, NOT tensile forces."

   Our collaborator has done extensive work to investigate the disturbed hemodynamics and deteriorated cardiac growth following LAL. They used echocardiography, micro-CT and Computational Fluid Dynamics (CFD) to quantify the hemodynamic change caused by LAL, and this work has been just published.

   "Effect of left atrial ligation-driven altered inflow hemodynamics on embryonic heart development: clues for prenatal progression of hypoplastic left heart syndrome." *Biomech. Model. Mechanobiol.* 2021 Apr;20(2):733-750. doi: 10.1007/s10237-020-01413-5.

   The CFD simulation shows LAL procedure caused a decrease in Wall Shear Stress (WSS) over AV cushions. At HH21, WSS on the superior and inferior sides of cushions was $0.48 \pm 0.029\ Pa$ and $0.52 \pm 0.076\ Pa$ in the LAL group, much less than the WSS of $0.78 \pm 0.029\ Pa$ and $1.2 \pm 0.44\ Pa$ in control. WSS is the friction applied by flowing blood on cushions when AV canals are open, the decreased WSS leads to limited tensile stress on bulged cushions at early development. However, the disruptive WSS did not influence the compression during cushion when the canal is closed. In conclusion, this study is consistent with our hypothesis that the LAL procedure delayed the transition from a compression dominated mechanical environment to a tension dominated environment.

2. In terms of the concern regarding the endothelial vs. mesenchymal cells.

   In addition to PECAM, we have tested a variety of markers, including VE-Cadherin, Isolectin-B4, as well as Sox9 and Vimentin. We found Sox9 and Vimentin could be expressed by both cell types. Here we use VE-Cadherin to label endothelial cells, as shown in Figure S6. VE-Cadherin positive cells were mainly distributed in the outer region, and absent in the inner region. This data verifies our assumption in the manuscript that endothelial cells remained on the surface while mesenchymal cells were inside tissues. Given the result shown in the manuscript that pSER-19 was present at or near the surface layers of the cushion while pSmad1/5 was observed throughout the cushions, endothelial contractility may drive the cushion compaction while mesenchymal proliferation may determine the cushion size.

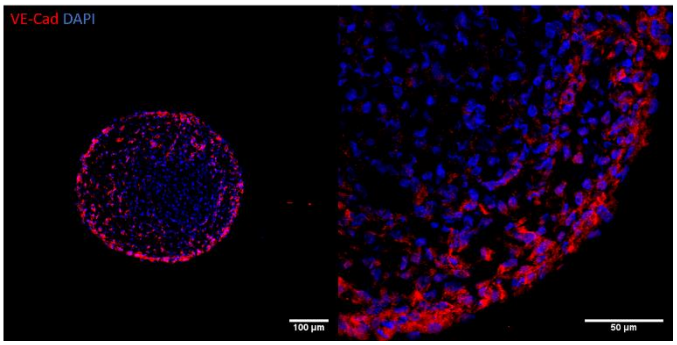

Figure S6. VE-Cadherin labeling shows most endothelial cells remained on the surface of cushions.

3. To further decouple the influence of sucrose on cushion growth, we designed two additional experiments.

   In the first experiment, we prepared 4 groups of culture media: 1. Hypotonic medium, with an osmolarity around 174 mOSM/kg; 2. Normal medium, the osmolarity is around 295 mOSM/kg. 3. Hypertonic medium (sucrose), its osmolarity is around 446 mOSM/kg and adjusted by adding sucrose. 4. Hypertonic medium (NaCl), its osmolarity is also around 446 mOSM/kg but adjusted by adding NaCl. We cultured HH25 AV cushions in the above media for 24 hours, following the same protocol described in the manuscript. The cushions were then fixed by 4% PFA, cryosectioned, and stained with pHH3.

   The result is shown in Figure S7A. Both hypertonic media, regardless of sucrose or NaCl added, promoted significantly higher percentages of pHH3 positive cells in cushions. The difference between sucrose added and NaCl added hypertonic media was not significant. This result suggests hypertonic media promote cell proliferation independent of sucrose.

   In the second experiment, we added the same amount of sucrose (0.14M) in a regular or diluted culture medium to create the higher osmolarity medium (446 mOSM/kg) and the lower osmolarity medium (325 mOSM/kg). We ran the ex-vivo AV cushion culture using these two media and examined cell proliferation. The result is shown in Figure S7B. With the same amount of sucrose in the media, higher osmolarity has a significantly ($p<0.01$) higher percentage of pHH3 positive cells. This result shows sucrose alone did not lead to a significant difference in cushion proliferation.

   These results support that sucrose did not contribute to cushion proliferation.

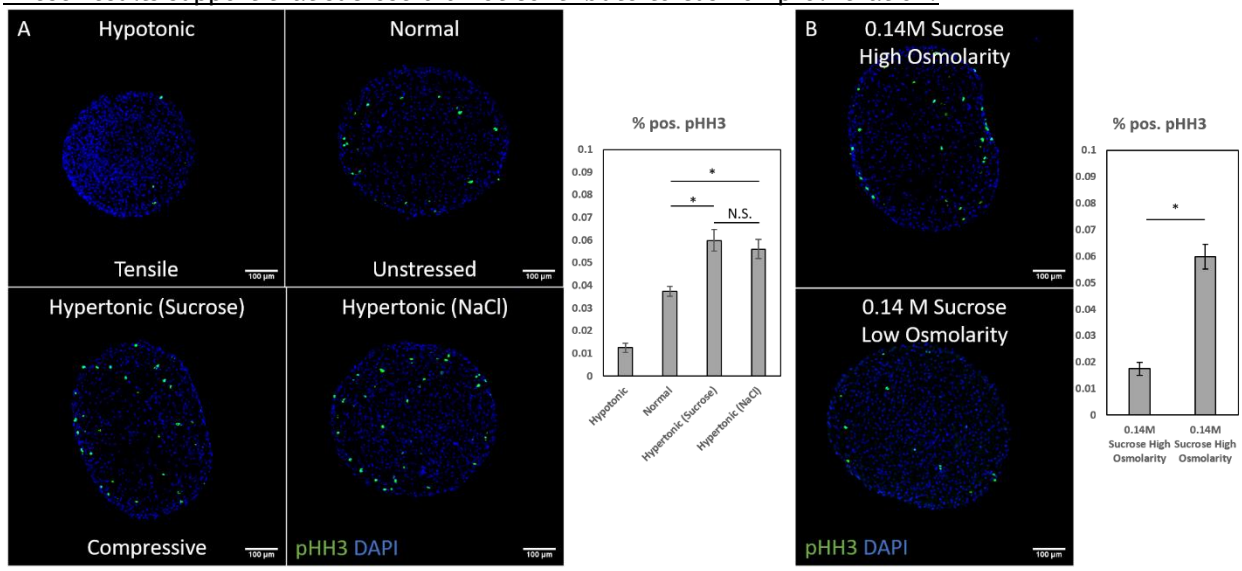

Figure S7. Addition of sucrose did not have a significant impact on cell proliferation. (A) Representative IF images of pHH3 in HH25 cushions cultured under osmotic stress for 24 hours, and quantification of pHH3 positive cells, n = 4-5, SD shown. (B) Representative IF images of pHH3 in HH25 cushions cultured in media with same amount of sucrose added but different osmolarity for 24 hours, and quantification of pHH3 positive cells, n = 4-5, SD shown.

Third decision letter

MS ID#: DEVELOP/2020/196519

MS TITLE: Hydrostatic Mechanical Stress Regulates Growth and Maturation of The Atrioventricular Valve

AUTHORS: David M Bassen, Mingkun Wang, Duc Pham, Shuofei Sun, Rashmi Rao, Rishabh Singh, and Jonathan Butcher
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 3

Advance summary and potential significance to field

The study by Bassen et al. investigates the role of hydrostatic mechanical stress in the development of the cardiac atrioventricular valve.
Interestingly, different types of stress lead to different effects on valve development, with compressive forces driving growth and tensile stresses inducing compaction. These effects are mediated by different molecular pathways: BMP signaling induced by compressive stress and MLC2 contractility induced by tensile stress. This study uncovers a previously unappreciated difference in the roles of different mechanical pressures in valve morphogenesis. Osmotic stress also leads to condensation and elongation through the collagen matrix.

Comments for the author

I thank the authors for their revisions, and have no further questions. The resubmitted manuscript is suitable for publication.