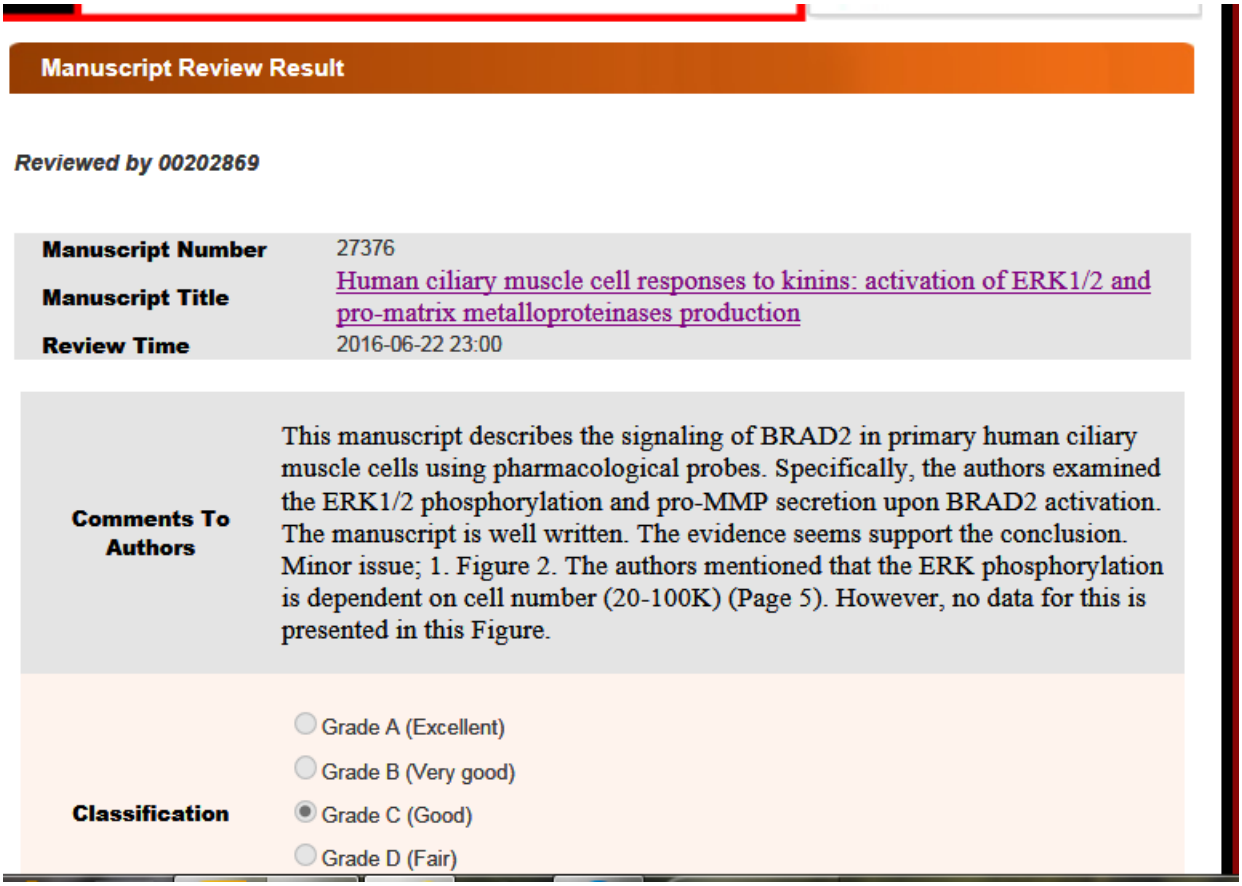
WJO MS # 27376 Reviewers' Comments and Author Responses

REVIEWER # 1 [202869]- Comments



Manuscript Review Result

Reviewed by 00202869

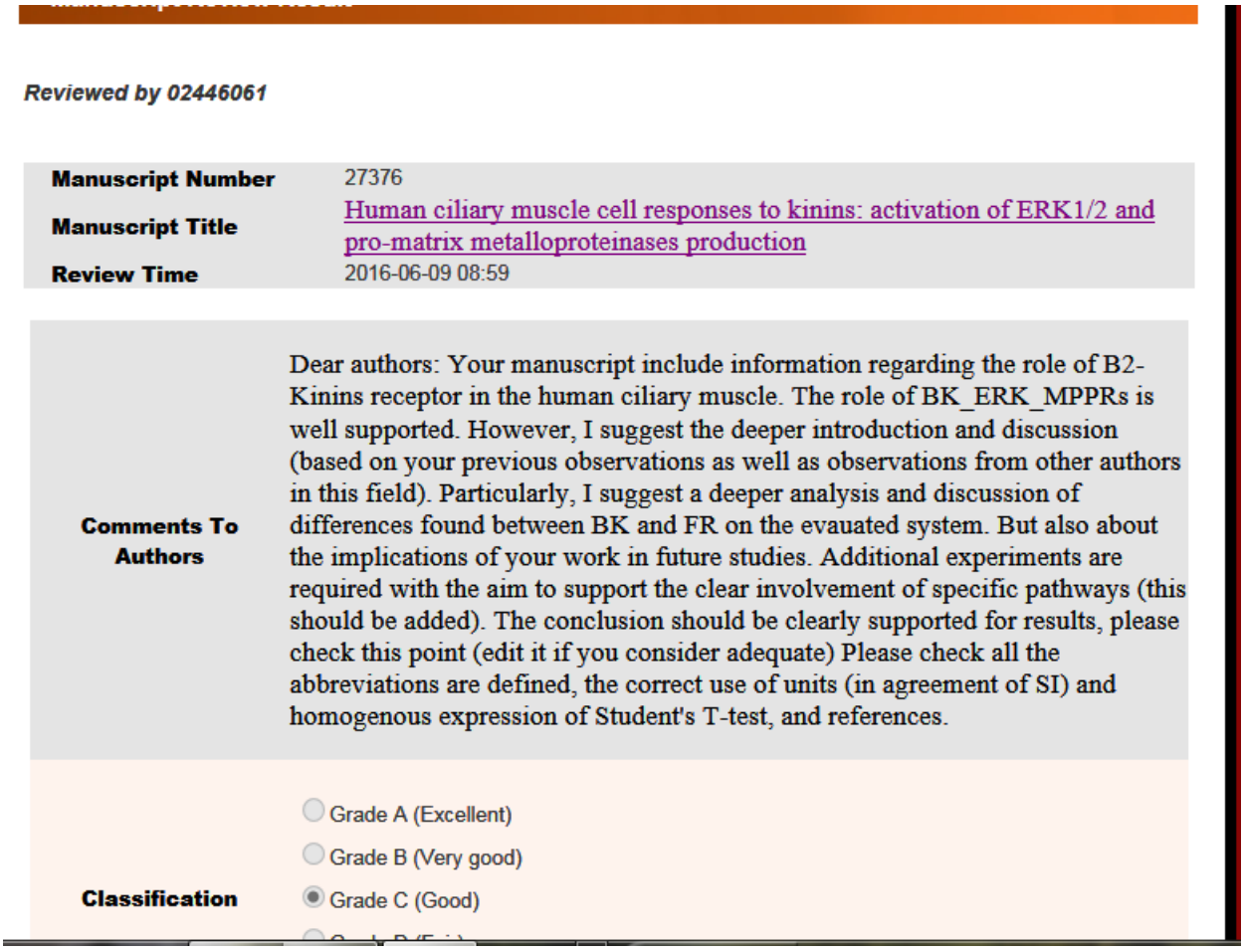
Manuscript Number	27376
Manuscript Title	Human ciliary muscle cell responses to kinins: activation of ERK1/2 and pro-matrix metalloproteinases production
Review Time	2016-06-22 23:00

Comments To Authors	This manuscript describes the signaling of BRAD2 in primary human ciliary muscle cells using pharmacological probes. Specifically, the authors examined the ERK1/2 phosphorylation and pro-MMP secretion upon BRAD2 activation. The manuscript is well written. The evidence seems support the conclusion. Minor issue; 1. Figure 2. The authors mentioned that the ERK phosphorylation is dependent on cell number (20-100K) (Page 5). However, no data for this is presented in this Figure.
Classification	○ Grade A (Excellent) ○ Grade B (Very good) ◉ Grade C (Good) ○ Grade D (Fair)

Authors' Responses to Reviewer #1 [202869]:

We agreed with the reviewer's point above and have deleted the mention to the dependence on cell number in the Results section.

Reviewer # 2 [2446061] – Comments



Reviewed by 02446061

Manuscript Number	27376
Manuscript Title	Human ciliary muscle cell responses to kinins: activation of ERK1/2 and pro-matrix metalloproteinases production
Review Time	2016-06-09 08:59

Comments To Authors

Dear authors: Your manuscript include information regarding the role of B2-Kinins receptor in the human ciliary muscle. The role of BK_ERK_MPPRs is well supported. However, I suggest the deeper introduction and discussion (based on your previous observations as well as observations from other authors in this field). Particularly, I suggest a deeper analysis and discussion of differences found between BK and FR on the evaluated system. But also about the implications of your work in future studies. Additional experiments are required with the aim to support the clear involvement of specific pathways (this should be added). The conclusion should be clearly supported for results, please check this point (edit it if you consider adequate) Please check all the abbreviations are defined, the correct use of units (in agreement of SI) and homogenous expression of Student's T-test, and references.

Classification

- ○ Grade A (Excellent)
- ○ Grade B (Very good)
- ◉ Grade C (Good)

Authors' Responses to Reviewer #2 [2446061]:

1. Regarding the request for a deeper Introduction and Discussion: we feel that the depth and breadth of our studies only necessitate a short succinct Introduction and Discussion which the current manuscript contains. We feel both these sections of the manuscript are adequate and don't wish to add too much unnecessary information or undue speculation.

2. We agreed with the reviewer on the use of SI units and have corrected them as requested.

Reviewer # 3 [2522131] – Comments

Reviewed by 02544131

Manuscript Number	27376
Manuscript Title	Human ciliary muscle cell responses to kinins: activation of ERK1/2 and pro-matrix metalloproteinases production
Review Time	2016-06-06 22:42

Comments To Authors

The manuscript "human ciliary muscl cell responses to kinins: Activation of ERK1/2 and pro-matrix metalloproteinases production" by Sharif et al. is a mechanistic study describing the molecular sequences by which activated bradykinin receptors initiate a rapid signalling cascade through ERK1/2 which in turn activate the MMP-1, -2 and -3 production leading to subsequent events (previously published by the authors). The topic of study is good no doubt but not supported by the way of presentation. I would like to point out few points and only after incorporating those points the manuscript might be considered for publication. Major point is the precise data acquisition on the MMPs. According to the abstract the data were obtained by immunoblot analysis. Therefore substantial additional information is needed. 1. The description of antibodies used (rabbit polyclonal anti-pro-MMP Abs) is the only information provided. However for the reader would be helpful if the authors provide information on recognition domain and producer-company and from which company the Abs were purchased. 2. The results are from western blots, however, there are no WB-presented and how the relative increase of the MMPs pro-forms were calculated. (ImageJ?) 3. The analysed cell supernatants were concentrated by Centricon spin coulmns but the authors provide no information on total protein content. What was the column MW-cut-off and what was the reference protein

Comments To Authors

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Classification

- ○ Grade A (Excellent)
- ○ Grade B (Very good)
- ◉ Grade C (Good)

Authors' Responses to Reviewer #3 [2522131]:

Q. 1 The description of antibodies used……the Abs were purchased.

Response: Anti-MMP-1, anti-MMP-2, and anti-MMP-3 antibodies were raised using synthetic peptide towards N-terminal of human MMP-1, MMP-2, or MMP-3 as stated by manufacturers. This information is included in the revised manuscript. Additionally, the catalogue number for each antibody, dilution, and sources of antibodies are provided in the revised manuscript.

Q. 2 The results are from western blots, however, there are no WB-presented and how the relative increase of the MMPs pro-forms were calculated (Image J?)

Response: As per suggestion, actual bands of WB are now included in each revised figure. The intensities of each band were measured by densitometry using Biorad Versa Doc program. The band intensities (arbitrary units) were normalized with total cellular protein. This normalization to total cellular protein was used to correct for the differences in the number of cells within each experimental assay for MMP secretion studies. It is also included in the Method's section.

Q. 3 The analyzed cell supernatants were concentrated by centricon spin columns but the authors provide no information on total protein content. What was the column MW-cut-off and what was the reference protein to assess in the individual samples the differences in secreted pro-MMPs?

Response: Medium (cell supernatant) was concentrated by using an ultrafiltration centrifugal concentrator (10 kDa cutoff; Amicon Beverly, MA) and adjusted to a final concentration ratio of 10:1. Equivalent volumes (40 L) of medium were loaded onto 10% SDS-polyacrylamide gels followed by transfer to a nitrocellulose membrane. The membranes were then probed with anti-pro-MMP-1, anti-pro-MMP-2, or anti-pro-MMP-3 antibodies overnight at 4°C. Bands were visualized by the addition of secondary antibody which is HRP-conjugated at dilution of 1:3000. The reference proteins were pro-MMP-1, pro-MMP-2, and pro-MMP-3 and each pro-MMP was identified using purified positive control of pro-MMP-1, pro-MMP-2, and pro-MMP-3 running parallel to the samples in 10% SDS-PAGE and Western blotting. Finally, the band intensities were visualized using ECL reagents. The band intensities were quantified by densitometry as arbitrary unit and normalized with total cellular protein. This part is also included in the Method's section.

Q. 4 Why were only the pro-forms determined? Additionally, would be helpful to run an in gel-zymography to identify all active forms of MMP2 and demonstrate at the same time that MMP-9 and its forms are not secreted.

Response: We measured pro-forms of MMPs because it gives a broader picture about the changes occurring to MMPs secretion in response to a drug treatment. It is important to emphasize that many pro-forms of MMPs will also show up in zymogram, considering this limitation we chose to measure pro-forms of the MMPs using highly selective antibodies for each pro-MMP (e.g.; Anti-pro-MMP-1, Anti-pro-MMP-2, and Anti-pro-MMP-3). None of our experiments have shown any secretion of pro-MMP-9 which was measured by Western blotting (data not shown).

Dear Editor,

We hope that all the responses to Reviewers' questions/ comments are satisfactory and that the revised manuscript meets your approval and acceptance.

We look forward to receiving your decision soon.

Sincerely,

Naj

Najam Sharif, PhD