The lncRNA *MEG3* mediates renal cell cancer progression by regulating *ST3Gal1* transcription and EGFR sialylation

Aihong Gong, Xinyu Zhao, Yue Pan, Yu Qi, Shuangda Li, Yiran Huang, Yanru Guo, Xia Qi, Wei Zheng and Li Jia
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Original submission

First decision letter

MS ID#: JOCES/2020/244020

MS TITLE: LncRNA MEG3 mediates renal cell cancer progression by regulating ST3Gal1 transcription and EGFR sialylation via PI3K/AKT pathway

AUTHORS: Aihong Gong, Xinyu Zhao, Yue Pan, Yu Qi, Shuangda Li, Yiran Huang, Yanru Guo, Xia Qi, Wei Zheng, and Li Jia
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 raise a number of substantial criticisms that prevent me from accepting the paper at this stage. As you will see they raise serious concerns regarding the data in particular the lack of quantitation (migration assays, immunoblots, etc...) throughout the figures and the over-reliance on one cell line (786-0). It would be of particular importance to address these concerns. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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 research found that MEG3 was a tumor suppressor to regulate ST3Gal1 by interacting with c-Jun. In addition, the investigation connected EGFR sialylation with EGFR phosphorylation. The result of this research is precise, but to some extent, the results were only gained through 786-O cells in vitro.

Comments for the author

There are still some concerns listed below,
1.In Fig. 2C and Fig. 2D, the difference between negative control and MEG3/siMEG3 is not very clear.
2.The expressional relationship of ST3Gal1 and MEG3 should be statisticed.
3.The annotation and figure sequence are wrong in Fig. 3 4.In the whole experiment, 786-O cell line is the only discussed cell line while the results of FISH in Fig. 4E suddenly included ACHN
5.According to ChIP and RIP, c-Jun directly bound to ST3Gal1 promoter and MEG3 but there is no further discussion of the complex

Reviewer 2

Advance summary and potential significance to field

Int the manuscript, "LncRNA MEG3 mediates renal cell cancer progression by regulating ST3Gal1 transcription and EGFR sialylation via PI3K/AKT pathway" Gong et al., associate MEG3 levels with RCC and try to demonstrate that its overexpression represses cancer cell growth and knocking it down increases growth and migration. Results were confirmed in vivo with tail vein metastasis assays and flank tumor injections using cells expressing MEG3 or knocking down MEG3. ST3Gal1 was found to corelate with MEG3 expression in RCC and normal tissue by immunostaining. Expression of MEG3 and ST3Gal1 positively correlated with MEG3 expression in siRNA and knockdown experiments in cells. Authors provide evidence that cJun represses ST3Gal1 transcription by directly binding its promoter; were siRNA to c-Jun increases ST3Gal1 levels. They also show that MEG3 interacts with cJun and suggest by MEG3 knockdown that MEG3 modulates cJun levels downregulating it. Authors show ST3Gal1 effects EGFR phosphorylation.

Comments for the author

Although interesting much of the data is obtained with one cell line and is not quantitated in many cases. In other examples some of the conclusions appear unfounded, for instance, the evidence that the PI3K/AKT pathway is downstream of MEG3 regulation of EGFR sialyation is sparse.

The manuscript needs to be edited and rewritten in some places, including the abstract. There are many places where experiments are unclear and need to explained better. Overall this manuscript needs multiple corroborating experiments to be ready for publication.

Specific comments:

1. Authors state, "As shown in Fig. 1C, 293T exhibited high level of MEG3, while RCC cell lines presented lower MEG3 level." This is just relatively high. These are normalized values with no information of read counts across multiple tissues and cells.

2. Authors state, "These results indicated that low level of MEG3 might affect the progression of RCC." This suggests that low levels are associated with RCC, there is no information about how it might regulate progression at this point. Did the authors validate the in situ with positive and negative controls? Not clear here and there is nothing mentioned in the methods.

3. Migration and invasion effects of MEG3 are not quantitated and in the example shown the scratch test assay results are unconvincing in terms of MEG3 alteration of migratory properties.

4. In figure 5 authors state that they want to confirm previous results with ST3Gal1, showing it blocks EGF binding and EGFR dimerization. They did not measure this, they only measured EGFR phosphorylation level +/- ST3Gal1; there are multiple mechanisms by which pEGFR levels could be modulated. None of the results in Fig5 are quantitated.

5. Results for figure 5 mentions MAL affinity without spelling out that this is a lectin binding assay. This needs to be clearer in the results section. Results in Fig 5C and D appear pretty minimal, maybe in the case of 5D non-existent.

6. Overexpression and siRNA experiments were primarily done with one cell line 786-0 cells and should be repeated in at least two cell lines.

Minor

Authors state, “whereas knockdown of MEG3 obviously promoted the cell proliferation in vitro”, don’t use obviously here.

Authors state in the introduction, “…and stimulates the activation of PIP3” do they mean PI3K?

In figure legend 4K, “RIP assay was taken to evaluate specific” spell out RIP assay.

Please rewrite, “As shown in Fig. 4J,K, altered MEG3 regulated the expression of c-Jun and interaction with c-Jun.”

First revision

Author response to reviewers' comments

Reviewer #1:

Advance Summary and Potential Significance to Field: The research found that MEG3 was a tumor suppressor to regulate ST3Gal1 by interacting with c-Jun. In addition, the investigation connected EGFR sialylation with EGFR phosphorylation. The result of this research is precise, but to some extent, the results were only gained through 786-O cells in vitro.

Answer: Thanks for your thorough comments. We had finished part of the experiment using ACHN cells in vitro (please see the Fig.S1-ACHN).

Question 1: In Fig. 2C and Fig. 2D, the difference between negative control and MEG3/siMEG is not very clear.

Answer: Thanks for the question. Vector was the control of MEG3 overexpression, and si-NC was the control of MEG3 down-expression.

Question 2: The expressional relationship of ST3Gal1 and MEG3 should be statisticed.

Answer: Thank you for your suggestion. We had added the expressional relationship of ST3Gal1 and MEG3 in Fig.S2. The sentence was shown on page 7 lines 13-14.

Question 3: The annotation and figure sequence are wrong in Fig. 3.

Answer: Thanks for the question. We had revised the annotation and figure sequence in Fig. 3.

Question 4: In the whole experiment, 786-O cell line is the only discussed cell line while the results of FISH in Fig. 4E suddenly included ACHN.

Answer: Thanks for your comment. We had misspelled the Fig.4E and deleted ACHN cells.

Question 5: According to ChIP and RIP, c-Jun directly bound to ST3Gal1 promoter and MEG3, but there is no further discussion of the complex

Answer: Thanks for your comments. We had added the discussion of the complex in discussion part on page 10 lines 1-3.

Reviewer #2:

Question 1: Although interesting much of the data is obtained with one cell line and is not quantitated in many cases. In other examples some of the conclusions appear unfounded, for instance, the evidence that the PI3K/AKT pathway is downstream of MEG3 regulation of EGFR sialyation is sparse.

Answer: Yes, thank you for the instruction. We had added the quantitation of Fig.2C-2F and Fig.5. MEG3 regulated ST3Gal1 level, which mediated EGFR sialyation. It has been reported that EGFR phosphorylation was suppressed after EGFR sialylation, while EGFR signaling was a complicated network regulated by its phosphorylation. Furthermore, Datta et al showed that phospho-EGFR-Y920 could dock PI3K, which inactivated the apoptotic cascade via Bad (Bcl2 associated death factor) and caspase-9.(Datta et al., 1997). So, it was reasonable to conclude that the promotional effects of MEG3 on RCC progression could at least be partially mediated via elevated EGFR-mediated PI3K/AKT pathway.

Question 2: The manuscript needs to be edited and rewritten in some places, including the abstract. There are many places where experiments are unclear and need to explain better. Overall this manuscript needs multiple corroborating experiments to be ready for publication.

Answer: Yes, we had our paper edited by a person fluent in English to correct spelling and grammar errors and to clarify confusing sentences of this manuscript.

Question 3: Authors state, “As shown in Fig. 1C, 293T exhibited high level of MEG3, while RCC cell lines presented lower MEG3 level.” This is just relatively high. These are normalized values with no information of read counts across multiple tissues and cells.

Answer: Thank you for your comments. We had added the data of multiple tissues and cells on page 5 line 20.

Question 4: Authors state, “These results indicated that low level of MEG3 might affect the progression of RCC.” This suggests that low levels are associated with RCC, there is no information about how it might regulate progression at this point. Did the authors validate the in situ with positive and negative controls? Not clear here and there is nothing mentioned in the methods.

Answer: Thanks for your comment. We were the wrong writing. It should be “low level of MEG3 might be associated with RCC”.

Question 5: Migration and invasion effects of MEG3 are not quantitated and in the example shown the scratch test assay results are unconvincing in terms of MEG3 alteration of migratory properties.

Answer: Thank you for your advice. We had added the quantitation of invasion and scratch test in Fig 2C, D, E, F.

Question 6: In figure 5 authors state that they want to confirm previous results with ST3Gal1, showing it blocks EGF binding and EGFR dimerization. They did not measure this, they only measured EGFR phosphorylation level +/- ST3Gal1; there are multiple mechanisms by which pEGFR levels could be modulated. None of the results in Fig5 are quantitated.

Answer: Thank you for the instruction. The results in Fig5 had been quantitated. We had added the discussion on page 11 lines 17-18.

Question 7: Results for figure 5 mentions MAL affinity without spelling out that this is a lectin binding assay. This needs to be clearer in the results section. Results in Fig 5C and D appear pretty minimal, maybe in the case of 5D non-existent.

Answer: Thank you for the instruction. We agree with you. We revised the sentences on Page 9 line 6, and revised Fig.5D, which showed that alteration of MEG3 impacted the activity of PI3K/AKT signaling.

Question 8: Overexpression and siRNA experiments were primarily done with one cell line 786-0 cells and should be repeated in at least two cell lines.

Answer: Thanks for your thorough comments. We had finished part of the experiment using ACHN cells in vitro (please see the Fig.S1-ACHN).

Minor issues:

Question 1: Authors state, "whereas knockdown of MEG3 obviously promoted the cell proliferation in vitro", don't use obviously here.

Answer: Thanks for your advice. We had deleted "obviously".

Question 2: Authors state in the introduction, "…and stimulates the activation of PIP3" do they mean PI3K?
Answer: Yes, thank you for the instruction. We were the wrong writing.

Question 3: In figure legend 4K, "RIP assay was taken to evaluate specific" spell out RIP assay.

Answer: Thanks for your comment. We had spelled out RIP assay in Fig 4K legend.

Question 4: Please rewrite, "As shown in Fig. 4J, K, altered MEG3 regulated the expression of c-Jun and interaction with c-Jun."

Answer: Thanks for your comment. We had rewritten the sentence.

Second decision letter

MS ID#: JOCES/2020/244020

MS TITLE: LncRNA MEG3 mediates renal cell cancer progression by regulating ST3Gal1 transcription and EGFR sialylation via PI3K/AKT pathway

AUTHORS: Aihong Gong, Xinyu Zhao, Yue Pan, Yu Qi, Shuangda Li, Yiran Huang, Yanru Guo, Xia Qi, Wei Zheng, and Li Jia
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, reviewer 2 still raisees a number of substantial criticisms that prevent me from accepting the paper at this stage. I have to agree with the reviewer that the data should be replicated in more than one cell line and that appropriate quantitfication should be performed. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 research offers a novel research that it linked glycosyl transferase and LncRNA to clarify the regulational mechanism glycosylation modification of EGFR in cancer progression.

Comments for the author

Although the answer to my question 1 is not what I want to ask, the revised data in figure 2 have gave me the answer.

Reviewer 2

Advance summary and potential significance to field

This is a marginal advance and the authors did not address previous concerns with additional experimental evidence.

Comments for the author

The authors have tried to address each point either by reanalyzing previously provided data or rewriting the manuscript. No new experimental data is added to the manuscript. The following issues have not been addressed satisfactorily.

Original Question 1: Although interesting much of the data is obtained with one cell line and is not quantitated in many cases. In other examples some of the conclusions appear unfounded, for instance, the evidence that the PI3K/AKT pathway is downstream of MEG3 regulation of EGFR sialyation is sparse.
Authors' answer: Yes, thank you for the instruction. We had added the quantitation of Fig.2C-2F and Fig.5. MEG3 regulated ST3Gal1 level, which mediated EGFR sialyation. It has been reported that EGFR phosphorylation was suppressed after EGFR sialylation, while EGFR signaling was a complicated network regulated by its phosphorylation. Furthermore, Datta et al showed that phospho-EGFR-Y920 could dock PI3K, which inactivated the apoptotic cascade via Bad (Bcl2 associated death factor) and caspase-9. (Datta et al.,
1997). So, it was reasonable to conclude that the promotional effects of MEG3 on RCC progression could at least be partially mediated via elevated EGFR-mediated PI3K/AKT pathway.
Reviewer's remaining concern: More cell lines have not been included in the manuscript to support their major conclusions. No additional evidence for PI3K pathway is provided in this revision.

Question 3: Authors state, “As shown in Fig. 1C, 293T exhibited high level of MEG3, while RCC cell lines presented lower MEG3 level.” This is just relatively high. These are normalized values with no information of read counts across multiple tissues and cells.
Authors’ answer: Thank you for your comments. We had added the data of multiple tissues and cells on page 5 line 20.
Reviewer’s remaining concern: Authors still only show relative levels; they just state it in the text what was already show in Fig. 1C. The information of read counts across the lines already shown is still lacking. Also rationale for using 293T as control line, which apparently is used for normalization remains tenuous.

Question 6: In figure 5 authors state that they want to confirm previous results with ST3Gal1, showing it blocks EGF binding and EGFR dimerization.
They did not measure this, they only measured EGFR phosphorylation level +/- ST3Gal1; there are multiple mechanisms by which pEGFR levels could be modulated. None of the results in Fig5 are quantitated.
Authors’ answer: Thank you for the instruction. The results in Fig5 had been quantitated. We had added the discussion on page 11 lines 17-18.
Reviewer’s remaining concern: No additional experimental support provided in this revision.

Question 7: Results for figure 5 mentions MAL affinity without spelling out that this is a lectin binding assay. This needs to be clearer in the results section. Results in Fig 5C and D appear pretty minimal, maybe in the case of 5D non-existent.
Answer: Thank you for the instruction. We agree with you. We revised the sentences on Page 9 line 6, and revised Fig.5D, which showed that alteration of MEG3 impacted the activity of PI3K/AKT signaling.
Reviewer’s remaining concern: Data in Fig. 5C,D still looks pretty minimal.
Also the quantification in Fig. 5 is not clear what it is normalized to? In some instances, a data value is close to 1. Should one condition at least have one value at 1 as reference? Are values normalized to reference, if yes, which one (control?) or is the band with highest intensity is used for normalizing. Also, please be advised that it is the quantification of western blot intensity and not relative protein expression.
Pertinent to Fig. 5, it earlier escaped notice: what is the top blot in Figs. 5A, B as there is no protein attributed to that band and just labeled as phospho-tyrosine? Even in the manuscript, that band is simply referred to as phosphor-tyrosine.

Question 8: Overexpression and siRNA experiments were primarily done with one cell line 786-0 cells and should be repeated in at least two cell lines.
Authors’ answer: Thanks for your thorough comments. We had finished part of the experiment using ACHN cells in vitro (please see the Fig. ACNH), which would be published in the next article.
Reviewer’s remaining concern: ACHN figure shared in the response letter should be included in the manuscript to confirm the results in at least two cell lines.

Second revision

Author response to reviewers' comments

Re: Manuscript ID JOCES/2020/244020 "LncRNA MEG3 mediates renal cell cancer progression by regulating ST3Gal1 transcription and EGFR sialylation via PI3K/AKT pathway".

Reviewer #2:

Question 1: Advance summary and potential significance to field This is a marginal advance and the authors did not address previous concerns with additional experimental evidence.

Answer: Thanks for your comment. We had finished the experiment using ACHN cells in vitro (please see the supplementary Fig.S1 and Fig.S2-ACHN).

Question 2: More cell lines have not been included in the manuscript to support their major conclusions. No additional evidence for PI3K pathway is provided in this revision.

Answer: Thanks for your comment. We had finished the experiment using ACHN cells in vitro (please see the supplementary Fig.S1 and Fig.S2-ACHN).

Question 3: Authors still only show relative levels; they just state it in the text what was already shown in Fig. 1C. The information of read counts across the lines already shown is still lacking. Also, rationale for using 293T as control line, which apparently is used for normalization remains tenuous.

Answer: Thank you for your comments. The data was only shown relative levels in Fig.1C.

Question 4: In figure 5 authors state that they want to confirm previous results with ST3Gal1, showing it blocks EGF binding and EGFR dimerization. They did not measure this, they only measured EGFR phosphorylation level +/- ST3Gal1; there are multiple mechanisms by which pEGFR levels could be modulated. None of the results in Fig5 are quantitated. Reviewer's remaining concern: No additional experimental support provided in this revision.

Answer: Thank you for the instruction. The results in Fig5 had been quantitated. We had added the discussion on page 11 lines 17-18 and no additional experimental support was provided in this revision. We would study these multiple mechanisms by pEGFR levels modulated in the future.

Question 5: Data in Fig. 5C, D still looks pretty minimal. Also the quantification in Fig. 5 is not clear what it is normalized to? In some instances, a data value is close to 1. Should one condition at least have one value at 1 as reference? Are values normalized to reference, if yes, which one (control?) or is the band with highest intensity is used for normalizing. Also, please be advised that it is the quantification of western blot intensity and not relative protein expression. Pertinent to Fig. 5, it earlier escaped notice: what is the top blot in Figs. 5A, B as there is no protein attributed to that band and just labeled as phospho-tyrosine? Even in the manuscript, that band is simply referred to as phosphor-tyrosine.

Answer: Thank you for the instruction. Fig. 5 showed relative protein expression. A data value was the ratio of each detected protein relative to the internal reference GAPDH. In Fig.5A, B, the band of phospho-tyrosine was wrong and deleted.

Question 6: ACHN figure shared in the response letter should be included in the manuscript to confirm the results in at least two cell lines.

Answer: Thanks for your thorough comments. We had added ACHN Figure in the manuscript (please see the supplementary Fig.S1-ACHN and Fig.S3-ACHN).

Third decision letter

MS ID#: JOCES/2020/244020

MS TITLE: LncRNA MEG3 mediates renal cell cancer progression by regulating ST3Gal1 transcription and EGFR sialylation via PI3K/AKT pathway

AUTHORS: Aihong Gong, Xinyu Zhao, Yue Pan, Yu Qi, Shuangda Li, Yiran Huang, Yanru Guo, Xia Qi, Wei Zheng, and Li Jia
ARTICLE TYPE: Research Article

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