THE EMBO JOURNAL

Manuscript EMBO-2010-75675

# Repression of VEGFA by CA-rich element-binding microRNAs is modulated by hnRNP L

Faegheh Jafarifar, Peng Yao, Sandeepa M. Eswarappa and Paul L. Fox

*Corresponding author: Paul L. Fox, Cleveland Clinic*

**Review timeline:**

| | |
|---|---|
| Submission date: | 13 August 2010 |
| Editorial Decision: | 17 September 2010 |
| Revision received: | 15 December 2010 |
| Editorial Decision: | 04 January 2011 |
| Revision received: | 10 January 2011 |
| Editorial Decision: | 21 January 2011 |
| Accepted: | 21 January 2011 |

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 September 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. I apologise that it has taken longer than usual to have your study evaluated but I have been out of the office for the several days. I have received the reports from three referees that have evaluated your study, which they find to be interesting and potentially important. They ask for a number of issues to be addressed these include if hnRNP L may interact with mRNAs in nucleus before export (Ref #1) and the effect of depletion of hnRNP L on the hypoxia dependent regulation of the VEGF mRNA (Ref #2 and #3). Unless you already have the data, the global analysis of miRNAs on VEGF expression is not required for publication here. Should be able to address these issues we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

 

Editor
The EMBO Journal

---
REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This study on miRNAs and hnRNP L binding the CARE element on the VEGFA mRNA adds to the small but growing list of RNA binding protein / miRNA co-modulation of post-transcriptional gene expression. Overall I find the study to be very interesting, the data crisp and the conclusions well-supported by the experiments. I think it will be of considerable interest to a broad scientific audience. I only have a few comments/suggestions to polish the study:

1. One caveat to the model that is presented is that the authors should try to address / rule out that hnRNP L interacts with mRNAs in the nucleus (and then get transported to the cytoplasm) rather than compete with miRNA binding to the CARE element in the cytoplasm. This could perhaps be addressed by a co-immunoprecipitation analysis ala Fig. 5D using the nuclear fractions under normal and hypoxic conditions.

2. Fig. 5A: qRT-PCR (or an alternative more quantitative means of assessment) should be used to confirm the RT-PCR data on a lack of change in hnRNP L

3. Previous work from the Lynch lab demonstrated dynamic changes in post-translational modification of hnRNP L using 2D gels. Does hnRNP L undergo any modifications under hypoxic conditions which could contribute to its subcellular localization / increase in RNA binding?

4. Minor Issues:

a. To make it easier for the reader, the authors should state in the legends specifically what the asterisks refer to in their quantitative data.

b. Several places in the discussion refer to miRNAs as 'miRs'. This jargon should be either clarified (or perhaps changed to miRNAs to assist the general EMBO readership)

c. There are several typos in the Materials and Methods section (e.g. Santa Cruz and New England Biolabs are presented without spaces)

d. Fig. 5A: The legend has the panels backwards (i.e. the RT-PCR study is on the left, not the right as stated in the legend).

Referee #2 (Remarks to the Author):

In their manuscript, Jafarifar et al., explore the regulation of vegfa gene by miRNAs and RBPs. Vegfa expression is negatively regulated by miR-297 and 299 which are regulated by hnRNPL during hypoxia.

In general, the manuscript is written well, and the experiments are well controlled. Moreover, the observations are interesting, important, and fit within the scope of EMBO J. However, a few issues need to be resolved before publication.

1) Figure 3B and C and D are missing control anti-miR-297 and 299 mutants. Preferably, mutants should be no more than 2 nucleotides in the seed sequence. 3D misses also a control immunoblot

 

equal amounts of extracts - definitely also because the unspecific band just above vegfa that seems to behave similarly to vegfA itself.

2) Figure 6, I miss here two controls. One, a mutant of hnRNP L that shows that RNA binding domain is responsible for its effect on vegfa expression. Second, I would like to see how the expression of vegfa is changes when hnRNP L expression is suppressed. Best would be to use 2 independent functional knockdown oligoes. One would expect that in response to hypoxia, vegfa fails to be induced. This is to my mind a critical experiment to do.

3) Somehow the observations presented here should be coupled to biological measurements following hypoxia. Cell cycle effects or survival of cells following hypoxia should be affected by hnRNP L/miRNA manipulations.

Referee #3 (Remarks to the Author):

The manuscript "Repression of VEGFA by CA-rich element-binding microRNAs is modulated by hnRNP L" by Jafarifar and colleagues describes a novel regulatory mechanism in which the interplay between miRNA and RNA-binding proteins influences expression of the central hypoxia-inducible angiogenic protein, VEGF. The experimental approach is generally sound and dissected in detail.

Questions:

1) Fig 2, it would be even stronger if a antimiR approach was included. It would address the endogenous effects of CARE binding miRs. Also, by overexpressing hnRNP-L in normoxia and hypoxia, can the effect of outcompete these miRs?

2) Fig 4: I "Hypoxia overcomes miRNA-mediated inhibition of VEGFA expression". I fail to notice any change between hypoxia and normoxia in the control lanes, where no miRs are manipulated. In this particular system why are these candidate regulatory components relevant? Also, it would be of interest to see the effects of the corresponding antimiRs in the same experiment.

3) Fig 6, the effect of overexpressing c-myc-hnRNP L on the overall VEGF abundance is not impressive, but one needs to accept that such variations may be biologically relevant. I would be very interested to see the effects of an siRNA against hnRNP-L. These should definitely be addressed. Inactivation of hnRNP-L should prevent the increase of VEGF in hypoxia, or at least mitigate it.

4) Northerns are semiquantitative, it would be useful to present a Quantitative Real time PCR -based analysis that shows similar results in normoxia versus hypoxia.

General:

1) One wonders if this mechanism is restricted to monocytes, as VEGF is also abundantly produced by the tumor cells within solid tumors and robustly regulated in hypoxia in the majority of cancer cell lines. A proof of concept experiment in at least one cancer cell line would help.

2) Given the fact that there is a very significant variation between the miR target prediction programs, it is entirely possible that the miRs discussed in the manuscript are only part of the story. One approach may be to perform global inactivation of endogenous miR maturation by siRNA against Dicer/Drosha. This may shed light into the global role of miRs on VEGF expression and functional interactions with RNP-L for example.

 

1st Revision - authors' response 15 December 2010

We are grateful to the reviewers for their thoughtful and critical comments. Our point-by-point responses follow:

Referee #1:

1. The referee suggests we address the possibility that hnRNP L interacts with target mRNAs in the nucleus and then is transported to the cytoplasm. We have addressed this issue by a new co-immunoprecipitation experiment as proposed by the reviewer with an additional control for a distinct hnRNP (A1) known to interact with mRNAs in the nucleus (Figure 5E). Our results indicate that the interaction of hnRNP L with VEGFA mRNA is cytoplasmic and hypoxia-dependent.

2. As requested by the referee we now show qRT-PCR results indicating a lack of change in hnRNP L mRNA expression in hypoxia (Figure 5A).

3. The referee commented on the possibility of hypoxia-mediated post-translational modification of hnRNP L that contributes to cellular re-localization. We agree that this is a strong possibility, but have not been able to detect a modification by mass spectrometry or with phospho-specific antibodies (not shown). However, we show that overexpression of hnRNP L can modulate microRNA activity and VEGFA expression in normoxia, thus showing a hypoxia-mediated modification of hnRNPL is not required for this activity (Figure 6A-C).

4. Minor issues:

a. The asterisks shown in the quantitative data in several figures are now defined in the legends. b. For simplicity, we now use the term ìmiRNAsî instead of ìmiRsî, except when discussing a specific miRNA, e.g, miR-297.

c. The noted typographical errors in the Materials and Methods have been corrected.

d. The error in the legend of Figure 5A has been corrected.

Referee #2:

1. As suggested by the referee, we have added controls using anti-miR-297 and -299 mutants (with 2 altered nucleotides in the seed sequence) in Figure 3B-D. The Referee also notes the presence of two bands in our VEGFA immuboblot suggests we include a control to show equal loading of extracts; however, this experiment is an immunoprecipitation of equal amounts of conditioned media with VEGFA monoclonal antibody and thus an appropriate loading control is not available. In the new experiment shown, the minor band is included in the quantification but it does not significantly alter the result.

2. The referee suggests we show that the activity of hnRNP L in hypoxia is suppressed by knockdown oligos targeting hnRNP L. We have done this experiment and have observed the expected result, namely, knockdown of hnRNP L decreases VEGFA expression in the presence of hypoxia and miR-299. However, an additional control experiment revealed that hnRNP L knockdown in hypoxic cells reduces VEGFA expression by itself, i.e., even in the absence of the inhibitory miRNA. This result is expected since knockdown of hnRNP L markedly destabilizes VEGFA mRNA (Shih & Claffey, 1999). Thus, an hnRNP L knockdown approach cannot be used to investigate its role in reversing the activity of miRNA and the results not included in the manuscript. This activity of hnRNP L is supported by other experiments, namely, inactivation by mutation of the

 

hnRNP L binding site in the VEGFA 3'UTR (Figure 1C), hypoxia-mediated translocation of hnRNP L to cytoplasm (Figure 5B,C), hypoxia-stimulated binding of hnRNP L to the VEGFA 3'UTR (Figure 5E), and reversal of miRNA activity by overexpression of hnRNP L (Figure 6A-C).
The referee suggests we show results using a mutant of hnRNP L lacking the RNA binding domain. Unfortunately, this approach has the same shortcoming as hnRNP L knockdown since inhibition of binding to the VEGFA 3'UTR will destabilize VEGFA mRNA in hypoxia.

3. The referee suggests we couple our observations to biological measurements such as cell cycle effects or survival of cells during hypoxia. The effects of miRNAs on VEGFA expression in our studies are modest, consistent with their well-established function as fine-tuners of gene regulation, and to date we have not established a role of these miRNAs in cell function.

Referee #3:

1. The referee suggests we use an anti-miRNA approach to address the endogenous effects of CARE binding miRs. We have added these results to Figure 3B-D. The referee also asks whether overexpression of hnRNP L can outcompete the inhibitory activity of miRs in normoxia. We show this result in Figure 6A-C.

2. The referee notes that VEGFA expression is not increased in hypoxia in our experiments, even in the absence of manipulation of miRNAs. We have expanded our discussion of this important point (Discussion, end of 4th paragraph). We speculate that additional inhibitory mechanisms might be oper¨ative in hypoxic myeloid cells that are balanced by the suppression of miRNA inhibitory activity by hnRNP L, and suggest that an interplay of regulatory mechanisms maintains myeloid cell VEGFA expression at a constant level in both normoxia and hypoxia. Interestingly, hypoxia increases VEGFA expression in HEK293T cells compared to normoxic cells, even in the presence of inhibitory miRNAs, thus indicating the potential for this mechanism to influence total VEGFA expression in non-myeloid cells.

3. The referee asks whether inactivation of hnRNP L prevents or mitigates the increase of VEGFA in hypoxia. As discussed in our response to Referee #2 (point #2), this experiment was done but not included in the manuscript since the results are confounded by the decrease in VEGFA mRNA stability and protein expression caused by hnRNP L inactivation.

4. As suggested by the referee, to improve quantification we have included qRTPCR-based analyses of microRNAs and hnRNP L that shows similar expression in normoxia and hypoxia (Figures 4C and 5A, respectively).

The referee asks whether the microRNA-mediated mechanism described here is restricted to monocytic cells as VEGFA is also abundantly produced by the tumor cells and cancer cell lines. In a new experiment we show that HEK293T cells, a mildly tumorigenic transformed cell line, also express miR-297 and VEGFA expression is repressed by miRNAs during normoxia but not hypoxia (Supplementary Figure S1).

We agree with the referee that other miRNAs beyond those investigated here might interact with hnRNP L and influence VEGFA expression. However, the insights gained from global inactivation of endogenous miRNA by siRNA to Dicer/Drosha would give only limited insight without extensive investigation comparable to that for the miRNAs described here, and thus have not been addressed. We note that the Editor commented that a global analysis of miRNAs is not required for publication.

 

2nd Editorial Decision 04 January 2011

Your revised manuscript has been reviewed once more by two of the original referees who find that you have satisfactorily addressed the original concerns raised. Referee #3 has some remaining issues regarding some of the figures that need to be addressed prior to publication. Pending satisfactory minor revision, we would be happy to publish your manuscript in the EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Stan Gorski
Editor
The EMBO Journal

---
REFEREE COMMENTS

Referee #2 (Remarks to the Author):

The revised version addressed all my concerns. I recommend acceptance.

Referee #3 (Remarks to the Author):

The revised version of the manuscript has addressed my concerns, it is a solid new model adequately supported by experiments.

I have only one concern before publication: The presence of what appears to be blank blots/PCR in the figures, specifically: Fig 5D (GAPDH), 5F (GADPH again), 6H (again GAPDH), 5E ( VEGF mRNA, poor signal, barely visible). Even if the assay/pulldown/PCR was negative for these particular mRNAs, there should be at least one positive control. Completely blank figures are not informative and should not be published in this form.

2nd Revision - authors' response 10 January 2011

Response to Referees' comments:

We are grateful that all three reviewers indicated that we have satisfactorily addressed their major concerns. Referee #3 raises minor issues concerning data presentation, and these are addressed below.

Referee #3:

*The Referee notes "the presence of what appears to be blank blots/PCR in the figures, specifically:*

 

*Fig 5D (GAPDH), 5F (GADPH again), 6H (again GAPDH), 5E ( VEGF mRNA, poor signal, barely visible). Even if the assay/pulldown/PCR was negative for these particular mRNAs, there should be at least one positive control. Completely blank figures are not informative and should not be published in this form."*

Response:

We have addressed these issues as follows:

Fig. 5D (GAPDH): We have clarified that indeed there is a positive control for the "blank blot" of GAPDH. We have rearranged the blot to more clearly show the positive control next to the ìblank blotî. In addition, we have re-labeled the positive control as ìInputî to clarify the relationship between the blots, and also clarified the results in the text.

Fig. 5F, 6H (GAPDH again): We regret that these panels were inadvertatnly duplicated in Figures 5 and 6. The data are now in Figure 6A only (we assume that the Referee meant Figure 6A, rather than 6H, since this figure only contains A-C). As we did for Fig. 5D, above, we have rearranged the blot to show the positive control next to the ìblank blotî, and clarified the label and text.

Fig. 5E (VEGF mRNA, poor signal, barely visible). We have replaced this panel with a new blot from the same experiment that shows the upper bands (exon-specific RT-PCR) more clearly. The difficulty in presenting these data is due to the much greater signal from VEGFA pre-mRNA (lower bands) compared to mature VEGFA mRNA (upper bands). If the Referee (or Editor) feels that the visibility of these bands remains insufficient, we would be glad to divide this panel into two panels (and increase the contrast of the upper bands only), or invert the scale of the gels in Fig. 5E which renders the bands much more visible. We also removed a ìblankî gel (RT-PCR for anti-Flag) from this figure; this blank negative control lacked a positive control and did not substantially contribute to the results.

Additional correspondence (editor) 18 January 2011

I have been going through the data in your revised manuscript and find that you have satisfactorily addressed the concerns raised by referee #3. However, during this process I have noticed that in Fig 6c it seems that some of the lanes of the gel have been spliced together and I would be very grateful if you could inform me how the figure was assembled.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence (author) 18 January 2011

Thanks for the good news about our manuscript.

You are correct that we have reordered the lanes in Fig. 6C. This was done to make the order of the lanes the same in each of the two transfection conditions. In the original gels, the controls are next to each other in the center of the figure instead of on the left for each condition. I was afraid that this might be a little confusing for the reader (it's a little confusing to me!) so we moved the control lane for the "Vector" experiment to the left of the figure. I have attached a revised PDF showing the

 

original unspliced version. I will be glad to use either, but prefer the rearranged version. Your advice would be appreciated.

Additional correspondence (editor) 19 January 2011

It is fine to keep the figure in the rearranged form, but we would need to have black lines drawn beside the rearranged lanes, to indicate that they are not consecutive on the original gel.

We would also need to add a sentence in the figure legend stating something along the lines of:
"Please note that the lanes in Fig 6c were run on the same gel but rearranged for clarity."

If you send us the new figure and a sentence we can add it to the manuscript before accepting it, alternatively if you wish you could make the changes yourself.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence (author) 19 January 2011

Thanks for the rapid response. As you suggested, we have re-drawn Figure 6C with the addition of thin black vertical lines to indicate where the lanes were spliced. We have modified the legend to read: "Lysates were subjected to immunoblot analysis with anti-c-Myc tag, VEGFA, and GAPDH antibodies (the samples were run on the same gel but the lanes rearranged for clarity)". The revised manuscript is attached.

I've taken this opportunity to attach a slightly revised version of Figure 3. In the original Figure panels 3C and 3D, the lanes (from the same gel) were rearranged so that the two wild-type anti-miRNAs preceded the mutant anti-miRNAs. Rather than add black lines to this figure as well, I have sent the original figures with un-edited gels. I'm confident that the readership will not have a problem seeing the mutant controls before the wild-type (but I find it a bit inelegant).

Please let me know if you need additional information and I apologize for any inconvenience we might have caused.

3rd Editorial Decision 21 January 2011

Thank you for incorporating all the changes into the manuscript, I can now accept it for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

 

Editor
The EMBO Journal

 