The long noncoding RNA *Meg3* regulates myoblast plasticity and muscle regeneration through epithelial-mesenchymal transition

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MS TITLE: The long noncoding RNA Meg3 regulates myoblast plasticity and muscle regeneration through epithelial-mesenchymal transition

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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 attend to all of the reviewers' comments and 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. 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

This paper studies the role of the lncRNA Meg3 on myogenesis and tissue repair. They show that Meg3 is enriched in satellite cells and mesenchymal cells in skeletal muscle, and is regulated during differentiation and regeneration. The main claim is that Meg3 is important for EMT during differentiation and this is supported by the cell culture and RNA-seq data. They also perform in vivo experiments with shMeg3, and while these are less clean due to lack of definivite knowledge about which cell types are affected by the knockdown, the results suggest a similar effect on satellite cells during regeneration. This work defines the role of Meg3 during muscle differentiation but also highlights the important of EMT. Overall, the paper is well written and logically articulated.

Comments for the author

-The sequence of cell populations for the various genes in Fig. 1A should be consistent. Sat cells then mes. cells are listed for Meg3 but this is reversed for Rian.

-Was the RNA-IP for Ezh2 (Fig. 1D) performed on proliferating or differentiating myoblasts? Does this interaction change during differentiation? While Meg3 levels go down, there could still be sufficient Meg3 for interaction with Ezh2 during differentiation.

-The authors do not show data for d7 differentiation (related to Fig. 2). It is not clear why they mention it but do not display the data. There are a few other cases of 'data not shown'.

-The experiments designed to rescue Meg3 reduction with expression of human MEG3 are important to show the phenotype is due to Meg3. These are bit complicated because the Meg3 shRNA also recognizes human MEG3. It is not clear why endogenous Meg3 is reduced in the shLacZ + MEG3 sample and why Meg3 is increased in the shMeg3 + MEG3 sample. Can the authors explain these results?

-Did the authors investigate expression of other RNAs associated with the Meg3 polycistronic transcript? This could help their argument that the phenotype in shMeg3 cells is specific to reduction of Meg3. It is also important because the authors use the rationale the Dlk1-Dio locus expression levels are associated with mitochondrial activity.

-Scale bars on all images would be optimal. Currently there is only '20x' listed.

-Often a one-way ANOVA should be used (such as Fig. 2D) instead of a student's t-test when there are comparisons between more than two groups.

Reviewer 2

Advance summary and potential significance to field

Strengths of this paper are in defining the role of Meg3 in these processes, and the convergence of in vitro and in vivo data. Meg3 is part of a complex of genes that are regulated in complex epigenetic ways, and understanding its role in development is important.

Comments for the author

In article, Dill et al address role of the long noncoding RNA Meg3 in muscle differentiation, using tissue culture model and in vivo using a muscle injury model. These authors show that muscle differentiation is diminished in Meg3 shRNA, due to a combination of reduced viability, reduced proliferation, and reduced ability to undergo myogenic differentiation. By transcriptomic analysis, authors show epithelial-to-mesenchymal transition markers are up regulated in knockdowns, and defects in muscle differentiation may be attributable to increased TGFbeta signaling and downstream kinase activity. It is interesting to see that similar transcriptomic changes are also observed in in vivo model.

Strengths of this paper are in defining the role of Meg3 in these processes, and the convergence of in vitro and in vivo data. Meg3 is part of a complex of genes that are regulated in complex epigenetic ways, and understanding its role in development is important.

There are some overall concerns:
1. The role of Meg3 specifically in muscles is not especially clear. If shRNA has an overall negative impact upon cell viability, then we would see many of same results. What makes the role of Meg3 in muscle more unique to that in other tissues?

2. Reader is left with a lack of understanding of the mechanisms used by Meg3 to regulate gene expression and therefore development. In Figure 1D, authors show interaction of Meg3 with repressor component Ezh2, and this suggests that overall Meg3 functions to repress gene expression (this experiment would benefit from a negative control with a different lncRNA). But this observation is not linked with other findings in the manuscript, and does not explain the large number of genes down regulated in the shRNA. Demonstration of Meg3 interacting with promoters of some genes studied in the later figures, such as TGFbeta genes, would provide better mechanistic understanding.

3. In several places, changes in readouts for experiments are modest and suggest that other processes are also affected, but not investigated. In Figure 6A, LY treatment rescued Meg3 shRNA, but not all the way back to control levels. In Figure 7B, SB treatment improved some parameters but not others. These results suggest that Meg3 affects many different processes (supported by the transcriptome data), but there is not a complete understanding of what is happening. As a result, some parts of the paper are descriptive and not mechanistic.

Other comments:
1. In Figure 2 where authors make stable lines expressing shRNA or MEG3, did the authors determine the relative copy numbers of each transfected gene to make sure that each sample could be compared?

2. Figure 5A right panel, the significance mark is not aligned properly.

3. Figure 6, the LY rescue experiments, do improvements result from improvements in viability, proliferation, or fusion, or combination of these things?

4. Figure 8B, add arrows to indicate scar tissue.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors identified that knockdown (KD) of lncRNA Meg3 in both C2C12 cells suppresses cell proliferation, increases cell apoptosis, decreases myogenic differentiation, and promotes cell migration and EMT. Meg3 KD also induces delayed muscle regeneration. Chemical inhibitors of TGFb ROCK1/2, and p38 MAPK restores myogenic differentiation after Meg3 KD in vitro. Therefore, the authors concluded that Meg3 is an important regulator for proper muscle differentiation via suppression of EMT.

Comments for the author

While this manuscript has some interesting observation, it is premature to support the author's conclusions.

Major issues:
1. EMT-mediated muscle differentiation or regeneration has not been documented yet. The authors identified the up-regulation of EMT-related genes and an increase in cell migration after KO of Meg3.

However, it is not so clear whether EMT is actively involved in muscle differentiation and regeneration. It is interesting finding that inhibition of TGFb, ROCK1/2, and p38 MAPK, which are downstream pathways of EMT restores myogenic differentiation after Meg3 KD. However, it is well known that inhibition of these signalings generally affects myogenic differentiation. Therefore, it is essential to describe how EMT is crucial for muscle differentiation and regeneration.

2. In Figure 8A, the reduction of Meg3 expression in TA muscle after shMeg3 injection is less than 30%. Therefore, it is not convincing that the effects seen in this Figure is due to down-regulation of Meg3 levels. Nevertheless, the reduction of cross-sectional area day 3, day 7, and day 14 after CTX injection are 50%, 20%, and 60%, respectively, in shMeg3 group. Therefore, please utilize an experimental protocol for which a significant reduction of Meg3 can be induced after KD in vivo. And then, please show the absolute number of the cross-sectional area of each muscle and the distribution of fiber sizes. In addition, please examine fibrosis detection by day 14 after CTx injection since PDGFRa+ cells were increased in shMeg3 group, and thus fibrosis may be increased in this group.

Minor issues:
1. Figure 1A should cite (Schaum et al. 2018).
2. In Figures 2 and 3, which differentiation date was used for immunostaining and other experiments?
Please indicate the dates in the figure legends.
3. Figure 2A showed the increased number of mononuclear myocytes while multinucleated myotubes are significantly reduced in Meg3 KD group, indicating that Meg3 KD blunts myogenic fusion. This observation is supported by RNA-seq data shown in Sup Figure 2 in which both Myomaker and Myomixer are down-
regulated in Meg3 KD group.
4. Figure 10D, the significant differences between each group are not convincing.

First revision

Author response to reviewers' comments

Reviewer 1 – we thank this reviewer for noting the importance of Meg3 in muscle differentiation and EMT, and that our paper was well written and logically articulated. This reviewer expressed a few concerns which we have now addressed with additional experiments as described below.

1. The sequence of cell populations for the various genes in Fig. 1A should be consistent. Sat cells then mes. cells are listed for Meg3 but this is reversed for Rian.

The order of cell populations on the Y-axis was originally organized by enrichment from most enriched (top) to least enriched (bottom). The suggestion of ordering by cell type – sat cells (top), mesench cells (next) - makes sense, and we have revised this part of the figure accordingly.

2. Was the RNA-IP for Ezh2 (Fig. 1D) performed on proliferating or differentiating myoblasts? Does this interaction change during differentiation? While Meg3 levels go down, there could still be sufficient Meg3 for interaction with Ezh2 during differentiation.

RNA-IP was performed in proliferating myoblasts, and we chose this time point based on enrichment of Meg3 transcripts. As suggested, we performed the RNA-IP on myotubes at differentiation day 3, and include these results in Sup. Fig. 1A to provide a more complete picture on how the epigenome might be regulated by Meg3 in muscle differentiation. Although we detected an interaction between Meg3 and Ezh2 in myotubes, this enrichment did not reach statistical significance, and coincided with Ezh2 cytoplasmic export at this timepoint (Sup. Fig. 7A). Taken together, these data suggest Meg3 interacts with Ezh2 in proliferating myoblasts, and this interaction becomes diminished with differentiation-induced Ezh2 export.

3. The authors do not show data for d7 differentiation (related to Fig. 2). It is not clear why they mention it but do not display the data. There are a few other cases of 'data not shown'

We did not show these data since the shMeg3 phenotype on d7 differentiation was largely similar to d3. Nevertheless, we agree with this reviewer, and for completeness sake we now include these data in Supplemental Figure 2B. We have imaged and quantified fusion index (actinin & myh4 immunofluorescence) to demonstrate that impaired differentiation persists on d7. Finally, in other sections of the manuscript we now show selected data, previously indicated as data not shown, as Supplemental Figures.

4. The experiments designed to rescue Meg3 reduction with expression of human MEG3 are important to show the phenotype is due to Meg3. These are bit complicated because the Meg3 shRNA also recognizes human MEG3. It is not clear why endogenous Meg3 is reduced in the shLacZ + MEG3 sample and why Meg3 is increased in the shMeg3 + MEG3 sample. Can the authors explain these results?

We agree with this reviewer that the ability of shMeg3 to recognize both mouse and human MEG3 presented challenges for the rescue, and that this approach resulted in some unusual effects on endogenous Meg3 expression, which we felt were important to convey. We feel we can provide reasonable interpretations of these confounding results.

Much of the unexpected results pertaining to endogenous Meg3 expression levels relates to the complex and poorly understood epigenetic auto-regulation of the Dlk1-Dio3 locus by MEG3. Counterintuitively, in embryonic stem cells MEG3 has been shown to recruit Ezh2 to an upstream differentially methylated region to maintain transcription of the locus by preventing recruitment and activity of DNA methyl transferases (Das PP et al. 2015). Thus, overexpression of MEG3 (in the shMeg3 background) could result in the upregulation of endogenous Meg3 through more efficient recruitment of Ezh2 and subsequent blocking of DNA methylation, thereby overriding the inhibitory effects of shMeg3. Alternatively, or perhaps in addition to the previously stated explanation, massive overexpression of MEG3 could feasibly serve as a highly efficient decoy for shMeg3, thereby preventing endogenous Meg3 transcripts from being targeted, and resulting in Meg3 levels comparable to wild type myoblasts (the slight increase is not statistically significant).

Based on the above model, MEG3 overexpression in the control shLacZ background would be expected to either upregulate or maintain expression of the locus (and endogenous Meg3). In this instance, without the negative effects of shMeg3, we observed that MEG3 enhances transcription of myogenic differentiation differentiation markers (Fig. 2G). We have shown that endogenous Meg3 are downregulated during myogenic differentiation (Fig. 1C). Taken together, we speculate that enhanced myogenesis triggered by MEG3 overexpression in a relatively normal background actually overrides the aforementioned autoregulatory effects of MEG3 on the locus ultimately resulting in Meg3 downregulation. It is likely that myogenic differentiation in shMeg3 myoblasts, while restored to some extent by MEG3 overexpression, is not in an enhanced state and insufficient to downregulate endogenous Meg3.

Because our work does not delve into the complicated autoregulatory mechanisms of the Dlk1-Dio3 locus, we feel these speculations should be limited in the manuscript. Nevertheless, since it provides some pertinent information regarding the rescue we have added brief text in the results section acknowledging the complex regulatory mechanisms.

5. Did the authors investigate expression of other RNAs associated with the Meg3 polycistronic transcript? This could help their argument that the phenotype in shMeg3 cells is specific to reduction of Meg3. It is also important because the authors use the rationale the Dlk1-Dio locus expression levels are associated with mitochondrial activity.

This is a good suggestion. We examined expression of other Dlk1-Dio3 locus RNAs in both shMeg3 cells and regenerating TA muscle, and found variable effects on other locus RNAs depending on the context. We observed downregulation of the lncRNAs Rian and Mirg in stable shMeg3 clones (Sup. Fig. 2A), but not in regenerating shMeg3 TA muscle (Sup. Fig. 8B). Interestingly, the microRNAs miR-410 and miR-495 were not affected in C2C12 cells (we did not examine expression of these miRNAs in TA muscle). It is unclear why the miRNAs were not affected, and may suggest complex

transcriptional / post-transcriptional processing of the polycistron. We now include these expression data in Supplemental Figure 2A.

The shMeg3 used in this manuscript has been shown previously to be specific for Meg3 (Mondal et al 2015), and our TA data is consistent with this specificity. Considering our C2C12 experiments utilized a stable knockdown approach, it is possible that chronic overexpression of shMeg3 could also target Meg3 sequence in the precursor transcript resulting in degradation of the entire precursor transcript in addition to mature Meg3 lncRNA. This prolonged shRNA targeting likely results in the downregulation of other RNAs in the locus but the rescue with MEG3. Alternatively, since Meg3 is required to maintain transcription of the locus as explained in our response to comment #4 its inhibition could result in downregulation of locus expression. Regardless of the putative mechanisms, the rescue experiments demonstrate that the phenotype is largely attributable to Meg3 knockdown, but we cannot rule out some minor effects due to the downregulation of these other locus RNAs.

6. Scale bars on all images would be optimal. Currently there is only '20x' listed.

We thank this reviewer for pointing out the lack of scale bars on some images. We have now included scale bars in all images.

7. Often a one-way ANOVA should be used (such as Fig. 2D) instead of a student's t-test when there are comparisons between more than two groups.

This is a valid point. Our rationale for using student's t-test instead of ANOVA was to perform a statistical pair-wise analyses, comparing each treatment group to the control group (often shLacZ). To evaluate for whether treatments significantly improved the shMeg3 phenotype, we also occasionally perform pairwise comparison between shMeg3 control vs shMeg3 treated. Nevertheless, a comparison between more than two groups is also a logical and important analysis to determine whether all samples in a set are equivalent. To this end, we performed ANOVA for samples in which there were more than one treatment group to initially confirm the presence of differences between samples. Subsequently, for samples with significant ANOVA p-value, we performed students T-test pairwise comparisons post-hoc to evaluate for differences between control and treatments.

Moreover, we have removed pairwise comparisons between two treatment groups (i.e. comparisons between green and purple bars), which distracted from the experimental objective of assessing for treatment-induced changes relative to untreated controls. We hope these revisions add clarity and focus to the manuscript.

Reviewer 2 - We thank this reviewer for pointing out the importance of defining the role of Meg3 in muscle and the strengths of using in vitro and in vivo data. This reviewer expressed some concerns regarding the number of processes affected in Meg3 mutants and the mechanism of Meg3 function. We have now addressed these concerns with additional experiments.

1. The role of Meg3 specifically in muscles is not especially clear. If shRNA has an overall negative impact upon cell viability, then we would see many of same results. What makes the role of Meg3 in muscle more unique to that in other tissues?

We acknowledge that some of our results pertaining to Meg3 in muscle parallel known functions of this lncRNA in other cellular contexts, such as suppressing EMT in cancer and facilitating neural progenitor cell differentiation. However, it is important to note that these analogous functions - particularly EMT - were not previously known to be regulated by Meg3 in muscle, and were identified de novo through unbiased transcriptomic and bioinformatic analyses (Figure 4). Along these lines, we show that Meg3 is a critical epigenetic regulator of cell plasticity through its regulation of EMT and PRC2 recruitment in muscle. Moreover, while EMT is known to regulate cancer metastasis, EMT is not well-known to influence myogenic differentiation. Given the importance of maintaining proper cell state for differentiation, we demonstrate a novel role for Meg3 in modulating myogenic differentiation via EMT pathways components. Finally, reduced viability in Meg3 knockdown was an unexpected (and distinct) result when compared with existing

literature, as Meg3 generally promotes apoptosis in proliferating non-muscle cells such as cancer cells, and likely points to a muscle-specific effect of this lncRNA in muscle progenitor cells.

2. Reader is left with a lack of understanding of the mechanisms used by Meg3 to regulate gene expression and therefore development. In Figure 1D, authors show interaction of Meg3 with repressor component Ezh2, and this suggests that overall Meg3 functions to repress gene expression (this experiment would benefit from a negative control with a different lncRNA). But this observation is not linked with other findings in the manuscript, and does not explain the large number of genes down regulated in the shRNA. Demonstration of Meg3 interacting with promoters of some genes studied in the later figures, such as TGFbeta genes, would provide better mechanistic understanding.

This is an excellent point. To improve the mechanistic scope of our manuscript, we have performed ChIP-qPCR to gain insight into Meg3-mediated muscle gene expression. Using 30 pooled samples of differentiated myotubes per group, we initially analyzed sonicates for aberrant Ezh2 occupancy. While we found that Ezh2-immunoprecipitated loci were generally low (Sup. Fig. 7B, EZH2 vs IgG mock), it was interesting to note that all detected instances of Ezh2 enrichment over mock were unique to shMeg3 immunoprecipitates, which coincides with reduced cytoplasmic export observed in shMeg3 myotubes (Sup. Fig. 7A). Since H3K27me3 is a functional readout of Ezh2 activity, we examined the same loci in H3K27me3 immunoprecipitates (Sup. Fig 7C). Notably, we found that a subset of loci TGFbeta signaling components exhibit reduced H3K27me3 enrichment (TGFB1, TGFB2, Smad2, Smad6), whereas H3K27me3 enrichment was detected in TGFbeta-binding proteins (LTBP2, LTBP4), as shown in new Figure 9A. Interestingly, we noticed that proliferating shMeg3 myoblasts displayed enriched H3K27me3, and was abolished with Unc1999, a chemical inhibitor of Ezh2 activity (new figure 9B). Finally, to demonstrate that Ezh2 contributes to the shMeg3 phenotype, we show shMeg3 myoblasts pre-treated with Ezh2 inhibitor differentiate with improved MYH4 expression and fusion index (new figure 9C). Taken together, these data provide mechanistic insights to the Meg3 knockdown phenotype.

3. In several places, changes in readouts for experiments are modest and suggest that other processes are also affected, but not investigated. In Figure 6A, LY treatment rescued Meg3 shRNA, but not all the way back to control levels. In Figure 7B, SB treatment improved some parameters but not others. These results suggest that Meg3 affects many different processes (supported by the transcriptome data), but there is not a complete understanding of what is happening. As a result, some parts of the paper are descriptive and not mechanistic.

We agree our results suggest that Meg3 regulates processes beyond TGFbeta signaling. Although this pathway was the most significantly dysregulated in the IPA, several other pathways were also significantly affected. Therefore, we examined three additional pathways that were significantly dysregulated in our transcriptomic and bioinformatic analyses: p53 (revised Figure 6A), Notch (Sup. Fig 5A), and Shh (Sup. Fig 5B). As shown in revised Figure 6, we inhibited p53 (it is upregulated in mutant cells) in shMeg3 myoblasts using the chemical inhibitor Pifithrin. This resulted in rescue of MYH4 expression, suggesting that it also plays a role in the phenotype – though it did not improve fusion index. In contrast, modulations by constitutively active Notch-ICD or Shh-N did not have a significant effect on the shMeg3 phenotype (see Supplemental Figure 5). Overall, these new data, along with the Ezh2-TGFbeta target genes ChIP experiments (see response to comment 2 and new Figure 9) reveal a more restricted regulatory role of Meg3 and provide a better mechanistic understanding of this lncRNA in muscle differentiation.

Other comments:

1. In Figure 2 where authors make stable lines expressing shRNA or MEG3, did the authors determine the relative copy numbers of each transfected gene to make sure that each sample could be compared?

This is a good point. Although we did not determine copy number of the plasmid, we quantified hMEG3 transgene expression for each sample with qPCR. For the stable lines, we focused on expression levels of the constructs by selecting samples exhibiting the most similar MEG3 transcript levels. It is worth noting that expression levels were slightly variable across all samples obtained but correlated with the extent of rescue.

2. Figure 5A right panel, the significance mark is not aligned properly.

We thank this reviewer for pointing out the misalignment. We have now revised Figure 5A accordingly.

3. Figure 6, the LY rescue experiments, do improvements result from improvements in viability, proliferation, or fusion, or combination of these things?

In new Sup. Fig. 6E, we show that cell viability did not improve with LY treatment, suggesting the cell death phenotype persists with Meg3 knockdown (it is important to note that viability significantly decreased in LY-treated shLacZ cells). Proliferation was examined by BrdU, and we show in Sup. Fig. 6D that LY treatment rescues the shMeg3 proliferation phenotype.

4. Figure 8B, add arrows to indicate scar tissue.

We have now added arrowheads to indicate scar tissue.

Reviewer 3 – We thank this reviewer for noting that our manuscript has interesting observations, but that it was premature to support our conclusions. In order to strengthen our model we have performed additional experiments that support the EMT phenotype and its importance in muscle differentiation and regeneration.

Major issues:

1. EMT-mediated muscle differentiation or regeneration has not been documented yet. The authors identified the up-regulation of EMT-related genes and an increase in cell migration after KO of Meg3. However, it is not so clear whether EMT is actively involved in muscle differentiation and regeneration. It is interesting finding that inhibition of TGFb, ROCK1/2, and p38 MAPK, which are downstream pathways of EMT, restores myogenic differentiation after Meg3 KD. However, it is well known that inhibition of these signaling generally affects myogenic differentiation. Therefore, it is essential to describe how EMT is crucial for muscle differentiation and regeneration.

The reviewer makes an important point regarding EMT and muscle differentiation. EMT is not generally associated with muscle differentiation (unlike embryonic myogenesis), and the roles of EMT markers in this process could be better articulated. Our observations, in addition to characterizing the role of Meg3 in skeletal muscle, add to the growing understanding of how EMT markers participate in myogenic differentiation. To better clarify the roles of EMT in muscle differentiation, we have revised the manuscript text to highlight the following points in the discussion, which we have summarized for the reviewer below:

Collectively, our data reinforces that EMT is crucial for muscle differentiation. As shown in Figure 5, stable shMeg3 myoblasts display cellular characteristics of EMT, namely enhanced mesenchymal-like migration behavior, and dysregulated epithelial and mesenchymal gene expression profiles suggesting a mesenchymal-like cell state. In addition, we show that Snai2, a major transcriptional regulator of EMT, represses epithelial genes in mesenchymal cells, but in the context of muscle, it has been shown that Snai2 occupies MyoD E-box targets. This has the overall effect of preventing premature differentiation in myoblasts. As we show in new Fig. 8, TGFβ-dependent Snai2 expression contributed to the fusion defect observed in shMeg3 cells. And this was further corroborated with Snai2 knockdown experiments. Moreover, our data demonstrates that Meg3 is required for Snai2 downregulation in proliferating myoblasts in vitro (revised Fig. 8A), and regenerating muscle (revised Fig. 12B-C).

The additional EMT genes analyzed in Meg3-deficiency such as vimentin and N-cadherin are known markers of mesenchymal cell properties. The EMT intermediate filament protein vimentin is associated with mesenchymal cell invasiveness, and in myogenesis appears to be repurposed for sarcomeric organization during muscle differentiation (serving as a transient placeholder for muscle-specific desmin). N-cadherin is also involved in mesenchymal cell invasiveness, and has temporally divergent roles in myogenic differentiation. In quiescent satellite cells, N-cadherin suppresses proliferation (a finding that parallels the reduced proliferation phenotype), whereas

during early differentiation, N-cadherin is an upstream activator of p38 and RhoA signaling, which (as the reviewer acknowledged) must be carefully regulated for myogenic differentiation. These observations suggest that regulation of EMT is important for proper muscle differentiation.

2. In Figure 8A, the reduction of Meg3 expression in TA muscle after shMeg3 injection is less than 30%. Therefore, it is not convincing that the effects seen in this Figure is due to down-regulation of Meg3 levels. Nevertheless, the reduction of cross-sectional area day 3, day 7, and day 14 after CTX injection are 50%, 20%, and 60%, respectively, in shMeg3 group. Therefore, please utilize an experimental protocol for which a significant reduction of Meg3 can be induced after KD in vivo. And then, please show the absolute number of the cross-sectional area of each muscle and the distribution of fiber sizes. In addition, please examine fibrosis detection by day 14 after CTx injection since PDGFRa+ cells were increased in shMeg3 group, and thus fibrosis may be increased in this group.

This is a valid point - because we examined Meg3 expression at 3 days post-injury and post-transduction, knockdown efficiency at the time of injection is masked by an abundance of Meg3 transcripts originating from the massive proliferation of untransduced muscle and non-muscle cells that occurs in the following days (refer to TA expression profiling Fig. 1B). Despite the low knockdown percentage on day 3, the phenotypic effects are likely due to downregulation of Meg3 for the following reasons. First, it is unlikely that the effects are due to injection or adenovirus because the shLacZ transduced animals displayed normal regeneration. Second, the shMeg3 is specific for Meg3 as confirmed by us (Sup. Fig 8A) and others (Mondal et al 2015). Third, and further reinforcement of a Meg3-specific effect, the dysregulated transcriptome and pathway analysis of regenerating shMeg3 muscle shows considerable overlap to that of stable shMeg3 cells which have robust knockdown of Meg3 (compare revised Fig. 4 to revised Fig. 11). A transgenic knockdown approach or genetic deletion experiments would not be possible within the time frame of revisions.

We thank this reviewer for suggesting cross-sectional area (CSA) of each muscle, and have performed this analysis and find the results entirely consistent with the global CSA results that revealed significantly reduced cross sectional area in shMeg3 muscle. We now include these data in Supplemental Figure 8B.

We agree with this reviewer that an increase in PDGFRa cells would result in increased fibrosis, and performed Sirius Red stain on regenerating shMeg3 TA to examine for fibrosis on Day 14, as suggested. We found a significant increase in fibrosis in these tissues, and these data are provided in new Figure 12E.

Minor issues:
1. Figure 1A should cite (Schaum et al. 2018).
We now include this citation.

2. In Figures 2 and 3, which differentiation date was used for immunostaining and other experiments? Please indicate the dates in the figure legends.
Throughout the manuscript, “myoblasts” refers to subconfluent/passaging C2C12 cells, and “myotubes” refers to day 3 differentiation. We have amended the legends accordingly.

3. Figure 2A showed the increased number of mononuclear myocytes while multinucleated myotubes are significantly reduced in Meg3 KD group, indicating that Meg3 KD blunts myogenic fusion. This observation is supported by RNA-seq data shown in Sup Figure 2 in which both Myomaker and Myomixer are down-regulated in Meg3 KD group.
While Myomaker and Myomixer trend downwards, abundance of these transcripts was not statistically significant in either in vitro or in vivo (p-value column, Sup Figure 2). We apologize for any confusion, and have modified the table to make statistically significant fusion transcripts stand out more by colorizing the bolded text (revised Sup. Fig. 3)

4. Figure 10D, the significant differences between each group are not convincing.
We agree that the differences in proportions of vimentin + cells were small, but statistically significant. This is attributed to the low variability within the treatment group. We have added an

additional panel that examines vimentin at day 14 +CTX, where we found increased populations of vimentin+ cells that were much more pronounced in shMeg3 muscle (revised Fig. 12D).

Second decision letter

MS ID#: DEVELOP/2020/194027

MS TITLE: The long noncoding RNA Meg3 regulates myoblast plasticity and muscle regeneration through epithelial-mesenchymal transition

AUTHORS: Tiffany L Dill, Alina Carroll, Amanda Pinheiro, Jiachen Gao, and Francisco J Naya
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This paper studies the role of the lncRNA Meg3 on myogenesis and tissue repair. They show that Meg3 is enriched in satellite cells and mesenchymal cells in skeletal muscle, and is regulated during differentiation and regeneration. The main claim is that Meg3 is important for EMT during differentiation and this is supported by the cell culture and RNA-seq data. They also perform in vivo experiments with shMeg3, and while these are less clean due to lack of definivite knowledge about which cell types are affected by the knockdown, the results suggest a similar effect on satellite cells during regeneration. This work defines the role of Meg3 during muscle differentiation but also highlights the important of EMT. Overall, the paper is well written and logically articulated.

Comments for the author

The authors have satisfied this reviewer.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addressed my concerns.

Comments for the author

The authors have satisfactorily addressed my concerns.

Reviewer 3

Advance summary and potential significance to field

This manuscript descrived Meg3 lncRNA which is involved in EMT and myogenic differentiation. The revised manuscript has been improved.

Comments for the author

The revised manuscript has been improved.