Author's response to reviews

Title: Higher BMP/Smad sensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model - Possible mechanism for their altered fate in vitro

Authors:

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Version: 2 Date: 2 August 2013

Author's response to reviews: see over
Dear Editor-in-Chief,

**Manuscript Re-submission (MS: 1837098406951723)**

Thank you very much for reviewing our full-length article on “Higher BMP/smadsensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model – Possible mechanism for their altered fate in vitro” by Pauline Po Yee LUI and Yin Mei WONG. We have revised the manuscript according to associate editor and the reviewers’ comments.

Our point-by-point responses to their questions are listed below:

**Associate Editor's Comments:**

**Abstract:**

1. The background section in the abstract is too long. The authors should make it shorter.

   The background section is shortened in the revised manuscript.

2. In the methods, the authors should state that they used rat patellar tendons in this study.

   This is added in the revised manuscript.

3. In the results, the authors should state there were no significant differences in mRNA levels of Bmp7 or Bmpr2 between TDSCs (CI) and TDSCs (HT).

   This is added in the revised manuscript.

**Background:**

No comments.

**Methods:**

Line 82-: Male Sprague Dawley rats, (6 weeks, weight 150-220 grams) were used. How many animals did the authors for each group?

Twelve rats were used. Six rats for the CI group and the other 6 rats for the HT group. This information is added in the revised manuscript.

Line 95-: The stem cell-related surface marker expression, clonogenicity and multi-lineage differentiation potential of isolated nucleated cells from the CI animal model and
healthy animals were confirmed as described previously before being used for the experiments in this study. The authors should briefly describe how to isolate TDSCs from the patellar tendon.

The procedure for the isolation of TDSCs from rat patellar tendon is added in the revised manuscript.

Line 111-: They were then treated with rhBMP-2 (100ng/ml) (Wyeth, Cambridge, MA, USA) in complete medium for 0, 15, 30 and 60 minutes at 37°C, 5% CO2. Why didn’t the authors examine the dose-dependent effects of rhBMP-2 application on the expressions of pSmad 1/5/8 and total Smad 1/5/8.

The pathological concentration of BMP-2 in tendinopathy was not known. The dose used in this study was chosen based on our previous study testing the effect of different concentrations of BMP-2 (0, 50, 100, 250, 500 and 1000 ng/ml) and 100ng/ml was the lowest dose that induced the osteogenic differentiation of TDSCs (unpublished results). BMP-2 at 100ng/ml promoted non-tenogenic (osteo-, chondro- and adiop-genic) differentiation, increased proteoglycan production but inhibited tendon-related marker expression in TDSCs (Rui et al., 2012). This same dose (100ng/ml) was also used in a previous study investigating the effect of BMP-2 on the osteogenic response as well as expression and translocation of pSmad 1/5/8 in tendon stem / progenitor cells (Bi et al., 2007). The use of pathological concentration of BMP-2 in tendinopathy for TDSC stimulation in vitro would yield more clinically relevant data. This information is added in the methodology section of the revised manuscript.


Results:
Line 186-: TDSCs (CI) expressed significantly higher mRNA levels of Bmp2 (2.2 fold, p=0.006), Bmp (1.7 fold, p=0.010); Bmpr1a (1.9 fold, p=0.004) and Bmpr1b (1.6 fold, p=0.016) but not Bmp7 (p=0.670) and Bmpr2 (p=0.873) compared to TDSCs (HT) (Figure 1). The authors should use ?BMP? and ?BMPR?, not ?Bmp? and ?Bmpr?, in the same manners to the methods section.

Bmp2, Bmp4, Bmp7 etc. are the GENE names for rat species. BMP-2, BMP-4, BMP-7 are the names of the PROTEINS. Therefore different symbols were used.
The addition of BMP-2 resulted in the increased expression and translocation of pSmad 1/5/8 to the cell nucleus in both TDSCs (Figure 5 B-D, F-H), suggesting that pSmad 1/5/8 was functional. Please delete suggesting that pSmad 1/5/8 was functional, since it's not a result.

Deleted as suggested

Discussion:

There was ectopic expression of BMP-2, BMP-4 and BMP-7 in clinical samples of tendinopathy [9-10] and collagenase-induced tendon injury animal model [11]. No expression of BMP-2, BMP-4 and BMP-7 was observed in the intact tendons [9,11].

Yes, this is correct.

The authors should discuss the reason why there were no significant differences in mRNA levels of Bmp7 between TDSCs (CI) and TDSCs (HT) in the present experiment.

The exact reason is not known. It might possibly be due to the different half-life of mRNA and protein. This is added in the revised manuscript and is quoted below:

“While we detected increased protein expression of BMP-7 and BMPR-II in TDSCs (CI) compared to TDSCs (HT), we failed to detect increased expression of their corresponding mRNA. It might be due to different half-lives of protein and mRNA.”

By exploring the mechanisms of altered fate of TDSCs (CI), the present study added further support to the role of chondro-osteogenic BMPs and the BMP/Smad signaling pathway in the pathogenesis of failed tendon healing and ectopic chondro-ossification. Why don’t the authors show that BMP/Smad signaling induces ectopic chondro-ossification by inhibition of BMP/Smad signaling in TDSCs (CI)?

Yes this is expected and we planned to do this in the future study. This is also added as one limitation of this study.

Conclusions

No comments

Reviewer’s report

Title: Higher BMP/smad sensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model – Possible mechanism for their altered fate in vitro
The submitted manuscript investigated the responsiveness of tendon-derived stem cells (TDSCs) obtained from healthy and collagenase-induced injured patellar tendons in rats to BMP-2 stimulation. The question was clear that was tested using established methods. Although overall the study was designed and conducted well and the manuscript was well written, the impact of the findings obtained is not very high. This is mainly because that the group of authors already showed in their previous studies that BMP-2 induces chondro-osteogenic differentiation of TDSCs, and thus, the present findings just provide one possible pathway of such differentiation, leaving a fundamental question why TDSCs from collagenase-induced injury tendons have different responsiveness from those from healthy tendons unknown.

I would like to supplement that BMP-2 also induced adipogenic differentiation of TDSCs (Rui et al., 2013).


I agreed with the reviewer that this study could not answered the questions why TDSCs (CI) have different responsiveness to BMP-2 stimulation compared to TDSCs (HT). However, it was our first step to understand the mechanisms of altered fate of TDSCs (CI). Both mechanical and biological factors might contribute to the altered fate of TDSCs (CI) and this study showed that increased expression of BMPs and BMP receptors as well as elevated BMP/Smad sensitivity of TDSCs (CI) could be one possible biological mechanism contributing to their increased non-tenogenic differentiation potential and hence altered fate. In addition, this study provided further support, in addition to our previous reports (Rui et al., 2011; Yee Lui et al., 2011), for the role of BMPs and the BMP/Smad signaling pathway in the pathogenesis of tendinopathy. This was discussed in the first paragraph of the original manuscript.

We speculated that tendon overuse (mechanical loading), the increased expression of BMPs as a result of mechanical loading, either produced by the cells or released from the extracellular matrix, might modulate the sensitivity of TDSCs to the BMP/Smad signaling pathway. Further research is required to confirm our speculation.

References:

Yee Lui PP, Wong YM, Rui YF, Lee YW, Chan LS, Chan KM: Expression of chondro-osteogenic BMPs in ossified failed tendon healing model of

Along with specific comments listed below, the authors may need to address to answer the question, to provide readers of the journal with an interesting insight of the etiology of tendinopathy.

- Major Compulsory Revisions

Methods

1. Page 10, Line 178. “Quantitative and semi-quantitative data was shown in boxplots.”
   Certainly, the authors used boxplots in Figures 1, 3, and 4, but the style of boxplots used in Figures 1 and 3 and that in Figure 4 seems different. In Figures 1 and 3, the maximum value, the minimum value, first and third quartiles, and median value were provided, while in Figure 4 plots are seemed just to proved the maximum and minimum values and median (mean?) value. In addition, there were error bars for x-axis in Figure 4 without any clear comments or explanation. Therefore, the authors must be consistent in using boxplots.

   I am sorry for the mistake in Figure 4. Figure 4 is not a boxplot but a plot showing the mean and SE. The graph was originally generated by SPSS showing the estimated marginal mean values only without the SE. However, one reviewer in the earlier submission required us to add the error bar which could not be shown automatically using SPSS. I hence plotted the graph using Excel. The extra bars for the x-axis were produced due to my lack of experience in using the software. I have corrected this in the revised manuscript.

2. Page 10, Line 182. The use of ANOVA for repeated measures may not be appropriate in this case, as the authors did not measure the same samples at different time points. Please consult it to a statistician.
   The use of ANOVA for repeated measures is correct. The experiment was repeated 4 times with cells isolated from different animals. However, same source of cells was used in each experiment (i.e. the WB data at different times after BMP-2 stimulation was from the cell source for each group). As the cells at different time points in each experiment was from the same rat, ANOVA for repeated measures with time as the within-subjects factor and treatment group as the between-subjects factor was used. This is clarified in the revised manuscript.

3. Page 10, Line 183. Statistical significance should be p<0.05, but not p # 0.05. Please rewrite Results section, Figure legends and Figures using a p < 0.05 criterion.

   This is corrected.

Discussion

1. Page 14, Lines 258-259; Page 7, Lines 45-46. “Results from this study open the door for human studies of the pathogenesis of tendinopathy.”
   It is not clear why and how the findings in the present study, using a rat model, are
related to human studies. The authors need to rewrite Conclusion as well as Conclusions in Abstract to be more precise.

I agreed with the reviewer and this sentence is deleted. The conclusion is modified in the revised manuscript and quoted below:

“The sensitization of the BMP/Smad pathway in TDSCs (CI) might account for their higher non-tenogenic differentiation potential and hence altered fate. It also provided further support of BMPs and the BMP/Smad signaling pathway in the pathogenesis of tendinopathy.”

Figures
1. Page 21, Line 374. Please provide the definitions of outlier and extreme value. Were these values included in statistical analysis?

Both outlier and extreme value are included in the statistical analysis. This is just a way to show the dispersion of the data.

The definition of extreme value and outlier are added in the data analysis section of the revised manuscript and is quoted below:

“For the boxplot, the lower, middle and upper boundaries of the box showed the 25th, 50th and 75th percentile of the dataset. Observation with value that was more than 3 box-length from the upper or lower edge of the box was shown as extreme value (*) if existed. Observation with value that was between 1.5 to 3 box-length from the upper or lower edge of the box was shown as outlier (o) if existed. The largest and smallest observations in the dataset that were not outliers or extreme values were shown as whiskers. If there were no outliers and extreme values, the whiskers represented the maximum and the minimum observations of the dataset.”

- Minor Essential Revisions

Background
1. Page 3, Lines 53-56. The sentence “It is characterized by ……. activity-related tendon pain.” is too long and unclear. Please divide it into 2 or 3 sentences to make it clear, and provide references to each of the characteristics.

Revised as suggested and it is quoted below:

“Histopathologically, tendinopathy is characterized by an increase of cellularity [1-3], vascularity [1], glycosaminoglycan deposition [1-2] and loss of matrix organization [1,4]. Tissue metaplasia, with the presence of chondrocyte phenotype [5] and occasional fatty and bony deposits [2,4,5], is observed.”
2. Page 3, Line 56-57. “As a result,…..with limited success.” Please provide references.

Two references were added in the revised manuscript.


3. Page 4, Line 61 to Page 5, Line 77. In this paragraph, the authors described past studies using animal models, although it is not clear which animal models were sued in these past studies.

This was the collagenase-induced (CI) tendon injury rat model as stated in first sentence of the second paragraph.

Methods

4. Page 5, Lines 94-95. Please provide more information on the isolation of TDSCs, at least a brief explanation of the isolation procedures.

The procedure for the isolation of TDSCs from the rat patellar tendon is added in the revised manuscript.

5. Page 5, Line 95 to Page 6, Line 98. Please provide the name(s) of stem cell markers examined.

Both TDSCs (CI) and TDSCs (HT) expressed CD44, CD90 and CD73. This information is added in the revised manuscript.

Discussion


7. The following reference is added in the revised manuscript:


Table

8. Page 21, Line 368. Table legend “Table showing the primer sequence,….” should be “The primer sequence,….”
Corrected, thank you very much

Figures
9. Page 27. Figure 5. Please provide the names for the column and line, so that readers can easily understand which photograph shows which condition.
This is corrected in the revised manuscript. Thanks

Minor issues not for publication
10. Page 1, Line 1, Title. “BMP/smad sensitivity…” is “BMP/Smad sensitivity…”?
   Corrected. Thank you very much.

   Corrected, thank you very much

12. Page 6, Line 105. “….Carlsbad, USA) at 37°C…” should be “…Carlsbad, USA) at 37°C…”.
   Corrected. Thank you very much.

13. Page 11, Line 193. “(Figure 4)” would be “(Figure 4A)”?
    should be A-C and this is corrected

14. Page 11, Line 197. “(Figure 4)” would be “(Figure 4A)”?
    Should be A-C and this is corrected

15. Page 11, Line 200. “(Figure 4)” would be “(Figure 4 B, C)”?
    corrected

- Discretionary Revisions

Abstract
1. Page 2. Lines 34-36. Methods. Indicate which animal model was used.
   This is added in the revised manuscript.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests.

Reviewer’s report
Title: Higher BMP/smad sensitivity of tendon-derived stem cells (TDSCs) isolated from the collagenase-induced tendon injury model – Possible mechanism for their altered fate in vitro
The pathogenesis of tendinopathy is unclear. The higher expression of BMPs was identified in clinical samples of tendinopathy and collagenase-induced (CI) tendon injury animal model. Tendon-derived stem cells (TDSCs) isolated from the CI animal model showed higher chondro-osteogenic differentiation potential and altered fate compared to TDSCs isolated from healthy animals. This study is to investigate the mechanism for their altered fate in vitro. The topic of this study is interesting to the readers of the journal. The study was well designed. The manuscript was well written.

The specific comments for this manuscript are:
1. Abstract: The conclusion of “Results from this study open the door for human studies of the pathogenesis of tendinopathy” (Line 45-46) should be rewritten carefully. There are 3 reasons: 1) The conclusions of this study were drawn from an in vitro cell study but not an in vivo study of tendinopathy; 2) The animal model in this study was achieved with collagenase-induced tendon injury but not repetitive overuse, the common etiological factor of tendinopathy; 3) Both lipid accumulation and calcification have been found in tendinopathic lesions. This study provides the potential evidence of calcification only.

I agreed with the reviewer in general and hence this statement is deleted in the revised manuscript. The conclusion is modified and is quoted below:

“The sensitization of the BMP/Smad pathway in TDSCs (CI) might account for their higher non-tenogenic differentiation potential and hence altered fate. It also provided further support of BMPs and the BMP/Smad signaling pathway in the pathogenesis of tendinopathy. “

However, I would like to supplement that BMP-2 also have adipogenic effect (Rui et al., 2013a) and our previous paper, while focusing on the osteogenic and chondrogenic differentiation of TDSCs (CI) and TDSCs (HT), actually also showed evidence of higher adipogenic differentiation of TDSCs (CI) compared to that in TDSCs (HT) (Rui et al., 2013b). However, since lipid accumulation was not consistently observed in the histology of the CI model in vivo, we did not focus on this aspect in our study.

References:

2. Methods: How many rats were used in this study? Was there specific reason to use beta-actin as control not other house-keeping genes?

Twelve rats were used in this study (Six for TDSC(CI) isolation and the other six rats for TDSC (HT) isolation). This information is added in the revised manuscript. Our previous results consistently showed that beta-actin showed stable expression under different treatment conditions (Tan et al., 2012a; Tan et al., 2012b; Rui et al., 2013; Lui PP et al., 2013; Rui et al., 2011), beta-actin was hence used as a marker in this study. This, however, did not exclude that other house-keeping genes would have also shown stable expression.

References:


3. Discussion: Please provide the explanation of inconsistent data of BMP7 and BMPR-II in Figures 1 and 3. The limitations of study should be addressed.

The exact reason is not known. It might possibly be due to the different half-life of mRNA and protein. This is added in the revised manuscript and is quoted below:

“While we detected increased protein expression of BMP-7 and BMPR-II in TDSCs (CI) compared to TDSCs (HT), we failed to detect increased expression of their corresponding mRNA. It might be due to different half-lives of protein and mRNA.”
The limitations of this study are discussed in the revised manuscript and are quoted below:

“This study has some limitations. First, the pathological concentration of BMP-2 in tendinopathy was not known. The dose used in this study was chosen based on our previous study [13] and another report [22]. The use of pathological concentration of BMP-2 in tendinopathy for TDSC stimulation in vitro would yield more clinically relevant data. This study was our first step to understand the mechanisms of non-tenogenic differentiation and hence altered fate of TDSCs (CI). A more fundamental question of why TDSCs (CI) have higher BMP and BMPR expression as well as elevated BMP/Smad sensitivity remains unanswered. We did not examine the fate of TDSCs (CI) by inhibiting the BMP/Smad signaling pathway in this study, the results of which would provide further support of BMPs and the BMP/Smad signaling pathway in regulating the fate of TDSCs (CI) and might shed lights on the pathogenesis and treatment of tendinopathy. Further study is required.”

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests.

If you have any queries about this study, please contact the corresponding author, Dr. PPY Lui at

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Thank you very much for your consideration and we are looking forward to hearing your reply.

Yours Sincerely,

[Signature]

Pauline Po-yee LUI
Corresponding Author