

Peer Review File

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RESPONSES TO COMMENTS OF REVIEWERS

Reviewer A

Authors compared miR expression in bladder of normal and SCI group isolated at different time points.

1) The authors should justify the use of t test instead of ANOVA for multigroup comparison. How do they plan to address the concern of multiple hypothesis testing in the claim of significance?

Reply: Thank you very much for your comments. It was our mistake that we had written the wrong statistical tests. Indeed, differential expression analysis of miRs obtained from NGS was performed by the DESeq2 (v. 1.16.1) which was an algorithm to examine differences between groups by using a generalized linear model and assuming a negative binomial distribution of RNA-Seq reads. Statistically differences in the levels of miRs verified by qRT-PCR between groups were determined by ANOVA using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). We re-did these statistical analyses and verified our results.

Changes in the text: we have modified our text as advised (see Page 6, line 16-20).

2) Authors should reconcile the reported time dependent changes in voiding function after SCI in published studies with the miR differences in SCI-1 and SCI-3 groups.

Reply: Thank you very much for your comments. As respectable reviewer requested, we reconciled the reported time dependent changes in voiding function after SCI with the miR differences in SCI-1 and SCI-3 groups. "Suprasacral SCI can abruptly disrupt intraspinal pathways and result in the "spinal shock" phase, during which the bladder is often atonic and areflexic and typically present with overflow incontinence (1). However, the relative concentration of collagen in rat bladders was reported to be significantly decreased in the first 10 days after SCI (2), which may be in agreement with the expression of miR-134-5p to a certain extent. Therefore, it is reasonable to presume that miR-134-5p might play a role in it. After spinal shock,

hypermechanosensitive C-fiber bladder wall afferents were activated gradually and urodynamic findings were mainly characterized by detrusor overactivity or detrusor-sphincter dyssynergia. miR-146a-5p may be involved in this stage of neurogenic bladder due to that it was significantly upregulated in the SCI-3 group compared to the SCI-1 group. In summary, it will be very interesting and meaningful to investigate the relationship between the dynamic change of these differentially expressed miRs and the different stages of neurogenic bladder.” These discussions were added in the manuscript.

Changes in the text: we have modified our text as advised (see Page 11, line 22 and Page 12, line 1-13).

3) what about the expression of miR-1949 reported in bladder of SCI rat Mol Med Rep. 2015 Aug;12(2):2849-57. The authors need to discuss why they could not measure it.
Reply: Thank you very much for your comments. Wang et al. reported that the expression of miR-1949 was significantly increased after the third month following SCI (3). However, there was no significant difference in the level of bladder miR-1949 between rats without SCI and those collected at 3 months following SCI. Indeed, our finding is consistent with the results of above research. We found that the expression of miR-1949 was not significantly increased in the first month following SCI compared with that in control group. Because only one month after SCI was investigated in our study. Therefore, this limitation was mentioned in the discussion and the finding reported by Wang et al. was also cited in this study.

Changes in the text: we have modified our text as advised (see Page 12, line 21 and Page 13, line 1-2).

4) The rationale for selecting female rats was not provided.

Reply: Thank you very much for your comments. As respectable reviewer requested, the rationale for selecting female rats was added in the manuscript. Female rats' urethras are shorter and more conducive to bladder evacuation via abdominal compression. Therefore, they were selected for this study.

Changes in the text: we have modified our text as advised (see Page 4, line 7-8).

Reviewer B

The goal of this study is to explore the expression of miRNAs in the neurogenic bladder of rats with SCI. Some recent studies have suggested associations between the level of specific miRNAs in bladder tissue and overactive bladder in patients. However, the expression of miRNAs in tissue from neurogenic bladder tissue has not been explored. Here, the authors use a rat model of spinal cord transection to evoke a neurogenic bladder phenotype. RNA isolated from bladder tissue harvested from injured and non-injured controls at 1, 2 and 4 weeks after injury were subjected to RNA sequencing. A subset of differentially expressed miRNAs was validated in the same tissues by qPCR. From this analysis, the authors have identified many differentially expressed miRNAs, both up- and down-regulated in bladder tissue from rats with SCI, among which miR-21-5p was the most significantly upregulated. Differential expression of miRNAs also appeared to evolve over time after injury. Gene ontology analysis identified several signaling pathways that would be impacted by differentially expressed miRNAs. Specific comments are noted below.

1) What was the impact of differential expression of specific miRNAs on target mRNAs or their encoded proteins? In other word are the changes in miRNA expression likely to be functionally meaningful by altering the expression of target genes?

Reply: Thank you very much for your comments. Just as respectable reviewer suggested, we will further study the changes of mRNAs targeted by these differentially expressed miRNAs and the impact of these differentially expressed miRNAs on target mRNAs or their encoded proteins. For example, miR-21-5p has been found to be the most significantly upregulated miR in this study. Furthermore, our further study tentatively finds that the levels of Smad7 mRNA and protein are significantly increased in bladders of SCI rats and inhibiting miR-21-5p could downregulate them. Importantly, inhibiting miR-21-5p is found to be able to significantly lessen bladder fibrosis. Due to that these results were not obtained before submitting this manuscript, they were not mentioned in this study and discussed as a limitation. “the interactions between miRs and mRNA were not explored. Thus, further experimental studies are needed to verify the proposed interactions and their roles in neurogenic bladder in the future.”

Changes in the text: we have modified our text as advised (see Page 12, line 18-19).

2) The miRNAs validated by qPCR show distinct patterns of expression over time. What are the implications of this?

Reply: Thank you very much for your comments. As respectable reviewer mentioned, the miRNAs validated by qRT-PCR show distinct patterns of expression over time. "Suprasacral SCI can abruptly disrupt intraspinal pathways and result in the "spinal shock" phase, during which the bladder is often atonic and areflexic and typically present with overflow incontinence (1). However, the relative concentration of collagen in rat bladders was reported to be significantly decreased in the first 10 days after SCI (2), which may be in agreement with the expression of miR-134-5p to a certain extent. Therefore, it is reasonable to presume that miR-134-5p might play a role in it. After spinal shock, hypermechanosensitive C-fiber bladder wall afferents were activated gradually and urodynamic findings were mainly characterized by detrusor overactivity or detrusor-sphincter dyssynergia. miR-146a-5p may be involved in this stage of neurogenic bladder due to that it was significantly upregulated in the SCI-3 group compared to the SCI-1 group. In summary, it will be very interesting and meaningful to investigate the relationship between the dynamic change of these differentially expressed miRs and the different stages of neurogenic bladder." These discussions were added in the manuscript.

Changes in the text: we have modified our text as advised (see Page 11, line 22 and Page 12, line 1-13).

3) How does differential miRNA expression compare with bladder function or other endpoints such as tissue remodeling in spinal cord injured rats?

Reply: Thank you very much for your comments. As respectable reviewer mentioned, bladder dysfunction after SCI involves bladder remodeling which was mainly shown as bladder fibrosis. Bladder fibrosis was characterized by the deposition of extracellular matrix and the increase of interstitial cells including fibroblasts and myofibroblasts. Indeed, our further study tentatively finds that deposition of collagens increases and bladder fibrosis gets worse progressively after SCI. Specifically, Author's Response figure 1 shows that collagens are blue with microscopy by Masson staining and their deposition is more and more over time. Furthermore, as mentioned in the discussion,

the levels of these differentially expressed miRs changed dynamically, which may indicate that they played predominant and dynamic role in different stages of bladder remodeling after SCI. For example, miR-21-5p maintained a high level of expression after SCI and may keep functioning in the progression of bladder fibrosis. Therefore, we will further investigate the relationship between the dynamic change of these differentially expressed miRs and the different stages of bladder remodeling.

Changes in the text: we have modified our text as advised (see Page 11, line 22 and Page 12, line 1-13).