Schwann cell reprogramming into repair cells increases miRNA-21 expression in exosomes promoting axonal growth

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DOI: 10.1242/jcs.239004

Editor: Giampietro Schiavo

Review timeline
Original submission: 6 September 2019
Editorial decision: 20 November 2019
First revision received: 24 March 2020
Accepted: 24 April 2020

Original submission

First decision letter

MS ID#: JOCES/2019/239004

MS TITLE: Schwann cell reprogramming into repair cells increases exosome-loaded miRNA-21 promoting axonal growth

AUTHORS: Rodrigo Lopez-Leal, Florencia Diaz-Viraque, Romina Catalan, Cristian Saquel, Anton Enright, Gregorio Iraola, and Felipe A Court
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript before further considering it for publication in JCS. Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The main findings of this manuscript are that rSC cells, but not dSC cells can promote neurite growth in DRG neurons. This is dependent on the expression of c-Jun and Sox2, which has been previously demonstrated. On the other hand, the authors show that exosomes secreted by these

cells are enriched in miRNA-21, whose inhibition impairs neurite growth in the presence of these exosomes. Finally, they find a relation with downregulation of PTEN in receiving neurons. The manuscript contributes to our understanding of how exosomes can promote neurite regeneration.

Comments for the author

This is a nice study demonstrating the function of exosome-derived miRNA-21 on neurite growth. The authors demonstrate that only exosomes derived from rSC cells generate this effect. They characterized c-Jun, Sox-2 and MBP expression to confirm that Fsk2 treatment can generate rSC cells. Next, they characterized that exosomes derived from Fsk2-treated cells promote neurite growth, but not those derived from cells that maintain MBP expression (dSCs). In general, it is a very good study, but the authors need to clarify some points.

1. In figure 3, they demonstrate that interference RNAs for c-Jun or Sox2 highly decrease the expression of their respective targets. However, it is not clear why in Figure 3B there is not a statistically significant difference in c-Jun expression between sh-c-Jun and sh-Sox2 conditions. Does sh-Sox2 decrease c-Jun expression? Similarly, while sh-Sox2 decreases Sox2 expression, it seems that sh-c-Jun increases Sox2 expression, while apparently no significant difference is found. Figure 3B shows the quantification of 3 experiments, some of them with a high deviation of data. It would be helpful to introduce one or two more experiments for Figure 3B to clarify whether suppression of c-Jun or Sox2 modifies the expression of the other transcription factor.

2. In figure 5, the authors show different miRNAs and the proportion of their expression, but later they focus on miRNA-21, that has been previously described as a promoting factor of neurite growth. It would be helpful, to give a higher value to miRNA-21, that authors show a qRT-PCR of other miRNAs enriched in exosomes, and the absence of their function through their inhibition.

3. Finally, regarding Figure 6, the authors demonstrate a decrease in PTEN mRNA but not its involvement in neurite growth in their experiments. It would be helpful to show a PTEN western blot in different conditions to show that PTEN protein expression is decreased, or that on inhibiting PI3-kinase, the miRNA-21 effect is blocked.

Reviewer 2

Advance summary and potential significance to field

This work adds to the knowledge about the potential mechanisms how Schwann cells can act as endogenous regenerators of the peripheral nervous system. The role of transcription factors such as c-jun has been well documented of importance for the transition of Schwann cells to the so-called repair phenotype and the authors have furthered this to explore their role in defining the exosomal part of the cell secretome. Specifically the role of exosomal miRNA in mediating the pro-regenerative effects of Schwann cells is new in this work.

Comments for the author

This is an easy to read manuscript (though I suggest that the authors do re-check for grammar consistency) and I have mainly comments related to methodology. The ability of exosomes and their miRNA as a mechanism for inducing neurite outgrowth is clear, however the relative importance of exosomes versus paracrine factors secreted from the Schwann cells is not. The authors need to directly compare the potency of conditioned medium from the repair Schwann cells with the effects of exosomes isolated from the conditioned medium i.e. how do exosomes isolated from a given volume compare with the potency of said volume of medium? It might be that the paracrine factors in the secretome completely override the effects of the exosomes. So how important is the exosome fraction of the secretome? This is a critical question for the wider field of exosome research. Linked to this, it is important that more details about preparation of the exosomes are included. What volume of medium was collected and how many exosomes (or protein levels as an indirect measure) were obtained per ml? Which passage of cells was used? Was medium pooled? How did the authors decide to use 120ng/ml? How variable was the exosome isolation procedure - was yield dependent on confluency/proliferation rates? Personal experience from our

laboratory suggests that protein content is not a reliable method for standardising experiments – do you have data for the number of particles used?
Minor comments:
Figure 1 – it would be better to set a fluorescence threshold limit and count and give data for the number of positive cells in each condition.
Figure 5 – are the RNA-seq data from one preparation or pooled preparations?
Figure 6D – why are there 6 replica data points on this graph – this doesn't appear to be consistent with the legend?
Discussion – it would be good to comment on the potential role of factors other than miRNAs in the exosomes – it is hard to believe that just one single miRNA is almost completely responsible for the effects on neurite outgrowth (Fig 6D).

First revision

Author response to reviewers' comments

Dear Dr Schiavo,

We thank the reviewers for their supportive comments and suggestions on our paper "Schwann cell reprogramming into repair cells increases exosome-loaded miRNA-21 promoting axonal growth".

We greatly appreciate the reviewer's comments on our work, and their positive comments related to our data. Including:

"This is a nice study demonstrating the function of exosome-derived miRNA-21 on neurite growth... In general, it is a very good study, but the authors need to clarify some points"
"The manuscript contributes to our understanding of how exosomes can promote neurite regeneration" (Reviewer 1)

"This work adds to the knowledge about the potential mechanisms how Schwann cells can act as endogenous regenerators of the peripheral nervous system" (Reviewer 2).

In order to approach the issues raised by the reviewers, we have performed a new set of experiments and data analysis, including:

- New experiments to increase the replicates for the experiments of knock-down of c-Jun and Sox2.

- We have inhibited two other miRNAs contained in exosomes, to demonstrate the specificity of miRNA-21 in the pro-growth effect of SC-derived exosomes.

- We have performed a new set of experiments using a PI3-kinase inhibitor to establish the involvement of this pathway in the exosome-activated neuronal growth.

- We have assesed the relative effect of SC-exosomes versus soluble factors in neuronal growth experiments.

- We have included data as a table for the quantification of exosome release by different techniques.

We hope that you will agree that addressing the critiques has strengthened this work, and we thank the comments provided as we feel the improvements we have incorporated into this revised manuscript clarifies the goal of this study.

In the paragraphs below, we provide specific responses in blue to each of the reviewers' comments.

With kind regards,
Felipe Court (BSc., MSc., Ph.D.)

Answers to reviewer's comments (in blue)

Reviewer 1 Advance Summary and Potential Significance to Field:

The main findings of this manuscript are that rSC cells, but not dSC cells, can promote neurite growth in DRG neurons. This is dependent on the expression of c-Jun and Sox2, which has been previously demonstrated. On the other hand, the authors show that exosomes secreted by these cells are enriched in miRNA-21, whose inhibition impairs neurite growth in the presence of these exosomes. Finally, they find a relation with downregulation of PTEN in receiving neurons. The manuscript contributes to our understanding of how exosomes can promote neurite regeneration.

Reviewer 1 Comments for the Author:

This is a nice study demonstrating the function of exosome-derived miRNA-21 on neurite growth. The authors demonstrate that only exosomes derived from rSC cells generate this effect. They characterized c-Jun, Sox-2 and MBP expression to confirm that Fsk2 treatment can generate rSC cells. Next, they characterized that exosomes derived from Fsk2-treated cells promote neurite growth, but not those derived from cells that maintain MBP expression (dSCs). In general, it is a very good study, but the authors need to clarify some points.

We thank the general and positive reviewers comments, we have approached experimentally all the issues raised by the reviewer, which have indeed strengthened the message of our paper, and defined critical issues not included in the original version.

1. In figure 3, they demonstrate that interference RNAs for c-Jun or Sox2 highly decrease the expression of their respective targets. However, it is not clear why in Figure 3B there is not a statistically significant difference in c-Jun expression between sh-c-Jun and sh-Sox2 conditions. Does sh-Sox2 decrease c-Jun expression? Similarly, while sh-Sox2 decreases Sox2 expression, it seems that sh-c-Jun increases Sox2 expression, while apparently not significant difference is found. Figure 3B shows the quantification of 3 experiments, some of them with a high deviation of data. It would be helpful to introduce one or two more experiments for Figure 3B to clarify whether suppression of c-Jun or Sox2 modifies the expression of the other transcription factor.

We agree with the reviewer, there was a problem with the labelling, and in addition the data was highly variable. We have now performed 3 more experiments for the shRNA set and re-quantified all the experiments again. From the new quantification is now clear by statistical analysis, but also by looking the individual experiments, that knock down of one transcription factor does not modify the expression of the other transcription factor. We have added this data to the revised manuscript and revised Figure 3.

2. In figure 5, the authors show different miRNAs and the proportion of their expression, but later they focus on miRNA-21, that has been previously described as a promoting factor of neurites growth. It would be helpful, to give a higher valueto miRNA-21, that authors show a qRT-PCR of other miRNAs enriched in exosomes, and the absence of their function through their inhibition.

We thank the reviewer for this suggestion. We have now selected two other miRNAs highly expressed in rSC-derived exosomes (miR-27b and miR10b, see Figure 5) and produced inhibitors for these miRNAs to then carry out experiments as described for miRNA-21. As shown in the revised manuscript (line 192-195) and revised Figure 6, inhibition of any of these two additional miRNAs does not have any effect on the pro-growth effect of rSC-derived exosomes, giving more strength for the role of miRNA-21 in the pro-growth effect of SC exosomes.

3. Finally, regarding Figure 6, authors demonstrate a decrease in PTEN mRNA, but not its involvement in neurite growth in their experiments. It would be helpful to show a PTEN western blot in different conditions to show that PTEN protein expression is decreased, or that on inhibiting PI3-kinase, the miRNA-21 effect is blocked.

We have followed the reviewer's suggestion and decided to test if the pro-growth effect of rSC exosomes is dependent on PI3-kinase. To this end we have used wortmannin, a PI3-kinase inhibitor. Indeed our data included in the revised manuscript (line 199-205) and revised Figure 6 demonstrate that this is the case, with wortmannin decreasing the pro-growth effect of rSC exosomes.

Interestingly, the growth of sensory neurons without exosome incubation is not affected by wortmannin, suggesting that rSC-exosomes activate a pathway to enhance neurite growth that is different to that used in basal, non-treated, conditions. We have added this into the discussion section of the revised manuscript (line 263-274).

Reviewer 2 Advance Summary and Potential Significance to Field:

This work adds to the knowledge about the potential mechanisms how Schwann cells can act as endogenous regenerators of the peripheral nervous system. The role of transcription factors such as c-jun has been well documented of importance for the transition of Schwann cells to the so-called repair phenotype and the authors have furthered this to explore their role in defining the exosomal part of the cell secretome. Specifically the role of exosomal miRNA in mediating the pro-regenerative effects of Schwann cells is new in this work.

Reviewer 2 Comments for the Author:

This is an easy to read manuscript (though I suggest that the authors do re-check for grammar consistency) and I have mainly comments related to methodology. The ability of exosomes and their miRNA as a mechanism for inducing neurite outgrowth is clear, however the relative importance of exosomes versus paracrine factors secreted from the Schwann cells is not. The authors need to directly compare the potency of conditioned medium from the repair Schwann cells with the effects of exosomes isolated from the conditioned medium i.e. how do exosomes isolated from a given volume compare with the potency of same volume of medium? It might be that the paracrine factors in the secretome completely override the effects of the exosomes. So how important is the exosome fraction of the secretome? This is a critical question for the wider field of exosome research.

We thank the positive reviewer comments. We have checked more carefully for grammar consistency.

Regarding the relative comparison between exosomes and SC paracrine factors, we have performed the suggested experiment to directly compare the pro-growth effect of rSC-derived exosomes and SC-conditioned medium depleted of exosomes. The results turn out to be quite interesting as, in the *in vitro* conditions used, the enhancement of axonal regeneration is mostly dependent on SC-exosomes, with no contribution from SC soluble factors. Is important to consider that these neurons without exosomes are growing in a medium that contains neurobasal medium and supplemented NGF, a neurotrophin that activates survival and regeneration of sensory neurons. We have previously performed dose response curves, and indeed neurite growth is directly dependent on NGF concentration (unpublished) as has been described (Kaselis et al., 2014). We have added this data in the revised manuscript (line 120-123) and as a supplementary Figure 2 A and B. Importantly, in a new experiment performed during this review process, we demonstrate that inhibition of PI3-kinase using wortmannin inhibits the pro-growth effect of SC-exosomes but has no effect on neurite growth in culture medium without exosomes. Therefore, our interpretation is that the effects of paracrine factors secreted by glial cells are emulated by factors contained in the neurobasal medium plus NGF and that the effect of exosomes is to enhance neurite growth by activating another pro-regenerative signaling pathway, which is PTEN and PI3 kinase-dependent. We have added this data in line 199-205 of the revised manuscript and as Figure 6F,G.

Linked to this, it is important that more details about preparation of the exosomes are included. What volume of medium was collected and how many exosomes (or protein levels as an indirect measure) were obtained per ml? Which passage of cells was used? Was medium pooled? How did the authors decide to use 120ng/ml? How variable was the exosome isolation procedure -was yield dependent on confluency/proliferation rates? Personal experience from our laboratory suggests that protein content is not a reliable method for standardising experiments - do you have data for the number of particles used?

We have added all the requested information in the revised manuscript, most of it in a new table (Supplementary Table 1) and other relevant data in the method sections, including volume of medium collected and amount of exosomes obtained in protein levels and in number of exosomes, passage of the cells and pooled medium (line 299 and line 325).

We have previously performed a dose-response curve in our DRG growth paradigm and selected this concentration as the lower concentration that enhances regeneration at a significant level. We have also analyzed some numbers associated with 120ng/ml exosomes. For this we have calculated the number of particles isolated by our method (by nanosight) per ng of proteins. Although it is quite difficult to establish if we are working in a physiological range, due to the lack of detailed analysis of release and internalization rates in vivo, the numbers can help us have an idea of the feasibility for a population of cells to receive 12 ng of exosomes.

Estimations from cell populations (not published):

(1) 120ng = 1.16E+8 particles, approx. 50K neurons per DRG= **96 exosomes per neuron/hour.**
(2) Rate of exosome release by a single SC (measured in 1 hour period) is **3332 exosomes/hr.**

The isolation procedure was not particularly variable in terms of yield. Cells were maintained in comparable confluency between experiments, as proliferation rate seems to modify exosome yield (we have not performed a detailed analysis of this). In our experience, protein content is highly consistent between experiments when compared to extracellular vesicle numbers from the same preparation quantified using the nanosight. We have added this information in the supplementary table for all the experiments in SC and HEK cells (Supplementary Table 1).

Minor comments:

Figure 1 - it would be better to set a fluorescence threshold limit and count and give data for the number of positive cells in each condition.

We have performed the suggested analysis, and now the data in Figure 1 is expressed as % of the positive cells per condition.

Figure 5 - are the RNA-seq data from one preparation or pooled preparations?

The RNA-seq data is from pooled preparations, we have now added this information in the method section (line 416-418).

Figure 6D - why are there 6 replica data points on this graph - this doesn't appear to be consistent with the legend?

This was an error, we have corrected this in the corresponding legend.

Discussion - it would be good to comment on the potential role of factors other than miRNAs in the exosomes - it is hard to believe that just one single miRNA is almost completely responsible for the effects on neurite outgrowth (Fig 6D).

We have included a paragraph commenting on the potential role of other factors in the pro-growth effect of Schwann cells in the revised manuscript (line 263-274).

Second decision letter

MS ID#: JOCES/2019/239004

MS TITLE: Schwann cell reprogramming into repair cells increases exosome-loaded miRNA-21 promoting axonal growth

AUTHORS: Rodrigo Lopez-Leal, Florencia Diaz-Viraque, Romina Catalan, Cristian Saquel, Anton Enright, Gregorio Iraola, and Felipe A Court
ARTICLE TYPE: Research Article

I am very happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Comments for the author

The manuscript has been revised accordingly to reviewer comments, and the results and conclusions are consistent and reinforce the manuscript. All main aspects underlined by this reviewer have been solved and figures and text modified in this sense.

Reviewer 2

Advance summary and potential significance to field

This work adds to the knowledge about the potential mechanisms how Schwann cells can act as endogenous regenerators of the peripheral nervous system. The role of transcription factors such as c-jun has been well documented of importance for the transition of Schwann cells to the so-called repair phenotype, and the authors have furthered this to explore their role in defining the exosomal part of the cell secretome. Specifically the role of exosomal miRNA-21 in mediating the pro-regenerative effects of Schwann cells is new in this work.

Comments for the author

The authors have addressed the revisions requested and the manuscript is now suitable for publication.