Author’s response to reviews

Title: All-trans retinoic acid stimulates the secretion of TGF-β2 via the phospholipase C but not the adenylyl cyclase signaling pathway in retinal pigment epithelium cells

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Author’s response to reviews:

Author Reply to comments

From: "BMC Ophthalmology Editorial Office" <em@editorialmanager.com>
Date: September 7, 2018 at 2:46:18 AM GMT+8
To: "Zhihong Deng" <daneyldeng@126.com>
Subject: Your submission to BMC Ophthalmology - BOPH-D-18-00528

Reply-To: "BMC Ophthalmology Editorial Office" <princess.quitalan@biomedcentral.com>

BOPH-D-18-00528

Effect of U73122 and SQ22536 on all-trans retinoic acid in stimulating the secretion of TGF-β2 in human retinal pigment epithelium cells: a randomized controlled trial
Dear Dr Deng,

Your manuscript "Effect of U73122 and SQ22536 on all-trans retinoic acid in stimulating the secretion of TGF-β2 in human retinal pigment epithelium cells: a randomized controlled trial" (BOPH-D-18-00528) has been assessed by our reviewers. They have raised a number of points which we believe would improve the manuscript and may allow a revised version to be published in BMC Ophthalmology.

Their reports, together with any other comments, are below. Please also take a moment to check our website at https://boph.editorialmanager.com/ for any additional comments that were saved as attachments.

If you are able to fully address these points, we would encourage you to submit a revised manuscript to BMC Ophthalmology.

Once you have made the necessary corrections, please submit online at:

https://boph.editorialmanager.com/

If you have forgotten your password, please use the 'Send Login Details' link on the login page at https://boph.editorialmanager.com/. For security reasons, your password will be reset.

Please include a cover letter with a point-by-point response to the comments, describing any additional experiments that were carried out and including a detailed rebuttal of any criticisms or requested revisions that you disagreed with. Please also ensure that all changes to the manuscript are indicated in the text by highlighting or using track changes.
Please also ensure that your revised manuscript conforms to the journal style, which can be found at the Submission Guidelines on the journal homepage.

A decision will be made once we have received your revised manuscript, which we expect by 06 Oct 2018.

Please note that you will not be able to add, remove, or change the order of authors once the editor has accepted your manuscript for publication. Any proposed changes to the authorship must be requested during peer-review, and adhere to our criteria for authorship as outlined in BioMed Central's policies. To request a change in authorship, please download the 'Request for change in authorship form' which can be found here - http://www.biomedcentral.com/about/editorialpolicies#authorship. Please note that incomplete forms will be rejected. Your request will be taken into consideration by the editor, and you will be advised whether any changes will be permitted. Please be aware that we may investigate, or ask your institute to investigate, any unauthorized attempts to change authorship or discrepancies in authorship between the submitted and revised versions of your manuscript.

I look forward to receiving your revised manuscript and please do not hesitate to contact us if you have any questions.

Best wishes,

shusheng wang

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Technical Comments:

Title page:
We have noted that the authors' order provided on the title page, differs to the one on file in the editorial manager system. Please ensure that the authors' order and their details match in the manuscript file and in the editorial manager system.

Reply: In the editorial manager system, there is only one corresponding author we can choose. In our research, Dr Deng and Dr Tan have made a great contribution both. Dr Deng and Dr Tan are the co-corrresponding authors of this paper.

Method:

As cell lines obtained commercially, please also state catalogue/identification number.

The human RPE cell line D407 was purchased from the Retinal Cell Biology Laboratory, University of South Carolina (USA). We have published another paper about the study of D407.


Funding:

We note that you have not included a ‘Funding’ section in the Declarations. All sources of funding for the research reported should be declared. If no funding was obtained for your study we still require this section to be included with the statement “No funding was obtained for this study”.

Reply: We added this section in the revised manuscript.

Acknowledgements:

We note that you have not included an acknowledgements section in the Declarations. If you have no acknowledgements please put ‘Not Applicable’ in this section.
Reply: We added this section in the revised manuscript.

Figure legends:

Please remove the figure legends embedded within the figure files. All figure titles/legends should be listed under a separate heading of 'Figure Legends' after the References.

Reply: We have removed the figure legends embedded within the figure files and added the section named 'Figure Legends' after the References.

Editor Comments:

BMC Ophthalmology operates a policy of open peer review, which means that you will be able to see the names of the reviewers who provided the reports via the online peer review system. We encourage you to also view the reports there, via the action links on the left-hand side of the page, to see the names of the reviewers.

Reviewer reports:

Wenchuan Wu (Reviewer 1): This study investigated the involvement of the phospholiase C, but not adenylyl cyclase, signaling pathway in the all-trans retinoic acid (ATRA)-stimulated TGF-beta2 expression and secretion in human retinal pigment epithelial (RPE) cells. The major flaw in this study is the insufficient quality in all experiments. In brief, authors did not mention in Methodology the solvents used for ATRA and both inhibitors of phospholiase and adenylyl cyclase. For these agents are water insoluble and should be dissolved in DMSO, all in vitro experiments lacked appropriate solvent controls so that all the results could not be justified. Besides, other minor issues are also concerned as below:

Reply: Thank you for your insightful review in improving our manuscript and we carefully studied your comments and suggestions. We realized it is our neglect of the solvents in the methods. We did use the DMSO as a solvent to dissolve the ATRA, U73122 and SQ22536 actually. We added the details of solvents in the revised manuscript. And the Group D set as solvent controls, we revised the statements and added the details of the experiment as follows:
“Group D: Control cells were cultured under identical conditions with the addition of an equivalent volume of DMSO only.”

1. Manuscript title is not informative, because it does not reveal the study conclusion directly. Moreover, the subtitle "a randomized controlled trial" is redundant and misleading.

Reply: Thank you for your advice and the corresponding modification have been made. the subtitle "a randomized controlled trial" has been removed.

2. Authors should rewrite Introduction by detailing the pathomechanistic significance of ATRA and TGF-beta2 in scleral remodeling and myopia. These information may rationalize why they design in vitro experiments this way.

Reply: the pathomechanistic significance of ATRA and TGF-beta2 in scleral remodeling and myopia has been detailed in the introduction.

3. In methodology (line 105), ELISA stands for enzyme-linked immnosorbent assay and should not follow by another assay. ELISA is well commercialized and a very common tool used for studies. Authors should show the kit maker and shorten the assay procedures.

Reply: Sorry for the negligence, we deleted the redundant “assay” and revised the assay procedures in the part of ELISA as follows: “Cell culture supernatants were collected, spun down to remove cell debris, and diluted 1:5 to measure TGF-β2 protein concentration using a 96-well human TGF-β2 ELISA kit (from Bender MedSystems) according to the manufacturers’ instructions strictly. The ELISA experiment was repeated three times.”

Khrishen Cunnusamy (Reviewer 2): Review of "Effect of U73122 and SQ22536 on all-trans retinoic acid in stimulating the secretion of TGF-β2 in human retinal pigment epithelium cells: a randomized controlled trial" manuscript.
This is an elegant and simple study which aims to investigate the difference between inhibitions of the phospholipase C versus inhibition of the adenylyl cyclase signaling pathways on the production of TGFβ2. In that respect, the study uses two compounds which differentially act on the respective pathways that may potentially regulate the production of TGFβ2. SQ22536 acts as an adenylyl cyclase inhibitor, while U73122 operates as a phospholipase C inhibitor, and a no treatment group complements the study. The use of RPE cells further reinforces the adequacy of this investigation. Based on the results of the dose response experiment, TGFβ2 expression appears to be regulated by phospholipase C and not by adenylyl cyclase expression. However, important questions such as ultrastructural changes induced by the modulating effect of TGFβ2 expression still need to be addressed. Accordingly, the authors of this manuscript should attempt to respond to the following points:

1. The author of the article should consider the use of an additional agent that inhibits exclusively the phospholipase C pathway to further validate the claim from the original findings with U73122.

Reply: Thank you for your insightful review in improving our manuscript and we carefully studied your comments and suggestions. Firstly, this is a good question and we considered it before research. We did a lot of work in choosing inhibitors of phospholipase C (PLC) signaling pathway and adenylyl cyclase signaling pathway. We found that D609 and U73122 are the popular inhibitors of phospholipase C in other research, the target of D609 is Phosphatidylcholine-Specific Phospholipase C (PC-PLC), but the key enzyme in the phospholipase C signaling pathway is phosphatidylinositol-specific phospholipase-C (PI-PLC), especially PI-PLCβ. We think D609 is not the best choice in inhibiting phospholipase C signaling pathway. we found the U73122 has been used as inhibitor of phospholipase C signaling pathway in a lot of studies. [1-4]


2. The same should be attempted with SQ22536 as it pertains to the inhibition of adenylyl cyclase to see if similar results are obtained.

Reply: Same as U73122, we try to choose a selective inhibitor of the adenylyl cyclase which is a key enzyme in the adenylyl cyclase signaling pathway. We found that Bithionol and SQ22536 are AC selective inhibitors of adenylyl cyclase signaling pathway. The target of Bithionol is soluble adenylyl cyclase (sAC). However, the key factor in the adenylyl cyclase signaling pathway is transmembrane adenylyl cyclases (tmACs), and we could not find the selective inhibitor of Transmembrane adenylyl cyclases (tmACs). We found some evidence to support it as follows:

“In mammals, two distinct families of adenylyl cyclase synthesize the nearly universal second messenger cAMP. Transmembrane adenylyl cyclases (tmACs) are obligatory membrane proteins regulated by heterotrimeric G proteins; they mediate intracellular responses to extracellular signals such as hormones and neurotransmitters. In contrast, soluble adenylyl cyclase (sAC) is specifically targeted to intracellular domains and organelles, where it is positioned to provide the second messenger activating the intracellular and intra-organelar targets of cAMP.” From the article “Buck J, Levin LR: The role of soluble adenylyl cyclase in health and disease Preface. Biochim Biophys Acta-Mol Basis Dis 2014, 1842(12):2533-2534.”

We also found the SQ22536 has been used as inhibitor of adenylyl cyclase (AC) signaling pathway in couple of studies:


It is creditable that we use the SQ22536 as the AC selective inhibitor.

3. The author should further demonstrate that those agents are effectively inhibiting both of those pathways are indeed working as suggested.

Reply: Inhibitors of intracellular signaling events, including enzyme inhibitors, are often used to investigate signal transduction pathways. As is said above, U73122 has been widely used as an inhibitor of phospholipase C, the enzyme mediating phosphoinositide hydrolysis. we sought to selectively block signaling through the phospholipase C pathway with U73122. The SQ22536 has been widely used as an inhibitor of AC, the enzyme increasing the level of cAMP. We sought to selectively block signaling through the AC pathway with SQ22536. Some studies were published to reveal the mechanism of them, we listed them as follows:


4. In addition to inhibiting those pathways, the authors should also investigate the effects of inducing those pathways further on the amount of TGFβ2 produced.

Reply: Firstly, thank you for your advice to improve our manuscript, this is a good way to confirm the conclusion that we have made, but we think it is not suitable for our research. Our study aims to reveal the mechanism of which pathway involves secretion of TGFβ2 stimulated by ATRA. In our study, we found that ATRA stimulates the secretion of TGFβ2 via the phospholipase C signaling pathway. Exerting the inhibitors of those pathways to investigate the mechanism is simple and effective.
5. Validate the results with TGFB2 ELISA kits which can are readily available from Ebioscience or R&D biolabs. Since the TGFB2 is secreted, it should be readily quantified by the ELISAs.

Reply: we did use the TGFβ2 ELISA kits to quantify the secreted TGFβ2 which were purchased from Bender MedSystems (Austria). It has been mentioned in the manuscript.

6. Last but not least, the main problem is that the study does not conclusively show that modulating the expression of TGFB2 has a beneficial effect on the incidence of myopia - either ultra structurally or functionally. This study is done in vivo but is not be functionally validated i.e. does modulation of TGFβ2 signaling affect the incidence of myopia.

Reply: This study focuses the signal pathway of ATRA stimulating the secretion of TGF-β2 in RPE cells. The further study will focus on the effect of ATRA on the expression of scleral TGF-β2, refractive diopter and eye axial of guinea pig.