Author’s response to reviews

Title: Gremlin induces cell proliferation and extra cellular matrix accumulation in mouse mesangial cells exposed to high glucose via the ERK1/2 pathway

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Author's response to reviews: see over
Dear Editor,

We are very pleased to learn from your letter about revision for our manuscript entitled “Gremlin induces cell proliferation and accumulation of extra cellular matrix in mouse mesangial cells under high glucose via the ERK1/2 pathway”. Thank you for your attention and the reviewers for their helpful comments and advice. We have revised the manuscript according to the comments from the reviewers.

Response to Reviewer: Motoko Yanagita

Overall comment:

1) To study certain functions of gremlin, authors should test whether the functions are BMP independent.

Response: Thank you very much for your comments. A number of studies have reported that Gremlin can interact directly with cell surface proteins, such as Slit protein family members or cell-surface heparan-sulfate proteoglycans (HS-PGs), to alter cell function. This indicates a mechanism of action for Gremlin that is independent of BMP antagonism. Stabile et al. in Blood (2007) reported that Gremlin binds with high affinity to the surface of subcutaneous microvascular endothelial cells via uncharacterized cell-surface HS-PGs. Molar excess of BMP4 did not hinder Gremlin-proteoglycan binding, suggesting that different sites on the Gremlin protein, other than those binding BMPs, are involved. This Gremlin proteoglycan binding was shown to cause tyrosine phosphorylation of ERK1/2 in these systemic endothelial cells. Furthermore, Kim et al. in PLoS One (2012) reported that Gremlin induces cancer cell migration, proliferation and invasion through a BMP-independent pathway. HS-PG is the main component of the glomerular capillary basement membrane, mesangial cell and vascular wall, and plays an important role in the maintenance of their structural integrity. Our study confirms Gremlin induces ERK1/2 pathway activation in mesangial cells exposed to high glucose, which indicates that Gremlin might interact directly with mesangial cell surface HS-PG to alter cell function through a BMP-independent pathway.

2) Transfection efficiency was low in primary cultured mesangial cells, and authors are dealing with the mixed population: cells with plasmid and cells without plasmid. To analyze the function of gremlin, one should try the administration of recombinant Gremlin, because the protein is commercially available, or at least more efficient transfection method such as letrovirus.

Response: Thank you very much for your comment, suggesting that recombinant proteins and viral plasmids are more efficient transfection methods. The cells used in our study were a mesangial cell line. A number of researchers have successfully conducted mesangial cell transfection with liposome plasmid vectors, for example, Das et al. in Cell Signal (2011) and Zhang et al. in PLoS One (2010). Also, we have conducted previous studies transfecting mesangial cells with the megsin plasmid and megsin siRNA plasmid, reported by Wang et al. in Zhong Hua Shen Zang Bing Za Zhi (2011). Based on these previous findings, our study transfected mesangial cells with the Gremlin plasmid and Gremlin siRNA plasmid and the transfection efficiency was determined with western blotting and real-time PCR methods. Western blotting showed the Gremlin plasmid and Gremlin siRNA plasmid up-regulated and inhibited Gremlin protein expression, respectively. Real-time PCR showed the Gremlin plasmid and Gremlin siRNA plasmid up-regulated and inhibit Gremlin mRNA expression,
respectively. These results indicated the plasmids were effective and deemed suitable for the study.

Specific comments:

1) Figure 1: The induction of gremlin in HG condition is already reported by McMahon et al. in JBC (2000).
Response: Thank you very much for your comments. McMahon et al. in JBC (2000) previously reported high glucose-induced Gremlin expression. However, they only included two experimental groups: a normal group and a high glucose group (high glucose stimulation for 7 days), and failed to observe multiple time points of high glucose stimulation on Gremlin expression. Furthermore, they only observed Gremlin gene expression using northern blot analysis. In our study, we included five experimental groups: a normal group and high glucose stimulation groups, for 6h, 12h, 24h and 48h. Our results show that high glucose exposure induces Gremlin expression time dependently. Our study also observed, using western blotting and real-time PCR, that expression of Gremlin protein and mRNA were stimulated by high glucose exposure. These results indicate that high glucose up-regulates expression of Gremlin protein and mRNA.

2) Figure 2: The efficacy of Gremlin plasmid transfection was not high (A), and the induction of Gremlin expression was not prominent in western blotting (E). To determine the function of Gremlin, the administration of recombinant Gremlin protein is more reliable than the overexpression of plasmid.
Response: Thank you very much for your comment, suggesting that administration of recombinant Gremlin protein is more reliable than overexpression of plasmid. The transfection efficiencies of Gremlin plasmid and Gremlin siRNA plasmid in our study were 60 and 65%, respectively (A). The original images were not representative; therefore, we have replaced them with more representative images. Western blotting (E) analysis showed Gremlin plasmid up-regulated Gremlin protein expression. Again, the original image was not representative; therefore, we have replaced it with a more representative image.

3) Figure 3, 4: Intensity of PCNA staining is not a reliable marker for cell proliferation (A). Authors should try more quantitative methods to analyze cell proliferation (e.g. BrdU elisa assay). To my knowledge, the induction of TGF-b1 and CTGF by Gremlin is novel (B, C), whereas the induction of collagen accumulation by Gremlin is already reported (D).
Response: We agree with the reviewer’s opinion, and have replaced the PCNA staining method with BrdU ELISA for the analysis of cell proliferation. Thank you very much for your comment pointing out that the induction of collagen accumulation by Gremlin has already been reported. Zhang et al. in PLoS One (2010) reported that Gremlin siRNA plasmid inhibited high glucose-induced collagen accumulation. Conversely, our experiments show that Gremlin up-regulates high glucose-induced collagen accumulation, both positively and negatively, by promotion and inhibition of Gremlin expression, respectively.

4) Figure 5: Phosphorylation of ERK by Gremlin is already reported in endothelial cells by Stabile H et al. in Blood (2007). Enhancement of ERK phosphorylation by Gremlin overexpression was not significant in the gel in Figure 5B.
Response: Thank you very much for your comments. Stabile et al. in Blood (2007) reported that Gremlin up-regulated phosphorylated ERK (pERK) expression in endothelial cells. The aim of their study was to observe the effect of Gremlin on expression of pERK in
subcutaneous microvascular endothelial cells stimulated by recombinant murine Drm/Gremlin (rDrm) (50 ng/ml) for 0–60 min. The aim of our study was to observe the effect of high glucose stimulation (0–48 h) on expression of pERK in mouse glomerular mesangial cells (MMCs) and the effect of Gremlin on expression of pERK in MMCs under high glucose stimulation (24 h). Our results showed that high glucose up-regulated phosphorylation of ERK expression time dependently. Our results also showed that Gremlin plasmid up-regulated high glucose-induced phosphorylation of ERK in MMCs (Fig.5B). The original image was not representative; therefore, we have replaced it with a more representative image.

Response to Reviewer: Phillip Kantharidis

Major Compulsory Revisions

1. Many of the experiments look at the role of gremlin in the context of HG. Because the treatments with gremlin or si-Gremlin are concurrent with the addition of the HG stimulus, it is difficult to assess whether gremlin has an effect on its own, or whether it transiently enhances the effect of HG. These experiments should be conducted in normal glucose conditions and also in MCs adapted to HG for the specific effects of gremlin to be identified.

Response: We agree with the reviewer’s opinion that our experiments should be conducted under normal glucose conditions and also in mouse mesangial cells (MMCs) adapted to high glucose (HG), in order for the specific effects of Gremlin to be identified. We have revised this accordingly.

2. Because of the HG stimulus, the control experiments for HG exposure should be either mannitol or low-glucose as an osmotic control. It is well established that osmotic pressure can activate signalling through the MAP kinase pathways. In the absence of an osmotic control, the data is difficult to interpret.

Response: We agree with the reviewer’s opinion that the control experiments for HG exposure should be carried out with either mannitol or low-glucose as an osmotic control. We have revised this accordingly.

3. The transfection efficiency claimed for figure 2 appears to be exaggerated. At best it may be 20-30%, resulting in a 1.6 fold increase in gremlin expression. However the effect of the transfection of gremlin or the siRNA into MCs appears to be in every cell in Figures 3 and 4, as evidenced by the PCNA staining. Why the difference? Maybe a gremlin immuno could be performed on transfected cells to confirm the presence of gremlin in most cells.

Response: Thank you very much for your comments. The transfection efficiencies of Gremlin plasmid and Gremlin siRNA plasmid in our study were 60 and 65%, respectively (Fig.2A). The original images were not representative; therefore, we have replaced them with more representative images. In addition, we have replaced the PCNA staining method with a quantitative BrdU ELISA method to measure cell proliferation.

Minor revisions

At the end of the first paragraph of the results section the claim is made that gremlin is involved with the HG-induced effects in MCs. This claim is premature so early in the manuscript when the only thing the investigators have shown till now is that gremlin gets upregulated by HG.
Response: We agree with the reviewer’s opinion that this claim is premature in the manuscript, when the only key finding the investigators show to this point is up-regulation of Gremlin by HG exposure. We have modified the text accordingly.

We acknowledge the reviewer’s comments and suggestions very much, which are valuable in improving the quality of our manuscript.

Sincerely,
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