



Life Science Alliance

Muscarinic receptor M3 contributes to intestinal stem cell maintenance via EphB/ephrin-B signaling

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

June 3, 2021

Re: Life Science Alliance manuscript #LSA-2020-00962-T

Dr. Toshio Takahashi
Suntory Foundation for Life Sciences
8-1-1 Seikadai
Seika-cho
Kyoto, Kyoto 619-0284
Japan

Dear Dr. Takahashi,

Thank you for submitting your manuscript entitled "Muscarinic receptor M3 contributes to intestinal stem cell maintenance via EphB/ephrin-B signaling" to Life Science Alliance. The manuscript was assessed by an expert reviewer, whose comments are appended to this letter.

Please address the Reviewer's comments, including Paneth cell quantification and a toning down of the Conclusions.

To upload the revised version of your manuscript, please log in to your account:

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While you are revising your manuscript, please also attend to the below editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

The typical timeframe for revisions is three months. Please note that papers are generally considered through only one revision cycle, so strong support from the referees on the revised version is needed for acceptance.

When submitting the revision, please include a letter addressing the reviewers' comments point by point.

We hope that the comments below will prove constructive as your work progresses.

Thank you for this interesting contribution to Life Science Alliance. We are looking forward to receiving your revised manuscript.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
<http://www.lsajournal.org>

A. THESE ITEMS ARE REQUIRED FOR REVISIONS

- A letter addressing the reviewers' comments point by point.
- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).
- High-resolution figure, supplementary figure and video files uploaded as individual files: See our detailed guidelines for preparing your production-ready images, <https://www.life-science-alliance.org/authors>
- Summary blurb (enter in submission system): A short text summarizing in a single sentence the study (max. 200 characters including spaces). This text is used in conjunction with the titles of papers, hence should be informative and complementary to the title and running title. It should describe the context and significance of the findings for a general readership; it should be written in the present tense and refer to the work in the third person. Author names should not be mentioned.

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We encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots and spreadsheets for the main figures of the manuscript. If you would like to add source data, we would welcome one PDF/Excel-file per figure for this information. These files will be linked online as supplementary "Source Data" files.

*****IMPORTANT:** It is Life Science Alliance policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Reviewer #1 (Comments to the Authors (Required)):

Well done study, written well. This work expands on this groups previous work demonstrating the endogenous (epithelial) cholinergic system controls homeostasis. Clear data support the M3 receptor inhibits Eph/Ephrin signaling with downstream upregulation of MEK, TNF and cell cycle when M3 is KO. Elegant in vitro and in vivo studies demonstrate the direct effect on ISC and expansion of TA compartment. I have no major critic's. Minor comments; would have expected to see Paneth cell quantification. Would also like to understand the role of the ENS better - limit comment in discussion only specifically given the similar in vitro data without signaling

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Thank you very much for your positive comments concerning our manuscript. I would like to answer your minor comments as follows.

Paneth cell quantification

(Answer): Concerning Paneth cell quantification, we carried out quantitative RT-PCR to evaluate the functional consequence of deletion of M3. mRNA level of Paneth cell marker gene (*Lysozyme*) was significantly enhanced in M3^{-/-} crypts (1.5 folds over WT crypts) (Fig. 3E). Furthermore, mRNA level of *EphB3* gene which is mainly expressed in Paneth cells was also enhanced compared with that of WT crypts (2 folds) (Fig. 5D). The data support increased numbers of ISCs with a proportionate increase in Paneth cells.

To have a fruitful discussion in our manuscript, I added a new paragraph as follows.

(New paragraph): The enlarged crypt morphology can be explained by a specific increase in the transit-amplifying compartment and by increased numbers of ISCs with a proportionate increase in transit-amplifying and Paneth cells. We identified the major intestinal epithelial cell populations according to their differential expression of EphB2 and marker gene expression. Loss of M3 caused profound increase in the population of the EphB2^{high} stem cell and EphB2^{med} progenitor compartment. In addition to their increased populations, we observed increased expression of ISC markers within EphB2^{high} and of progenitor markers within EphB2^{med} populations, supporting an expansion of each compartment upon loss of M3. We also observed increased expression of the marker genes for Paneth cells (*Lysozyme* and *EphB3*) as well as for enterocytes (*villin*) and goblet cells (*mucin-2*) in M3^{-/-} crypts, quantitatively. Paneth cells are the source of multiple stem cell growth factors (for example, Wnt3, EGF, and TGF- α) which are essential signals for stem cell maintenance (Sato *et al*, 2011). Indeed, co-culturing of sorted ISCs with Paneth cells dramatically improves organoid formation (Sato *et al*, 2011). Thus, the number of Paneth cell increased can induce to increase the number of ISCs in M3^{-/-} crypts. Collectively, the data support a direct effect of M3 on ISCs, which subsequently affects the size of ISC compartment and the crypt.

The role of the ENS

I agree with the reviewer's comment. Understanding the role of the ENS in the intestinal crypts is of key importance. According to the reviewer's comment, I added new sentences in the third paragraph in discussion and new references as follows.

(New sentences): In a more recent study, co-culture of a monolayer of organoid-grown differentiated cells with dissociated adult mouse ENS cells shows that the epithelial cell density increases by 40% (Puzan *et al*, 2018). Especially, chromogranin A-positive epithelial cells increased, suggesting enhancement of enteroendocrine cell population (Puzan *et al*. 2018). The data imply that epithelial lineage composition may ultimately benefit from the presence of the ENS. As one of the major pathways of excitatory transmission within ENS is mediated by cholinergic transmission (Galligan *et al*, 2000), neuronal ACh is expected to have the potential functional effects on crypt homeostasis.

(New references)

1) Puzan M, Hosic S, Ghio C, Koppes A (2018) Enteric nervous system regulation of intestinal stem cell differentiation and epithelial monolayer function. *Sci Rep* 8: 6313

2) Galligan JJ, LePard KJ, Schneider DA, Zhou X (2000) Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* 81: 97-103

The new link between M3 and EphB/ephrin-B signaling

To have a fruitful discussion in our manuscript, I added a new paragraph as follows. Additionally, I added new references as follows.

(New paragraph): The evidence that EphB and ephrin-B associate and interact with other cell surface receptors such as channel type receptors and G protein-coupled receptors suggests integration of different signaling routes or modulation of signal transmission. The N-methyl-D-aspartate (NMDA) receptor channel, which is a receptor for glutamate in the central nervous system, interacts with EphB at the cell surface; this interaction is mediated by the extracellular regions of the two receptors (Dalva *et al*, 2000). Bruno and coworkers (Bruno *et al*, 2005) have provided evidence for a novel type of interaction between ephrin-B2, NMDA receptors, and metabotropic glutamate 1 receptors, a new partner in the network in the developing brain. M3 and metabotropic glutamate 1 receptors are both coupled to Gq proteins, and their activation stimulates phosphatidylinositol hydrolysis with ensuing intracellular Ca²⁺ release and activation of protein kinase C (De Blasi *et al*, 2001). This is the first time that M3 signaling has controlled the EphB/ephrin-B system in the small intestine. However, the molecular details of the new link between M3 and EphB/ephrin-B signaling remain unclear.

(New references)

1) Dalva MB, Takasu MA, Lin MZ, Shamah SM, Hu L, Gale NW, Greenberg ME (2000) EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell* 103: 945-956

2) Bruno LC, Spinsanti P, Molinari G, Korkhov V, Esposito Z, Patanè M, Melchiorri D, Freissmuth M, Nicoletti F (2005) Interactions between ephrin-B and metabotropic glutamate 1 receptors in brain tissue and cultured neurons. *J Neurosci* 25: 2245-2254

3) De Blasi A, Conn PJ, Pin J, Nicoletti F (2001) Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol Sci* 22: 114-120

June 23, 2021

RE: Life Science Alliance Manuscript #LSA-2020-00962-TR

Dear Dr. Takahashi,

Thank you for submitting your revised manuscript entitled "Muscarinic receptor M3 contributes to intestinal stem cell maintenance via EphB/ephrin-B signaling". We would be happy to publish your paper in Life Science Alliance pending final revisions necessary to meet our formatting guidelines.

Along with points mentioned below, please tend to the following:

- please use the [10 author names, et al.] format in your references (i.e. limit the author names to the first 10)
- LSA allows supplementary figures, but no EV Figures; please update your callouts for the Supplementary Figures in the manuscript Fig EV1A=Fig S1A; while supplementary figures use the system supplementary Fig S1;
- please add your table legends to the main manuscript text after the main and supplementary figure legends
- please add callouts for Figures 4C and from S3-S10 A and B to your main manuscript text
- please add a Data Availability Statement, including the deposited NextGen sequencing data mentioned in the Materials and Methods.

If you are planning a press release on your work, please inform us immediately to allow informing our production team and scheduling a release date.

To upload the final version of your manuscript, please log in to your account:

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You will be guided to complete the submission of your revised manuscript and to fill in all necessary information. Please get in touch in case you do not know or remember your login name.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. FINAL FILES:

These items are required for acceptance.

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure, supplementary figure and video files uploaded as individual files: See our detailed guidelines for preparing your production-ready images, <https://www.life-science-alliance.org/authors>

-- Summary blurb (enter in submission system): A short text summarizing in a single sentence the study (max. 200 characters including spaces). This text is used in conjunction with the titles of papers, hence should be informative and complementary to the title. It should describe the context and significance of the findings for a general readership; it should be written in the present tense

and refer to the work in the third person. Author names should not be mentioned.

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****Submission of a paper that does not conform to Life Science Alliance guidelines will delay the acceptance of your manuscript.****

****It is Life Science Alliance policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.****

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****Reviews, decision letters, and point-by-point responses associated with peer-review at Life Science Alliance will be published online, alongside the manuscript. If you do want to opt out of having the reviewer reports and your point-by-point responses displayed, please let us know immediately.****

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Thank you for this interesting contribution, we look forward to publishing your paper in Life Science Alliance.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance
<http://www.lsjournal.org>

July 1, 2021

RE: Life Science Alliance Manuscript #LSA-2020-00962-TRR

Author information redacted

Dear Dr. Takahashi,

Thank you for submitting your Research Article entitled "Muscarinic receptor M3 contributes to intestinal stem cell maintenance via EphB/ephrin-B signaling". It is a pleasure to let you know that your manuscript is now accepted for publication in Life Science Alliance. Congratulations on this interesting work.

The final published version of your manuscript will be deposited by us to PubMed Central upon online publication.

Your manuscript will now progress through copyediting and proofing. It is journal policy that authors provide original data upon request.

Reviews, decision letters, and point-by-point responses associated with peer-review at Life Science Alliance will be published online, alongside the manuscript. If you do want to opt out of having the reviewer reports and your point-by-point responses displayed, please let us know immediately.

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Again, congratulations on a very nice paper. I hope you found the review process to be constructive and are pleased with how the manuscript was handled editorially. We look forward to future exciting submissions from your lab.

Sincerely,

Eric Sawey, PhD
Executive Editor
Life Science Alliance

