To Editor, World Journal of Translational Medicine

We appreciate your kindly giving us an opportunity to submit a revised version of our manuscript (NO.: 20825). Here we are sending the point-by-point responses to the reviewers' comments. Thanks to the valuable comments from reviewers, we believe that our manuscript has been greatly up-graded.

Comments given by Reviewer 693245

In this study, the authors have identified that p38 alpha MAPK is a crucial downstream effector of RGS5 in type I-type II VECs conversion. This finding provides a new strategy in the drug discovery for the treatment of ischemic disease. In general, this is a quite interesting and nice study. Experiments were well designed with appropriate controls and executed. Conclusions are significant and justified based on the high quality data. This manuscript certainly deserves to be published.
Classification: Grade A
Language evaluation: Grade A
Conclusion: Accept

Comments given by Reviewer 1299180

This is a manuscript that follows up a study that is being considered by the same journal on the topics of different types of vascular endothelial cells. The first study (not published yet) described the differential expression of RGS5 in two types of vascular endothelial cells. It was not quite clear whether the differential expression of RGS5 is indeed the cause of the distinctive phenotypes of the two types of vascular endothelial cells since the study has not been published. In the current study, it appears the authors set to explore what mediates the effect of RGS5. Accordingly, they compared kinase activity using an array available from Cell Signaling Technology between the two types of cells. As a result, they identified the activity of p38 is consistently different. Using p38 inhibitor as well as RGS5 overexpression, the authors suggested a model that RGS5 regulates type II to type I conversion via inhibiting p38 activity. The work could be of interest but the quality of some data could have been improved.

Classification: Grade C

Language evaluation: Grade B

Conclusion: Major revision

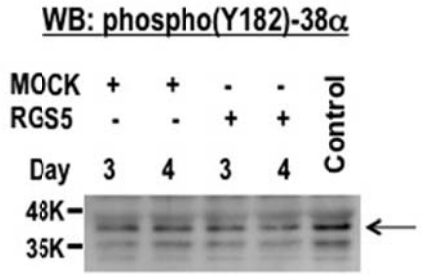
Major Comments:

Comment 1

Figure 3, it was unclear why the authors chose to using phosphor(T180)-p38alpha rather than the phosphor(T180/Y182)-p38alpha, since only the dually phosphorylated is in the active state.

Response 1

We performed Western blotting using anti-phosphor(Y182)-p38alpha (i.e. a rabbit polyclonal anti-p38 (phospho Y182) antibody; ab47363, Abcam plc.) and found that the introduction of an RGS5 expression vector did not significantly affect the level of tyrosine phosphorylation at Y182 (*see* below) although it suppressed threonine phosphorylation at T180 (figure 3A). Thus, we concluded that RGS5, which is an inhibitory molecule against S1P/S1P1-dependent signaling as we showed in the current study, specifically suppressed the threonine phosphorylation at T180. As the reviewer pointed, only the dually phosphorylated form of p38alpha is active. Therefore, it was concluded that RGS5 suppressed p38alpha activity by specifically lowering the level of T180 phosphorylation.



Comment 2

The authors put heavy emphasis on a potential positive feedback between RGS5 up-regulation and p38-alpha inhibition. However, the effect of p38 inhibition on RGS5 expression is rather modest as shown in Figure 3C.

Response 2

As the reviewer pointed, p38-inhibitor-mediated increments in RGS5 expression may not be so large (i.e. 1.65-fold increments). However, the results were statistically significant ($P < 0.000001$). Moreover, increments in RGS5 expression by subculture stress-dependent stress were not so large either (i.e. 1.73-fold increments *per* subculture) as calculated from the result of "3.0-fold increments *per* twice subcultures from P2 to P4" as shown in Fig. 4F in Reference #1 (Nishio et al., World J Transl Med). Nevertheless, repetitive subcultures inevitably induced "type-II to type-I conversion" together with increments in RGS5 expression (Reference #1). Thus, a modest but definite increment in RGS5 expression is of great significance because it brings about substantial outcomes when it is accumulated.

Collectively, it is highly reasonable to think that a modest increment in RGS5 expression by p38 inhibition creates a positive feedback loop between "RGS5 induction" and "p38 inhibition" to promote a one directional progression of degeneration-associated "type-II to type-I conversion".

Comment 3

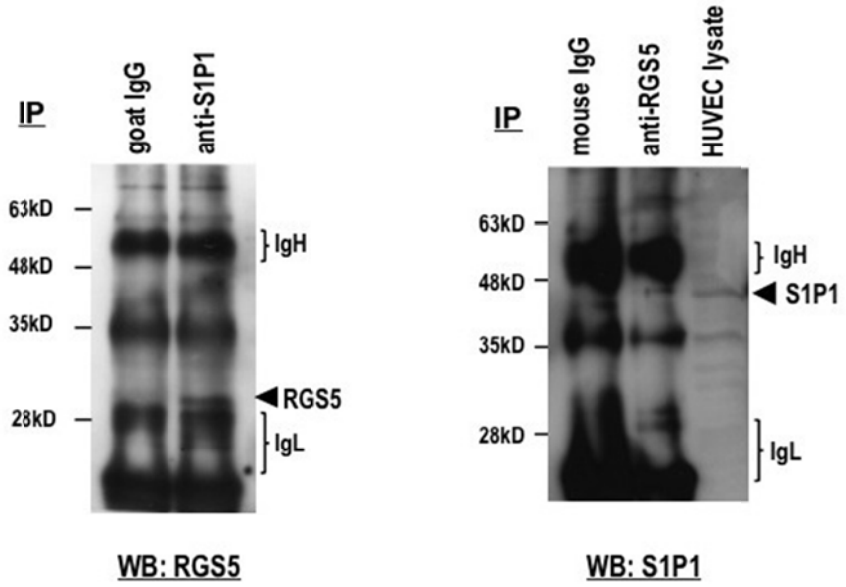
The quality of co-immunoprecipitation results as shown in Figure 4 is completely unacceptable. In most cases, it is virtually impossible to identify a specific band. Using TrueBlot IP/Western might help minimize the interference of heavy/light chain on the signal.

Response 3

Contrary to the judgment of the reviewer, we do not think that the quality of co-immunoprecipitation results as shown in Figure 4 was completely unacceptable. Indeed, we had performed the experiments multiple times and confirmed the reproducibility of the results.

Indeed, we re-performed the experiments using using TrueBlot(R):Anti-Rabbit IgG HRP and TrueBlot(R):Anti-Goat IgG HRP (Rockland Immunochemicals Inc., Pottstown, PA, USA) according to the reviewer's suggestion; however, we only obtained similar results (see below). Honestly, quality of data was not significantly improved or even down-graded (right).

In conclusion, our results sufficiently show that RGS5 was co-precipitated with S1P1 in type-I human VECs.



Comment 4

The results reported here are related to a finding reported in JBC by Jin Y et al. entitled "RGS5, a hypoxia-inducible apoptotic stimulator in endothelial cells". The authors should compare and contrast their results with these earlier related studies.

Response 4

According to the comment of the reviewer, we added descriptions regarding the comparison between the result by Jin Y et al and our finding in the 5th paragraph in Discussion.

Comment 5

On page 3, the last paragraph, "Interestingly, p38alpha MAPK inhibitor treatments suppressed the induction of RGS5 gene" should read "Interestingly, p38alpha MAPK inhibitor treatments enhanced the induction of RGS5 gene"

Response 5

Thanks to the comment of the reviewer, we were able to correct the erroneous description.

Comment 6

To firmly establish a role of p38 in this process, results from p38 activator in combination with RGS5 overexpression should be employed.

Response 6

Although the issue that the reviewer pointed is interesting, the requested experiment is considered as neither realistic nor practicable by the following reasons. Firstly, there are no specific activators for p38 MAPK. Although U-46619 (CAS 56985-40-1) has been being introduced as a p38 MAPK activator by Santa Cruz Biotechnology Inc., there is also a description that U-46619 is an activator of ERK 1 and ERK 2 (http://www.scbt.jp/datasheet-201242-u-46619.html). Secondly, an artificial p38 MAPK activation by stimulating cells with specific cytokines such as vascular endothelial growth factor (VEGF) is inappropriate either because VEGF activates diverse downstream events, and thus, cannot be considered as a p38 MAPK-specific activator at all. Thirdly, hyper-activation or inappropriate activation of p38 MAPK may bring about an unexpected or even unbeneficial outcome because a transient hyper-activation of p38 MAPK is reportedly observed under acute stressful conditions. As we described in Discussion, to preserve p38α MAPK activity at relatively higher levels by preventing the stress-induced reduction of its activity, but not to transport its activity at a hyperactive state, is important for human VECs to keep their healthy states.
Thus, “the result of from p38 activator in combination with RGS5 overexpression” is of little use in strengthening our finding. Instead, we believe that our RNAi-based RGS5 knockdown experiments along with the experiments using a p38 MAPK-specific inhibitor have sufficiently proven the involvement of the RGS5-p38 MAPK-signaling axis in phenotype determination of human VECs.

Comment 7

It would be more helpful to elaborate a bit on the proteomic kinase assay because not every reader is familiar with this method.

Response 7

According to the kind advice of the reviewer, we added description regarding the proteomic kinase assay at the end of the first paragraph in Results.