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 corrected the language and revised the manuscript according to the comments from the reviewers. Revisions in the text are shown using red colour for the changes.

Response to Reviewer: Phillip Kantharidis

The authors have not adequately addressed my concerns about the osmotic control in figures 1a and 1b, as well as in figure 5a.

The point of the paper is to demonstrate that HG induces gremlin which then induces TGFβ and CTGF to mediate downstream effects commonly seen in nephropathy. The absence of an osmotic control in these figures is a serious omission in the manuscript and the controls should be included.

Response: We agree with the reviewer’s opinion. Figures 1a and 1b, as well as figure 5a and their legends have been added the osmotic control in the manuscript. Please see line 11 in page 17, and line 34 in page 18. We have also added results of the osmotic control in Figures 1a and 1b, as well as figure 5a. Please see line 17 in page 7, line 26 in page 7, line 22 in page 9, and line 27 in page 9.

Response to Section Editor (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. We have added information to the Discussion to highlight BMP independent functions of Gremlin. Please see line 7 in page 12. “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, to alter cell function, indicating a mechanism of action for Gremlin that is independent of BMP antagonism. Chen et al [33] identified Slit1 and Slit2 as Gremlin-interacting proteins. BMP2 or BMP4 did not interfere with the Gremlin-Slit interaction, suggesting that the region of Gremlin protein responsible for BMP-binding is distinct from that interacting with Slit proteins. In a second study, Stabile et al [32] showed that Gremlin binds with high affinity to the surface of subcutaneous microvascular endothelial cells via uncharacterized cell-surface heparan-sulfate proteoglycans (HS-PGs). Molar excess of BMP4 did not hinder Gremlin-proteoglycan binding, again suggesting that different sites on the Gremlin protein, other than those binding BMPs, are involved. Furthermore, Gremlin proteoglycan binding was shown to cause tyrosine phosphorylation of ERK1/2 in these systemic endothelial cells. HS-PG is the main component of the glomerular capillary basement membrane, mesangial cell and vascular wall, and plays an important role in maintenance of their structural integrity. Our study shows that Gremlin induces ERK1/2 pathway activation in mesangial cells cultured under high glucose exposure, which indicates that Gremlin might interact directly with mesangial cell surface HS-PGs to alter cell function through BMP-independent pathways.”

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 F, et al. in Cell Signal (2011,
23(8):1311-1319) and Zhang Q, et al. in PLoS One (2010, 5(7):e11709). Also, we have
conducted previous studies transfecting mesangial cells with the megsin plasmid and megsin
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 R, et al. in J Biol Chem
(2000, 275(14):9901-9904) 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 nine experimental groups: a
normal group, normal plus 24.5 mM mannitol groups 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 (Figure 2A and 2B). The original images were not representative;
therefore, we have replaced them with more representative images. Western blotting (Figure
2E) 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 in Figures 3A and 4A. Figures 3A and 4A and their legends have been revised in the manuscript. Please see line 5 in page 18, and line 21 in page 18. We have also have added results of BrdU ELISA. Please see line 10 in page 8, and line 36 in page 8. Thank you very much for your comment pointing out that the induction of collagen accumulation by Gremlin has already been reported (Figure 3D and 4D). Zhang Q, et al. in PLoS One (2010, 5(7):e11709) 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 H, et al. in Blood (2007, 109(5):1834-1840) 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 (Fig.5). We have added information to the Discussion to highlight Phosphorylation of ERK by Gremlin. Please see line 39 in page 11. “Stabile et al [32] reported that Gremlin up-regulated pERK1/2 expression in subcutaneous microvascular endothelial cells (SIE) stimulated by recombinant murine Drm/ Gremlin (rDrm) (50 ng/ml) for 0–60 min.” 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.

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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