Re-organization of nucleolar architecture in myogenic differentiation

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MS TITLE: Re-organization of Nucleolar Architecture in Myogenic Differentiation

AUTHORS: Tetsuaki Miyake and John C McDermott
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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, both expert reviewers thought that the work was potentially quite interesting and significant but all also raised a number of concerns that must be dealt with before the manuscript can be reconsidered. Please address these issues as thoroughly as possible. In particular, you should perform quantification of the data and include information on sample size, statistics and significance. 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

Mechanisms underlying the progression of the myogenic program has focused extensively on epigenetic, transcriptional and post-transcriptional regulation of gene expression. Gene regulation is also achieved at the level of protein synthesis, with regulation of specific transcripts by

microRNAs, splicing and RNA binding proteins, along with pathways that regulate global protein synthesis, such as mTOR signaling. How the cell adapts to increased protein synthesis within a differentiation program remains incompletely understood.

This is an interesting manuscript that reports changes in nucleoli morphology and function during the differentiation of the C2C12 myogenic cell line. The authors report that progenitor cells have multiple small, spherical nucleoli, while differentiated myotubes typically have a single, large, irregularly shaped nucleoi. Changes in nucleoi morphology correlate with increased rRNA production and protein synthesis, and are dependent on mTOR activity.

Comments for the author

The following essential revisions should be addressed:
1. Throughout the manuscript and applicable to all presented data in Figures: The immunofluorescence data is high quality, but is often presented without quantification of results and in other Figures, sample size, statistics and significance are not reported.

- Figure 1F should include information on sample size (biological replicates etc), statistics and significance. Please provide more information in the y-axis.

- Figure 2 requires some quantification of the presented data with information on sample size, statistics and significance. It is unclear what three panels in (A) and (B) represent. In Figure 2B, there appears to be a fair amount of variability between images, suggested by the heatmap. Legends for the heatmap would be helpful.

- Figure 3 requires quantification of immunofluorescence analyses with sample size, statistics and significance reported.

- Figure 4B, C requires information on sample size, statistics and significance.
Some of these considerations are provided in Table 2,

- Figure 5B requires information on sample size, statistics and significance.

2. The data presented in Figure 5 appear to be C2C12 cells transfected with two plasmids one expressing GFP and the other expressing mTor-S2215Y. How do the authors know that an analyzed cell has been transfected with both plasmids? This consideration is further problematic given the low rates of transfection in C2C12 cell line. (Materials and methods should refer to both plasmids used in the study).

The scope of the study is within a context of a cellular model of C2C12 myogenic differentiation. The addition of data showing that nucleoli changes in morphology are a feature of progression of the myogenic program in vivo, for example in developing or regenerating skeletal muscle, would increase the impact of the work and might be feasible given the authors high quality IF data. If experimental revisions are not possible, suggestion to be clear about where within the myogenic program the C2C12 differentiation system lies when interpreting results.

Additional minor comments:

In my opinion the final statements in the abstract and the discussion do not reflect the scope of the work as addressing an interesting fundamental question.

pg 13, line 265, six bins are indicated the but the Figure 1F shows five.

Figure 1F is labeled with RC, MT and MB - suggestion to especially increase the font size for easier understanding of the Figure.

Reviewer 2

Advance summary and potential significance to field

The manuscript of Miyake and McDermott presents an interesting and novel dissection of nucleolar dynamics and structure during muscle cell differentiation. Nucleolar biology, is in general, a poorly understood area of cell biology, despite the centrality of this structure in regulating cell fate changes and adaptation to external cues. Nucleolar function in skeletal muscle biology is particularly noteworthy in that rRNA is highly active perhaps more so than any other tissue, owing to the very significant protein synthesis demands to sustain the core protein contractile apparatus that constitutes the sarcomere. The observations here are important and will be a welcome addition to the field of skeletal muscle biology and to those interested in cell differentiation in general. The data is of high quality, the interpretations are reasonable and grounded, with no overt concerns. Minor revisions are requested.

Comments for the author

Prior to publication the authors should address a number of minor issues, experimental and textual.

1. The current title of the paper is quite general. I would suggest the authors consider a more declarative title which reflects the discovery aspect of the manuscript, i.e. “mTOR is required to reorganize nucleolar architecture in myogenic differentiation”

2. In Figure 1, the authors provide positive and negative observations regarding nucleolar morphology, i.e. fibroblasts do not engage the muscle cell reorganization. This is the one portion of the manuscript that would benefit from supporting observations in a primary myoblast culture system. I do not discount the utility of the C2C12 cell system, however some supporting observations in differentiating primary myoblasts would greatly strengthen the core supposition of the manuscript (which could be achieved by replicating Figure 1 alone with the primary myoblast model).

3. Quantitation for Figure 2 would be informative. In the current manuscript this appears to be presented as an all or none phenomenon.

4. Figure 4 is somewhat difficult to interpret. Here, the authors have used rapamycin as an mTOR inhibitor to query the role of mTOR in nucleolar adaptation in differentiated myotubes (MTs). My only concern here is that rapamycin blockade of mTOR will generally suppress differentiation, which may or may not be related to nucleolar dynamics. To address this concern the authors should conduct an experiment where rapamycin is added at 2-3 days post low serum induction of differentiation. This temporal experiment would remove any general effect of rapamycin on early stages of the differentiation program and ask whether the requirements of the fusing myotube structure (and its protein synthesis demands) may also be dependent on mTOR independent of other early differentiating requirements not related to the nucleolus.

5. In Figure 5, it is not clear whether the authors measured nucleolar size in the mTOR gain of function experiments in the MTs. The author’s model would predict this and it would be an easy measure to document.

6. I do not see Table 2 in the main body of the text nor the supplemental data. Please add.

First revision

Author response to reviewers' comments

Response to reviewer comments:

Reviewer 1

COMMENT: Reviewer 1 Advance Summary and Potential Significance to Field: Mechanisms underlying the progression of the myogenic program has focused extensively on epigenetic, transcriptional and post-transcriptional regulation of gene expression. Gene regulation is also achieved at the level of protein synthesis, with regulation of specific transcripts by microRNAs, splicing and RNA binding proteins, along with pathways that regulate global protein synthesis, such as mTOR signaling. How the cell adapts to increased protein synthesis within a differentiation program remains incompletely understood. This is an interesting manuscript that reports changes in nucleoli morphology and function during the differentiation of the C2C12 myogenic cell line. The authors report that progenitor cells have multiple small, spherical nucleoli, while differentiated myotubes typically have a single, large, irregularly shaped nucleoi. Changes in nucleoi morphology correlate with increased rRNA production and protein synthesis, and are dependent on mTOR activity.

RESPONSE: We appreciate the reviewer’s comments regarding the context of our manuscript in the field of myogenesis and its potential interest to scientists working on nucleolar morphology and its role in the differentiation process.

COMMENT: Throughout the manuscript and applicable to all presented data in Figures: The immunofluorescence data is high quality, but is often presented without quantification of results and in other Figures, sample size, statistics and significance are not reported.

RESPONSE: we believe there was an error in the original file conversion sent to the reviewers since we had some quantification of the IF data in most figures. However, we have improved this in the revised manuscript and also added documentation of sample sizes and statistical analysis.

COMMENT: - Figure 1F should include information on sample size (biological replicates etc), statistics and significance. Please provide more information in the y-axis.

RESPONSE: Fig 1F All requested information is now included.

COMMENT: Figure 2 requires some quantification of the presented data with information on sample size, statistics and significance. It is unclear what three panels in (A) and (B) represent. In Figure 2B, there appears to be a fair amount of variability between images, suggested by the heatmap. Legends for the heatmap would be helpful.

RESPONSE: All requested information is now included in the Fig. In addition, we have added a 2.5D plot and line scan to aid in the visual representation of the data.

COMMENT: Figure 3 requires quantification of immunofluorescence analyses with samplesize, statistics and significance reported.

RESPONSE: All requested information is now included. In addition we have used arrows to point out RC and MTs.

COMMENT: Figure 4B, C requires information on sample size, statistics and significance. Some of these considerations are provided in Table 2.

RESPONSE: All requested information is now included. In addition, we added D (morphology), E (experimental design schematic), F (nucleolar size change), G (differentiation index) for DM4 (2days with Rapamycin).

COMMENT: Figure 5B requires information on sample size, statistics and significance.

RESPONSE: All requested information is now included. In addition we added I2500F data with arrows indicating transfected cells.

COMMENT: The data presented in Figure 5 appear to be C2C12 cells transfected with two plasmids one expressing GFP and the other expressing mTor-S2215Y. How do the authors know that an analyzed cell has been transfected with both plasmids? This consideration is further problematic given the low rates of transfection in C2C12 cell line. (Materials and methods should refer to both plasmids used in the study).

RESPONSE: We have documented, along with many other groups in the past, that plasmid transfection results in non-selective uptake of plasmids in the cells that become transfected. All of our data using fluorescently labelled proteins confirms this phenomenon. If this were not the case then there would be literally many thousands of manuscripts in the literature that would be artifactual since they rely on uptake being non-selective, ie a cell that is transfected takes up all of the plasmids in the transfection mix. There is no question that this is true. However, we do concede that in some cells the expression levels of transfected reagents do vary to some degree and this can result in some variability in the data.

COMMENT: The scope of the study is within a context of a cellular model of C2C12 myogenic differentiation. The addition of data showing that nucleoli changes in morphology are a feature of progression of the myogenic program in vivo, for example in developing or regenerating skeletal muscle, would increase the impact of the work, and might be feasible given the authors high quality IF data. If experimental revisions are not possible, suggestion to be clear about where within the myogenic program the C2C12 differentiation system lies when interpreting results.

RESPONSE: We fully agree with the reviewer that to have data in primary cells would improve the impact of our manuscript. We therefore delayed the re-submission of the manuscript to replicate the data in Fig 1 in primary mouse myoblasts (satellite cells) derived from hindlimb muscles. These data are now presented in Fig 2 and essentially confirm our observations using the C2C12 mouse derived cell line on nucleolar morphology changes during differentiation. Since C2C12 cells were originally clonally isolated from post-natal mouse skeletal muscle, we think that using murine primary hindlimb muscle myoblasts was the most relevant model to use to compliment the C2C12 data. We appreciate the reviewer’s comment to include this experimentation.

Additional minor comments:

COMMENT: In my opinion the final statements in the abstract and the discussion do not reflect the scope of the work as addressing an interesting fundamental question.

RESPONSE: The speculative point we are making here is that these changes in nucleolar morphology may be of importance in physiological processes and this needs to be assessed in further studies.

COMMENT: pg 13, line 265, six bins are indicated the but the Figure 1F shows five.

RESPONSE: We have changed to five categories.

COMMENT: Figure 1F is labeled with RC, MT and MB - suggestion to especially increase the font size for easier understanding of the Figure.

RESPONSE: Requested change is now included.

Reviewer 2

COMMENT: Advance Summary and Potential Significance to Field:

The manuscript of Miyake and McDermott presents an interesting and novel dissection of nucleolar dynamics and structure during muscle cell differentiation. Nucleolar biology, is in general, a poorly understood area of cell biology, despite the centrality of this structure in regulating cell fate changes and adaptation to external cues. Nucleolar function in skeletal muscle biology is particularly noteworthy in that rRNA is highly active, perhaps more so than any other tissue, owing

to the very significant protein synthesis demands to sustain the core protein contractile apparatus that constitutes the sarcomere. The observations here are important and will be a welcome addition to the field of skeletal muscle biology and to those interested in cell differentiation in general. The data is of high quality, the interpretations are reasonable and grounded, with no overt concerns. Minor revisions are requested.

RESPONSE: We appreciate the reviewer's comments regarding the work in this manuscript and the potential impact in the myogenesis field.

COMMENT: The current title of the paper is quite general. I would suggest the authors consider a more declarative title which reflects the discovery aspect of the manuscript, i.e. "mTOR is required to reorganize nucleolar architecture in myogenic differentiation"

RESPONSE: We deliberated over this comment quite extensively. In the end we decided that the main theme of our manuscript is the documentation of the nucleolar morphology changes during differentiation. While, we have some interesting and consistent observations that mTOR may be important in these changes, we are reluctant to make such a bold statement in the title of the manuscript. As indicated by the reviewers, mTOR plays many roles in the cell so to definitively conclude mTOR's direct regulatory involvement requires extensive proof. The additional experiments that we have now added to the manuscript do further support this idea but we still feel that the main message is the morphology change and therefore would appreciate keeping the title as is.

COMMENT: In Figure 1, the authors provide positive and negative observations regarding nucleolar morphology, i.e. fibroblasts do not engage the muscle cell reorganization. This is the one portion of the manuscript that would benefit from supporting observations in a primary myoblast culture system. I do not discount the utility of the C2C12 cell system, however some supporting observations in differentiating primary myoblasts would greatly strengthen the core supposition of the manuscript (which could be achieved by replicating Figure 1 alone with the primary myoblast model).

RESPONSE: As referred to above, we have now replicated the data in Fig 1 (using C2C12 myoblasts) with data derived from mouse primary myoblasts (satellite cells). These data are now included in Fig 2.

COMMENT: Quantitation for Figure 2 would be informative. In the current manuscript this appears to be presented as an all or none phenomenon.

RESPONSE: We have added additional extensive quantitation as requested and indicated to reviewer 1 above.

COMMENT: Figure 4 is somewhat difficult to interpret. Here, the authors have used rapamycin as an mTOR inhibitor to query the role of mTOR in nucleolar adaptation in differentiated myotubes (MTs). My only concern here is that rapamycin blockade of mTOR will generally suppress differentiation, which may or may not be related to nucleolar dynamics. To address this concern the authors should conduct an experiment where rapamycin is added at 2-3 days post low serum induction of differentiation. This temporal experiment would remove any general effect of rapamycin on early stages of the differentiation program and ask whether the requirements of the fusing myotube structure (and its protein synthesis demands) may also be dependent on mTOR independent of other early differentiating requirements not related to the nucleolus.

RESPONSE: We agree with the reviewer and we carried out the experiment proposed in which we added Rapamycin at 2days after DM. The results are quite striking in that the differentiation program is not inhibited to any degree but the nucleolar re-organization is impacted to a much greater extent. These data are now included in Fig 5E . We appreciate the reviewer's suggestion since it has allowed us to begin to tease apart the effect of mTOR on nucleolar re-organization and its effect on differentiation in general.

COMMENT: In Figure 5, it is not clear whether the authors measured nucleolar size in the mTOR gain of function experiments in the MTs. The author's model would predict this and it would be an easy

measure to document.

RESPONSE: We measured the effects of the gain of function mutants in myoblasts since we predicted that GoF would result in nucleolar re-organization and expansion and this is supported in the data (new Fig 6, previously Fig 5). However, it is very difficult to maintain exogenous expression of plasmids for 4-5 days in differentiating cells to observe the effects of this in MT's (the cells can only be transfected at the MB stage). We also did not expect that mTOR GoF in MT's would result in any differences since the MTs naturally expand the nucleoli in the normal transition to multinucleated MTs.

COMMENT: I do not see Table 2 in the main body of the text nor the supplemental data. Please add.

RESPONSE: the requested change is now included and Table 2 has been replaced.

Reviewer 3

COMMENT: Mainly based on immunostaining and fluorescent microscopic imaging, Miyake and McDermott reported in this manuscript that the size and morphology of nucleoli change during myoblast differentiation in culture: from multiple small and rounded nucleoli in undifferentiated myoblasts to one large and irregular-shaped nucleolus in differentiated myotubes. They showed that such changes are dictated by mTOR signaling. Inhibition of mTOR by rapamycin prevented such structural changes in nucleoli, whereas a hyperactivated mutant of mTOR enhanced such changes.

RESPONSE: Thank you for this synopsis of our manuscript.

COMMENT: Overall, I am afraid that this manuscript failed to report anything interesting or informative. Importantly, the main finding of the manuscript that the size and morphology of nucleoli change during myoblast differentiation is not novel at all: it has been reported in the paper by Ohira et al in 2011 (see Figure 3C and Figure 4B and 4C in Biosci. Biotechnol. Biochem., 75 (6), 1085–1089, 2011).

RESPONSE: The opinion that there is nothing interesting or informative in our manuscript is obviously not shared by the other reviewers nor other members of the myogenesis community who I have presented this work to in the last 6 months. Nevertheless, we respect anyone's personal opinion but don't agree with it at all. More specifically, we would contend that we have an extensive documentation of nucleolar morphology changes in myogenic differentiation using state of the art methodology that is also linked to changes in nucleolar function. In the manuscript cited (published in in Biosci. Biotechnol. Biochem which has been cited twice since 2011) there is data from 2 nuclei (one nucleus in MB and one in MT) in Figure 3C. The data in Figure 4B and C document quantitative effects of creatine supplementation as far as we can ascertain. Even though this data is not at all comprehensive and is essentially unknown in the myogenesis community, we have now referenced this manuscript which will allow readers to form their own opinions of the work. In addition, we would point out that the limited information in this manuscript does nevertheless support the main theme of our manuscript.

COMMENT: Moreover, it has also been well-established that mTOR is indispensable for myoblast differentiation in culture (e.g., Coolican et al, J Biol Chem 1997). Thus, it is not surprising that inhibition of mTOR by rapamycin blocks the size and morphological changes of nucleoli that associate with the differentiation process.

RESPONSE: We agree that mTOR plays several roles in myogenic cells thus making it difficult to tease apart primary and secondary effects. We have now referenced the Coolican manuscript regarding the role of mTOR in myogenic differentiation. Certainly, the reviewer is correct that multiple studies have documented that mTOR is indispensable for MB differentiation. However, the statement "it is not surprising that inhibition of mTOR by rapamycin blocks the size and morphological changes of nucleoli" is a patently teleogical argument that, quite frankly, has no place in a scientific review. Moreover, the statement is actually proven incorrect since in the new Fig 5E we indicate that Rapamycin treatment at 2 d following DM (in response to the other reviewer's request), still prevents nucleolar re-organization while having a marginal effect on the

differentiation. In addition, the effect of exogenous mTOR gain of function mutants indicates that mTOR activation results in nucleolar re-organization in MBs when differentiation is not initiated, thus suggesting that the effect of mTOR is independent of differentiation even if the two processes occur concurrently under normal differentiation.

COMMENT: Another obvious problem of the manuscript is the lack of quantitative analysis for all the changes shown in the figures.

RESPONSE: We respect the reviewers comment and, as alluded to above, we have made sure there is extensive quantification of the data in the revised version of the manuscript.

Second decision letter

MS ID#: JOCES/2022/260496

MS TITLE: Re-organization of Nucleolar Architecture in Myogenic Differentiation

AUTHORS: Tetsuaki Miyake and John C McDermott
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.