Author's response to reviews

Title: Angelica sinensis promotes myotube hypertrophy through PI3K/Akt/mTOR pathway

Authors:

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Author's response to reviews: see over
We, the authors, thank the reviewer for the valuable comments. We have revised the content of the article. These are the corrections that have been made and the answers for the questions.

**Referee 1: David Cameron-Smith**

**Reviewer's report:**
The present manuscript shows the beneficial effect of Angelica sinensis (AS) in myotube diameter and that this effect is through increased mTOR pathway activation. The paper is well conducted with proper controls being carried for most of the experiments. However the paper should be revised, and only accepted if major concerns stated below are properly addressed or explained.

1. **Major compulsory revisions:**
   1. Although the study shows that AS increase myotube diameter and that it seems to be mediated via mTOR pathway, it does not address or provide data whether it is direct effect on mTOR pathway or enhancing mTOR pathway by facilitating IGF-1 binding to its receptor. Throughout the serum was used in all treatment media, this it is possible that AS exerts its actions through facilitating the action of the growth factors within this media. Parallel studies in serum free conditions are required before conclusions of actions can be drawn.

   **Response:** We greatly appreciate the comments of the reviewer. In this study, the 2% horse serum (HS) was used in all three groups ((1) NON group: DMEM containing 2% HS; (2) IGF-1 group: 10 ng/mL IGF-1 in 2% HS/DMEM; (3) AS group: 10 ng/mL Angelica sinensis in 2% HS/DMEM). Therefore, it cannot explain how AS affects muscle hypertrophy signaling pathways. However, the experimental data (Fig 2) showed that myotube diameter in the AS group was significantly thickened as compared to that of the NON group, but not IGF-1 group. Even if 2% horse serum were to contain some of IGF-1, AS-induced myotube hypertrophy was not entirely due to IGF-1 receptor and therefore would not have a major effect on the result. Assuredly, further study by using serum free medium is needed to investigate how the AS activates PI3K/Akt/mTOR pathway. We have discussed about experimental design, methodology, and suggestion in the manuscript (lines 318-327).

2. It is not clear in which media the chemicals were diluted in. It is only stated that “For the treatment, the stocks were diluted in the medium”. Which medium is that, growth media (containing serum) or serum free media?

   **Response:** We thank the comments of reviewer. Before treatment, we prepared the
medium with AS or IGF-1, then both were added separately to the cultured cell at a final concentration of 10 ng/ml in 2% HS/DMEM. This means that the medium contained 2% horse serum. We have added the detailed description in the revised manuscript (lines 118-119).

3. To induce differentiation, C2C12 are usually treated with horse serum (HS), it seems that it was not used in this study. How the cells were differentiated?
Response: We are sorry that we left out this important description. In this study, myotube formation was induced by DMEM containing 2% horse serum instead of fetal bovine serum. We have added this sentence in the revised manuscript (lines 103-105).

4. In the Abstract the claim that AS could be used in ergogenic aid for elderly and athlete is an overestimation of the results presented. The results from the study may at most indicate a potentially ergogenic effect on muscle, but further suggestion of which population it could aid is not possible to estimate at this point.
Response: We thank the comments of reviewer. We have deleted that sentence related to ergogenic aid and revised the manuscript.

5. Discussion needs further revision by the authors. The parallel made with exercise activation of Akt is inconsistent with the evidence that actions of exercise on mTOR signalling are Akt independent mechanism. Moreover the suggestion that Akt phosphorylation at different time points are due to de novo synthesis of growth factors for the 15 min time point and at 45 min due to autocrine/paracrine loop of IGF-1 are both groundless. Additionally, 45 min of treatment is not likely to increase expression of Akt protein as stated as a possible mechanism (“The second peak that appeared 45 min after AS treatment was possibly mediated by the autocrine/paracrine loop of IGF-1, because the expression of Akt (...) has been known to be increased by AS treat”).
Response: We greatly appreciate the comments of reviewer. We have deleted and revised. (lines 312-317 and 329-355).

1. **Minor essential revisions:**

1. The western blot membranes were stripped and reprobed using the same secondary antibody, did the authors tested for stripping efficiency? This is important because the phosphorylation data were normalized by the specific total proteins which when proteins are not efficiently removed may affect the densitometry of the second probing (total Akt and mTOR). In the methods it is stated that b-actin was
also verified. Do phosphorylation of Akt and mTOR remain the same when normalized by b-actin? The use of a different molecular weight protein (as b-actin) might be useful to clarify this.

**Response:** Yes, we did confirm the stripping efficiency before the experiment. β-actin was also examined after stripping. We have added the detailed description in the revised manuscript (figure legends) and showed the individual β-actin image in Figure 3 and 4.

2. Is the SD in the Pre bar representing variation within different experiments or different membranes? Please state.

**Response:** It represents the different experiments. We have added the detailed description in the revised manuscript (lines 195).

3. Figure 4A: it is missing ‘l’ ‘e’ on Relative at the figure legend.

**Response:** We thank the comments of reviewer. We have revised the Figure.

**Discretionary revisions**

1. Line 298: “de novo” should be in italic.

**Response:** We rewrote this part.

2. Keywords: Akt and mTOR is already on the title, no need to add them in the keywords.

**Response:** We thank the comments of reviewer. We have deleted “Akt” and “mTOR”.

1  **Level of interest:** An article of limited interest
1  **Quality of written English:** Acceptable
1  **Statistical review:** No, the manuscript does not need to be seen by a statistician.
Referee 2: Matthew Alexander

Reviewer's report:
The research article by Yeh and colleagues describes the characterization of Angelica sinensis (AS) (commonly referred to as dong quai), an herbal medicine used by athletes and the general population to improve athletic performance and overall stamina. The authors look directly at its functional role in muscle cells in cell culture, with an emphasis on a potential link between AS and myotube hypertrophy. The authors identified ferulic acid as being the main potent compound of AS via HPLC chromatography, and go on to characterize the expression levels of PI3K/AKT/mTOR signaling factors in C2C12 myoblasts/myotubes treated with AS and a series of PI3K/AKT/mTOR pathway inhibitors and/or activators.

1. **Major Essential Revisions:**

1. My main scientific criticism of this manuscript is the fact that the authors state that the PI3K/AKT/mTOR pathway is affected by AS treatment of myotubes, yet the authors look AKT and mTOR, yet not other aspects of this signaling pathway. Were the PI3K complex (p85, p110) components and or PTEN expression levels detected? Were the downstream signaling factors of PI3K/AKT/mTOR signaling pathway detected and quantified, namely 4E-BP1/phospho-4E-BP1 and/or S6K? The authors should look at the downstream signaling factors at either the RNA or protein levels following AS treatment of myotubes if they want to fully support their claim that AS is having its hypertrophic effects in myotubes directly through this pathway.

Response: We greatly appreciate the comments of reviewer. We understand that presenting the data of PI3K complex and PTEN is important for clearing the AS effects of PI3K/AKT/mTOR signaling. We also understand that examining the 4E-BP1 and S6K are powerful evidence for AS-induced muscle hypertrophy function. However, we currently do not have these data. In fact, we are conducting AS with exercise training to promote muscle hypertrophy in animal experiments. In our preliminary data, we found that exercise training with AS supplements for 6 weeks can significantly increase forelimb grip strength and weight-loaded swimming performance in mice. We will present the 4E-BP1 and S6K protein levels of skeletal muscle in the future experimental results. We wish the reviewer understand the limitations of this study, and give us a possibility to supplement important information in the future publishing.

2. I had an overall difficult time in reading this manuscript. Throughout the manuscript there are terms that are confusing to the reader (ex. proliferous property, page 5, line 78; do the authors mean promote cell proliferation?). I strongly
recommend the authors either have a native English speaker or carefully edit the manuscript for English grammar and sentence comprehension and clarity.

**Response:** We thank the suggestion of reviewer. We have carefully edited this manuscript for English grammar and sentence comprehension and clarity.

1. **Minor Essential Revisions:**

   1. The HPLC chromatogram is only discussed in the Methods section and not the Results. If the authors do not want to discuss this in the Results section, then they should move it to the Supplemental Data/Figures.

   **Response:** We greatly appreciate the suggestion of reviewer. We have moved the figures to the Supplemental Data.

   2. The myotube pictures in Figure 2 appear green tinted and are difficult to view and interpret. The authors should either adjust the filter settings on the images or retake the pictures for clarity.

   **Response:** We thank the suggestion of reviewer. We have adjusted the images and revised Figure.

   3. In the Methods the authors cite another paper for the description of how the differentiated myoblasts into myotubes, the authors should specifically state how (reduced serum percentage) and when they differentiated the myoblasts into myotubes in the Methods.

   **Response:** We are sorry that we have left out this important description. In this study, myotube formation was induced by DMEM containing 2% horse serum instead of fetal bovine serum. We have added this sentence in the revised manuscript (lines 103-105).

   4. Figure 4A typo, “Relativ” on y-axis change to “Relative”.

   **Response:** We greatly appreciate the correction of reviewer. We have done it.

   5. Figure 4 and 5 western blots. Given that some of the differences in blot band intensities appear to be minor, the authors should include a loading control. There is a reference to a Beta-actin antibody from Sigma used, but I don’t see it in the manuscript. Also, this is a minor point but the authors should be consistent in their references to certain companies (Cell Signaling Technology in Danvers, MA, USA or Beverly, MA?). Just one of the Cell Signaling Tech company citation is needed.

   **Response:** We thank the comments of reviewer. We have added the corresponding result of western blot analysis of Beta-actin in Figure upper panels. We have revised
the citation of company.

6. Minor comment, the authors state that they used “fresh growth medium” for the “NON” samples. There’s some discrepancy, was this growth or differentiation medium? The authors need to be specific and state this in the Methods or at very least the Figure legends.

Response: We thank the comments of the reviewer. The fresh growth medium is 2% HS/DMEM. We are sorry to have left out this necessary description; we have added the detailed description in the revised manuscript (lines 138, 139, 142, and 143) and Figure legends (Figure 1-4).

7. References/citations are needed on page 5, sentences on lines 66, 69.

Response: We thank the comments of reviewer. We have added the references in the revised manuscript.

8. pg17, line 295 Awkward sentence.

Response: We thank the comments of reviewer. We have revised the manuscript.

1 Level of interest: An article of importance in its field
1 Quality of written English: Not suitable for publication unless extensively edited
1 Statistical review: No, the manuscript does not need to be seen by a statistician.
Referee 3: Manuel Estrada
Reviewer's report:
In this study, the authors showed that Angelica sinencis extracts induce hypertrophy in the C2C12 myotube cell line via PI3K-Akt-mTOR signaling. The experiments are technically well done and the findings are interesting. I find the subject matter appealing and potentially important. The main concerns about this study are related to the lack of other hypertrophic parameters to check myotube hypertrophy. The study appears to be sound regards to the overall design and methodology.

1 Major Compulsory Revisions:
The authors should address potential off-target effects of pharmacological compounds. The experiments would be strengthened performing parallel experiments utilizing perturbation and other molecular techniques to reinforce the other approaches.

Response: The Angelica sinensis used in this study were commercial product from certified pharmaceutical factory. The main chemical constituents of Angelica roots are ferulic acid, ligustilide, angelicide, brefeldin A, butylidenephthalide, butyphthalide, succinic acid, nicotinic acid, uracil, and adenine. The constituents most often associated with the pharmacological activities of Angelica roots are ferulic acid and ligustilide (predominantly the Z-isomer), the former being able to inhibit platelet aggregation and serotonin release, the latter having significant antiasthmatic and spasmolytic activities. The levels of these two constituents are usually used as chemical markers for the quality control of Angelica roots. To confirm the quality of AS, we detect the main chemical constituents of AS. We hope reviewer could understand that we try to complete the experiment with certain limitations. We also strengthened the introduction of Angelica in the revised manuscript (lines 77-84 and 207).

Did the authors determine which minimal and maximal "AS" concentrations were used to induce myotube hypertrophy?
Response: We greatly appreciate the suggestion of reviewer. After XTT assay, the results indicated that AS was not harmful to myotubes at concentrations of 1, 10, 100 ng/mL for 72 h. We examined the myotubes at various concentrations of AS (10, 50, 100, 500, and 1000 ng/mL), and incubated for 72 h. Myotubes can be observed at 10, 50, 100, and 500 ng/mL, but not at 1000 ng/mL. We have added this results in supplement 2. The final manuscript presented only 10 ng/mL of AS, because of it is simply compared the myotubes hypertrophic efficiency on the same concentrations of IGF-1.
There are several well established methods to determine skeletal muscle hypertrophy; however the authors show just one hypertrophic parameter using light microscope for all their conclusions. The manuscript and scope would be strengthened if myotube hypertrophy is characterized by using an alternative parameter as determination of alpha-actin levels, total protein content, amino acid incorporation, for instance.

Response: We greatly appreciate the suggestion of reviewer. We have gave a definition of myotube hypertrophy in this study and added the detailed description in the revised manuscript (lines 229-230).

Why mTOR phosphorylation was determined at Ser2448? This should be discussed. Determination of either S6K1 or 4EBP1 phosphorylation (downstream targets) are selective parameters to evaluate mTOR activity.

Response: We greatly appreciate the suggestion of reviewer. Previous studies have shown that mTOR is a direct substrate for the Akt kinase and identified Ser<sup>2448</sup> as the Akt target site in mTOR. In addition, phosphorylation of mTOR at Ser<sup>2448</sup> is mediated by p70S6 kinase. We have added the discussed related to mTOR Ser<sup>2448</sup> and revised manuscript (lines 338-355).

1 **Minor Essential Revisions:**
Whereas the English language is relatively good, there is definitively room for improvement.

Response: We thank the suggestion of reviewer. We have invite carefully edit this manuscript for English grammar and sentence comprehension and clarity.

1 **Level of interest:** An article whose findings are important to those with closely related research interests.
1 **Quality of written English:** Not suitable for publication unless extensively edited.
1 **Statistical review:** No, the manuscript does not need to be seen by a statistician.