Response to reviewers
We thank our reviewers for their thoughtful and constructive comments. We have revised our manuscript in response to these critiques. Our point-by-point responses are below.

Reviewer 2:

The manuscript titled “Expression and distribution of activin-follistatin-inhibin axis in mouse urinary bladder” by Weiqun Yu et al. is both engaging and well-constructed. However, I have several observations and recommendations to enhance the clarity and impact of the study.

a. The authors should address how the findings from the mouse model might be extrapolated to the human bladder, highlighting potential implications and relevance to human physiology and pathology.

Response: *We have added a new Figure 9 showing upregulated AFI expression in human bladder cancer, highlighting the potential application in bladder cancer diagnosis and prognosis.*

b. Expanding on the potential clinical translation of the study’s findings would add considerable value, as it could provide a broader context for their significance.

Response: *please see the response to comment* ***a****. We have discussed the expression and potential functions of the AFI axis in human bladder cancer.*

c. The use of an antibody against CD34 raises the question of whether the authors evaluated the activin-follistatin-inhibin axis specifically within endothelial cells. Additionally, clarification is needed on whether all vessels were immunoreactive.

Response: *CD34 is a major biomarker for ENTPD2-positive or non-myofibroblasts interstitial cells in the bladder wall. Please refer to our previous publication: PMCID: PMC3492220 for more information. In the current study, it colocalizes with INHBA in the lamina propria (Figure 4), indicating INHBA+ cells are interstitial cells in bladder lamina propria. There are very few CD34+ non-interstitial cells in the bladder wall, which sits right beneath the urothelial cell layer. The identity of these CD34+ cells is not known yet, it could be the progenitor of endothelial cells or other stem cells. Thus, CD34 is not a major biomarker for endothelial cells in the bladder wall, and this can also be confirmed by single-cell sequencing data published previously: PMCID: PMC6830796: supplemental data 8.*

d. In Table 1, the column heading "Application" should be revised to "Application, dilution" to improve accuracy and consistency.

Response: *We have revised accordingly.*

e. In the Immunofluorescence analysis section, the authors report a 1:100 dilution for the

primary antibody. However, Table 1 indicates that all antibodies were diluted 1:100. Clarification is needed — did the authors test all antibodies with a single dilution, or were different dilutions used?

***Response**: The dilution is 1:100.*

f. Finally, details about image acquisition are absent. The authors should specify the equipment used for immunofluorescence imaging, including the microscope model and any associated software, to enhance reproducibility.

Response: We have added the requested information to the revised manuscript.

Reviewer 3:

I appreciate the authors for exploring the AF1 axis in the urinary system, especially the mouse bladder. The authors postulate that local production of INH, ACT, and ECT by local tissues (in this case, urothelial cells) could regulate a plethora of biological activities. While the manuscript presents intriguing evidence on the AF1 axis in the mouse bladder wall, it does not clearly delineate the biological implications of these findings in the current version. Specifically, the manuscript lacks experimental evidence or at least a detailed discussion on how the signaling events related to the AF1 axis can be distinctly separated from the influences of INH, ACT, and FST, which are primarily produced by the pituitary cells and gonads for sex and reproductive regulation.

***Response**: We have mentioned in the revised introduction that AFI can be produced locally, and our mRNA data showing the AFI axis in bladder tissue confirmed this idea. We have added a new figure to the revised manuscript showing upregulated AFI expression in human bladder cancer, highlighting the potential role of the AFI axis in bladder cancer diagnosis and prognosis.*

Additionally, while the authors discovered seven molecules of the AF1 axis in both male and female mouse bladders, they should discuss the relevance of these transcripts in the physiology and pathology of both sexes. To strengthen the manuscript, I recommend providing a more detailed analysis or discussion on separating these signaling pathways in the context of the mouse bladder.

***Response:** We have described and discussed male-female differences as follows in the results: "**We then compared gene expression levels between males and females. Ligand subunit genes Inha, Inhbe, and Fst exhibited higher expression in male than in female mouse bladder, whereas Inhba was expressed at higher levels in female than in male mouse bladder. The major receptor genes Acvr2b and Acvr1 were also expressed at higher levels in female than in male bladder (Figure 1A). The data suggest that female bladders might exhibit higher levels of activin signaling than male bladders, but the mechanism and physiological importance of this sexually dimorphic expression pattern remains unclear."*

Q1. What are the physiological triggers of the AF1 axis in the male and female bladder walls beyond tissue injury or cancer?- Revise the discussion accordingly.

Response: *The role of the AFI axis in bladder physiology (as well as in bladder injury and cancer) remains unknown. We agree that this important question requires further investigation. We speculate that the AFI axis may play a role in bladder tissue homeostasis and bladder cancer. We have revised the discussion accordingly.*

Q2. The authors could potentially investigate and demonstrate the dysregulation of the AF1 axis within the male or female urothelial system using an injury or cancer model, thereby providing more compelling evidence for the functional significance of these pathways. Alternatively, if such models are lacking, the authors should at least demonstrate the proof of functional complexes using co-immunoprecipitation assays. These will further support and strengthen the Western blot analysis and immunofluorescence data. For example, the authors suggested that "INHA and INHBA could dimerize to form Inhibin A, and INHBA could also form homodimers". The authors should provide evidence for these statements using co-immunoprecipitation with lysates from urothelial cells.

Response: *Thank you for this constructive suggestion. We have added a new figure showing upregulated AFI expression in human bladder cancer, highlighting the potential application in bladder cancer diagnosis and prognosis. We have cited references for the dimerization of INHA with INHBA to form inhibin A, and for INHBA self-dimerization to form activin A in non-bladder tissues. Inhibin receptor mechanisms of urothelial cells will be subjects of future experiments*

Q3. Please provide Western blot data to demonstrate the baseline phosphorylation status of ACVR2B, ACVR1B, and ACVR1C, and co-immunoprecipitation data for the ACVR2B/ACVR1B functional receptor complex.

Response: *The functional receptor complex ACVR2B/ACVR1B is well-established in the AFI axis. Activin binding to ACVR2B recruits ACVR1, forming a receptor complex that activates downstream signaling by phosphorylating Smad2/3 transcription factors. We have described this briefly in the Introduction.*

Q4. The working model proposed is unclear in the current format and needs further refinement and clarity to explain the relevance of these findings in the context of normal and disease-state physiology.

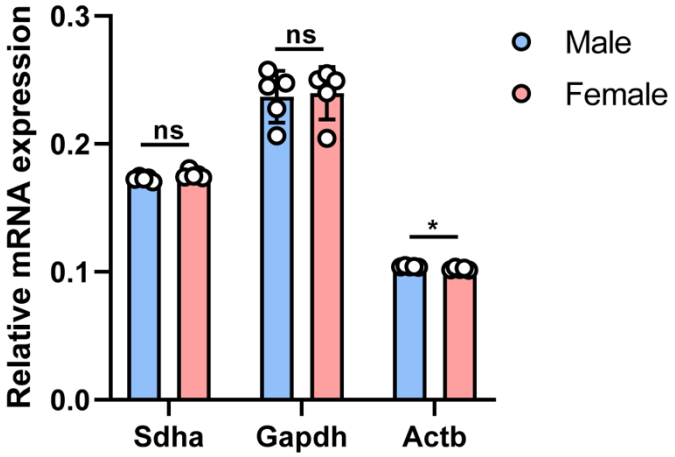
Response: *We have a detailed discussion of the model in paragraph 6 of the Discussion part. We have further discussed its potential involvement in human bladder cancer in paragraph 7 of the Discussion part in this revised version.*

Q5. Please provide quantitative plots of Western blots in Figure 3 A-D. Additionally, please provide a complete scan of Western blots for Figure 3 as supplementary data.

Response: *The requested information has been provided in Figure 2 and the supplementary data.*

Q6. Please provide melt curves for the quantitative RT-PCR data used in Figure 1 as supplementary data.

Response: *melt curves for all the genes are provided as supplementary data.*

Q7. For the RT-PCR data used in Figure 1, the authors have chosen SDHA as the single housekeeping gene for normalization. While SDHA is a widely used internal control, reliance on a single gene can potentially introduce bias due to variability in gene expression under different experimental conditions or between males and females. The authors should consider using at least three housekeeping genes and provide a revised figure.

Response: *We commonly use SDHA as a housekeeping gene for bladder tissue. We have previously tested three common housekeeping genes SDHA, GAPDH, and beta Actin. Our data reveal SDHA as the most reliable housekeeping gene in bladder tissue, while GAPDH shows a larger standard deviation in expression, and beta Actin exhibits different expression levels in the male and female bladder. Please see the figure below:*