miR-206 enforces a slow muscle phenotype

Kristen K. Bjorkman, Martin G. Guess, Brooke C. Harrison, Michael M. Polmear, Angela K. Peter and Leslie A. Leinwand
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AUTHORS: Kristen K Bjorkman, Martin Guess, Brooke C Harrison, Michael M Polmear, Angela K Peter, and Leslie A. Leinwand
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. 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

In this study, Bjorkman (re-)investigated the expression of miR-206 in striated muscles and examined a potential function of miR-206 in enforcing a slow phenotype in muscle fibers. The authors confirm previous work from several other groups, already reporting preferential expression of miR-206 in slow myofibers under physiological and pathophysiological conditions but added some further details such as higher expression of miR-206 in female compared to male TA muscles. To understand whether miR-206 has an effect on myofiber type specification, the authors took advantage of a previously described miR-206 knockout model.
Reanalysis of this model reveled male-specific increase of soleus muscle mass and a moderate shift from type I (slow oxidative) to type IIa (fast oxidative) myofibers in male mice. Furthermore, the authors analyzed a potential function of miR-206 in the heart muscle and observed increased LV mass in male but not female miR-206 mutants compared to WT after isoproterenol induced hypertrophy, although miR-206 is expressed at very low levels in the heart. Interestingly, the differences in LV mass between mutants and WT were correlated with increased expression of (fast) MYH6. The authors conclude that miR-206 might be necessary to enforce the slow skeletal muscle phenotype and might play a role for sex-differences in striated muscles.

The data included in the manuscript conform previously published findings but also provide additional information that might be interesting to understand the role of miR-206 in skeletal muscle fiber type specification and MYH switching in the heart. Unfortunately, the study lacks any mechanistic part and the correlative observation are often incomplete. Additional evidence is needed to support the conclusions.

Comments for the author

The most important deficit is the lack of any mechanistic studies. Given the advanced state of the micro RNA field and research on myomirs, mechanistic studies are necessary to understand HOW miR-206 might enforce a slow muscle phenotype.

The potential sex-specific effects of miR-206 were not analyzed rigorously. The reduced expression of miR-206 expression in aromatase null females is interesting but it unclear whether this is simply due to a shift in fiber type composition in this model. The authors observed a male-specific increase in soleus muscle mass and changes in fiber type composition in male soleus muscles. Surprisingly, a fiber typing was not provided for female soleus muscles. Is there no change in fibers types in female soleus muscle? If yes, why? For analysis of isoproterenol effects on hearts of miR-206 mutants a more thorough analysis was done only for male mice. Even if there is no difference in LV mass between female isoproterenol-treated miR-206 mutants vs. WT, what happens to the expression of MHC6? Is the expression changed in females as well but without an impact on LV mass? If there is no change in MHC6 expression, why is that so? The reasons for the sex-specific differences remain in the dark.

It would be interesting to understand why miR-206 is upregulated 3 days after initiation of voluntary running.

The authors refer to previous findings describing that endurance exercise induces a shift towards a slow twitch phenotype. However, in the current study it remains unclear whether the increase of miR-206 expression is paralleled by an increase in slow fibers.

The correlation between miR-206 expression and the slow muscle phenotype should be investigated at all time points and alternative explanations for the observed changes should be considered. Why is there no increase of miR-206 observed 7 or 14 days after initiation of running? It is necessary to include TA muscles in the analysis (see Allen 2001).

In regenerating TA after BaCl2 treatment the authors observe a robust increase of miR-206 expression, which corroborates previous findings. A new aspect, is the strong increase in pri-mir-206 after 3 days, which might be interesting and should be confirmed by northern blot analysis.

Further investigations of this phenomenon are required. Is this expression peak related to acquisition of fiber type identity?

The mass of soleus muscles is increased after loss of miR-206 and the authors claim to detect increased CSA of fibers. However, the changes in CSA do not seem to be statistically significant. Data showing trends that are not significant should be deleted from the manuscript. (Holds also true for the trend in the reduction of MHC7 expression)

The authors observe moderate changes in fiber type composition in the soleus after deletion of miR-206. However, these data are not fully confirmed by the molecular analysis. Loss of miR-206 induces counterintuitive changes in expression of Mef2c and Six1, but no changes in structural genes (with the exception of MHC IIa and IIx) are detected that confirm changes in fiber type identity. A more thorough molecular analysis with appropriate statistics is needed. The authors announced a discussion about the counterintuitive changes in Mef2c and Six1 expression in miR-206 mutants but only wrote rather enigmatically "…that fast oxidative fibers (Types IIa and IIx) are characterized by a stronger mitochondrial and electron transport chain signature than Type I, which is consistent with our overall change in gene expression and fiber type profiles". Why is that consistent with the unexpected increase of "slow" Mef2c? I do not get the point. Is Mef2c expressed at higher levels in type IIx compared to type I fibers? Seems not very likely.

The authors discussed that the lack of fiber type changes in the published miR-206/miR-133b double knockout mice (Boettger et al) compared to the miR-206 mutants in this study might be caused by opposing roles of miR-206/miR-133b. This is a highly exciting possibility, which should be studied further. Would a depletion of miR-133b in miR-206 mutants (e.g. by gapmers or antagomirs) prevent the fiber type shift?

Analysis of a heart phenotype in the germline miR-206 mutants is problematic in view. miR-206 is expressed at much higher levels in skeletal compared to heart muscles, raising the possibility that potential effects on the heart are secondarily mediated by changes in the skeletal musculature. To rule out the possibility and ensure cell autonomous effects, it would be necessary to specifically inactivate miR-206 in the heart. Such an approach might also help to exclude developmental defects. Along the same line: MHC6 and MHC7 levels in the heart are mainly regulated by the thyroid hormone T3. Can the authors rule out any thyroid dysfunction?
Is the effect of T3 exacerbated in miR-206 mutants?

I do have problems to appreciate the expression of miR-206 in the heart. Williams et al. and other groups convincingly reported that mir-206 is not detected in the heart (see Williams et al, figure S3), which corresponds to data shown in this study (figure S4). A four-fold increase of virtually nothing still means that expression is very low. In my view an absolute quantification of miR-206 molecules per cardiomyocyte is necessary together with appropriate controls and a comparison to other non-muscle cells (e.g. brain, smooth muscle, etc.). It is very hard to understand how extremely low levels of miR-206 exert a biological function, in particular when a highly related miRNA (i.e. miR-1) is abundantly present. As mentioned above, the authors should also include female mice when studying the acclaimed function of miR-206 in the heart. Even if there is no increase on LV mass, it would be highly informative to learn whether the same (moderate) increase of MHC6 expression occurs. Further molecular data are essential to support the suggested changes in heart structure or function after loss of miR-206.

Reviewer 2

Advance summary and potential significance to field

miR-206 is a muscle specific miRNA that was implicated in skeletal muscle differentiation and is enriched in slow muscles. Its expression was shown to be induced in muscle pathologies including Duchene muscular dystrophy and amyotrophic lateral sclerosis. The present study is aimed to investigate whether miR-206 drives the slow muscle phenotype and to analyze miR-206 expression in physiologic and pathological conditions that promote a slow muscle phenotype. The major findings are; 1. miR-206 expression is correlated with muscle fiber type; it is low in fast (TA) and high in slow muscles (Sol). 2. Its levels are higher in female TA than in male TA muscles. Female TA

muscle was shown before to contain a greater fraction of slow muscles than male TA. 3. Endurance exercise increased the levels of miR-206 in TA muscles 4. Levels of miR-206 are upregulated in fast but not in slow muscles of mdx mice compared to WT mice. Its expression is also upregulated in injured muscles. miR-206 expression is transcriptionally regulated after injury (reporter luciferase assay). 5. Analysis of miR-206 KO mice revealed, a- the mass and the cross sectional area of Soleus muscle is increases, b- the proportion of slow fibers of the soleus decreases and that of fast fibers increases, c- expression of genes associated with fast oxidative phenotype increases. 6. In a model of pressure overload, the levels of miR-206 as well as those of slow β MyHC (mRNA) were increased. 7.
In miR-206 KO mice, different approaches indicated cardiac dilation and systolic dysfunction. Interestingly, expression of several cardiac genes associated with pathologies were not modified. The expression of fast cardiac α MyHC was slightly elevated and that of slow β MyHC was slightly reduced relative to wild type mice. Overall, results of this study indicate that miR-206 is involved in the shift towards slow fibers occurring in both physiological and pathological conditions. This study is interesting, yet it suffers from several major weaknesses which are detailed below.

Comments for the author

Major points:
1. The definition of Slow and Fast muscles relates to their metabolic states and not only to MyHC isoforms. Yet, in this study, the authors investigated only the expression of myosin isoforms and did not analyze muscle metabolism. Change of slow to fast myosin isoforms should be accompanied with a parallel change of oxidative to glycolytic metabolism.
Metabolic analysis will greatly improve this study.
2. Lack of focus: This study is widespread and therefore very descriptive and lacking deep insight into the mechanism of miR-206-mediated shift towards slow fiber type. The study investigates different phenomena such as the gender effect of miR-206, its expression in different disease (mdx) and injury models, and the analysis of the effect of miR-206 expression on both skeletal muscles and cardiac muscles. The reader would benefit from a more focused and deep study. For example, a deep study of the effect of miR-206 on skeletal muscles fiber-type in endurance exercise or in disease state would be more informative.
3. Most of the expression analysis is based on qPCR analysis. This is especially noticeable for the cardiac myosin type (Figs 6, 7) and for the genes of slow and fast skeletal muscles (Fig 5). Conclusions of slow vs.
fast fiber type should be made based on quantitative analysis of immunoblots and immunostaining of histological muscle sections.
4. In several cases, the results do not support the conclusions made by the authors. Does the amount of fast type IIX fibers indeed increase in soleus of miR-206 KO mice (figure 4)? The differences in this case are not statistically significant (Fig 4B). Also, expression levels of troponins and metabolic factors (Myoglobin and Serca2a) do not support the shift of slow soleus fibers toward fast oxidative fibers in miR-206 KO mice (figure 5).
Can we be certain that the shift of fiber-type indeed occurs?
Minor points:
1. How would one explain the results of Fig 1C? The authors should explain why the levels of miR-206 decrease back to basal after 7 and 14 days of running. Why levels of miR-206 in exercising muscles are similar to those of sedentary muscles? How can this be explained in light of the different fate of muscle fibers?
2. Figure 2E: What is the explanation for the increase in luciferase activity of miR-206 enhancer in 7 days in PBS injected muscles (Sham).
3. Figure 2: In both Mdx mouse model and BaCl2 injury, muscle undergoes massive regeneration. Previous studies showed that miR-206 is upregulated in satellite cells following muscle injury (Liu et al , 2012 J Clin Invest.;122(6):2054-65). The authors need to show that miR-206 expression occurs in newly formed slow myofibers, and not merely in activated satellite cells.
4. Figure 6: The increase in the expression of β MyHC in hearts of isoproterenol-injected mice is not significant. Therefore, this result is not in line with the authors statement: “We saw a significant dose responsive increase in β MyHC mRNA…” (page 13).

Reviewer 3

Advance summary and potential significance to field

In the manuscript the authors show that miR-206 is induced in conditions promoting a slow oxidative muscle phenotype and provide evidence for a role of miR-206 in driving a slow muscle phenotype. Consistently, the authors find a fiber type shift towards a faster muscle phenotype in soleus muscle of miR-206 knockout male mice. Furthermore, miR-206 knockout male mice develop a dilated cardiomyopathy phenotype, which the authors hypothesize is associated with a shift in MyHC expression towards a faster phenotype. Interestingly these changes were only observed in male mice, suggesting a role of miR-206 in sexual dimorphism. The results are interesting and significantly advances the field, also suggesting miR-206 as a potential therapeutic target.

Comments for the author

The manuscript is interesting and clearly written. However, before publication various issues need to be addressed as detailed below:

Major comments:

Page 9, line 11: It is mentioned that miR-206 levels were higher in female TA compared to male. Why was this checked only in TA muscle. What about SOL and GP?

Page 9, line 18: “There was no change in the ArKO GP and a downward trend of similar magnitude in the ArKO soleus”
Since miR-206 expression was not significantly reduced in SOL and variable, this comment should be avoided unless close to significance. Also, since the previous sentence also mentions the expression of other miRs, it is not clear to what there is a similar trend. It would be better to write: “miR-206 levels were not altered in ArKO GP and SOL”, or similar.

Page 10, line 8: “We observed this induction in human DMD vastus lateralis biopsies, a normally mixed fiber type thigh muscle”
To put more emphasis on the fact that miR-206 was induced both in mouse and human DMD, it make the sentence more clear, it be better to write “Consistently, miR-206 was induced in vastus lateralis from human DMD patients, a thigh muscle with mixed fiber type”, or similar.
In Figure 2B, since there is usually a lot of variability in RNA levels in human biopsies, please show each individual measurement on the graph rather than a bar graph.
Page 10, line 10: “Accordingly, we found a stepwise increase in miR-206 expressing during a two week regeneration time course in BaCl2-injured TA”
This statement is initially confusing, as one would expect an initial high miR-206 level, which should then decrease. It then makes sense when one reads the next sentence describing the induced pri-miR-206 level which gradually decreases. To avoid confusion, it would be better to avoid writing “accordingly” or start the sentence by describing the pri-miR-206 level.

In Figure 3 and 4, how do you explain the increased CSA in 206KO SOL, while there was a reduction in the bigger type I fibers and increase in the smaller type IIA and possibly IIX fibers? Please measure the fiber type-
specific fiber size by costainings for MHC isoforms and laminin to determine whether there is a change in fiber type specific CSA to explain this.
What was the age of the mice? In the methods it is written that mice between 4-7 months of age were used but this is a very large age span. Please write the age of the mice used for each experiment in all figures.

It is written that there is no difference in muscle weight in female 206KO muscle. However, from Figure S3B there seems to be a trend towards to an increased muscle weight of SOL. This is based on the weight of only 3 female 206KO mice, which is not sufficient. Please increase the number of mice.

Were the measurement of muscle weight, fiber size and fiber type distribution done on littermates? Since muscle growth depends on litter sizes and other environmental factors, it is important that these experiments are done on littermates. This applies to Figure 3, 4 and S3.

Page 11, line 3 from the bottom: “Type IIX fibers are rare in the predominantly slow soleus, but we observed a striking increase in the fraction of these fast fibers in 206KO mice. While this was not statistically significant due to inter-animal variability, it is noteworthy (Fig. 4B)”
From Figure 4B it is clear that there is a huge variability in type IIX proportion, so since this observation is not statistically significant less emphasis should be put on it unless the authors will be able to get more consistent results by including additional mice and littermate controls.

In the last line of Page 11 it is written that there was a modest change in β-MyHC mRNA. However, according to Figure 4C, β-MyHC levels were unaltered, so this is not correct.

Figure 6: The title “miR-206 is associated with pathologic increases in slow MyHC expression in the LV” is unclear and does not correspond to the content of the figure. From the data there is no demonstration that miR-206 is directly associated with the increase in slow MyHC expression.

In Figure 7, the number of mice in the groups for echocardiography is to low. There should be at least 8 mice per group. Why was anterior wall thickness rather than posterior wall thickness measured? Posterior wall thickness is a more commonly used measurement. Please write the age of the mice that were analyzed by echocardiography. Is it possible that the females may develop a phenotype at a later age?

To determine whether the hypothesis is correct that the cardiac phenotype of 206KO male mice is related to the shift towards the expression of the faster β-MyHC, it should be determined whether the α-MyHC/β-MyHC ratio is unaltered in female mice.
From Figure S5C the heart rate is only little over 400 bpm, which is rather low. Usually it should be > 550 bpm, suggesting that the mice may have gotten too much isofluorane. In the methods it is mentioned that the mice got 2% isofluoreance. This is fine for making the mice sleep, but the amount should subsequently be reduced during the measurements.

Minor comments:

Page 4, line 2: Please correct “consructs”

On page 9, line 13, the abbreviations GP and SOL are introduced. However, while GP is used throughout the manuscript, soleus is not abbreviated. If using the GP abbreviation, the SOL abbreviation should be used as well.

Page 11, line 13: “This effect appears specific to lack of miR-206 as we did not observed any compensatory change in the closely related miR-1”
Please write “…in the expression of the closely related miR-1”

Page 12, line 13: “Expression of the slow-associated calcium-handling factor Serca2a did not change and there was a modest but significant increase in the oxygen-binding protein myoglobin (Mb)”
Please correct to “… but there was a modest…”

Figure S1: The description of S1B and C is inverted. Pleae correct. It is not necessary to write “We measured miRNA levels by qPCR as in Fig. 1” for each subfigure.

Figure 5 legend: Please write “N was 5-6 male mice per group”.

Page 13, line 6: Please write “we saw no significant dysregulation of other myomiRs”

Figure S2 and legend: in Figure S2B, please centralize the asterisks and daggers over the bars. There doesn't seem to be a statistical comparison between D1 and D0, which seem to be statistically different. Please add this. In the legend to Figure S2B, rather than writing “double daggers”, please write the symbol as for **.

In the legend to Figure S2E, please modify “The consensus is above the table” to “the consensus sequence is shown above the table”. Also, what does “Figure 2-figure supplement 1) mean? Does his refer to Figure 2A?
It is written that all experiments were performed in triplicate but in the methods it is written that each experiment was repeated at least twice. Please mention this also in the legend. This also applies for other legends for cellular studies.

Figure S5 legend second last line: Confusing sentence. What is meant with “as in Fig. 1?. Please correct to ”mRNA expression levels for cardiac stress markers in males, as indicated”, or similar.
Page 14, line 11: “The same miR-206 KO mouse studied here”
It would be better to write “The miR-206 KO mouse model studied here was previously shown to...”, or similar.

In the Materials and Methods, essentially every sentence starts with “We”. The reviewer understands that nowadays active language is generally preferred, but starting every sentence with “We” seems excessive.

Finally, commas are missing in many sentences.

First revision

Author response to reviewers' comments

MS ID#: JOCES/2019/243162
MS TITLE: miR-206 Enforces a Slow Muscle Phenotype
AUTHORS: Kristen K Bjorkman, Martin Guess, Brooke C Harrison, Michael M Polmear, Angela K Peter, and Leslie A. Leinwand
ARTICLE TYPE: Research Article

Reviewer 1 Advance Summary and Potential Significance to Field:
In this study, Bjorkman (re-)investigated the expression of miR-206 in striated muscles and examined a potential function of miR-206 in enforcing a slow phenotype in muscle fibers. The authors confirm previous work from several other groups, already reporting preferential expression of miR-206 in slow myofibers under physiological and pathophysiological conditions but added some further details such as higher expression of miR-206 in female compared to male TA muscles. To understand whether miR-206 has an effect on myofiber type specification, the authors took advantage of a previously described miR- 206 knockout model. Reanalysis of this model reveled male-specific increase of soleus muscle mass and a moderate shift from type I (slow oxidative) to type IIa (fast oxidative) myofibers in male mice.
Furthermore, the authors analyzed a potential function of miR-206 in the heart muscle and observed increased LV mass in male but not female miR-206 mutants compared to WT after isoproterenol induced hypertrophy, although miR-206 is expressed at very low levels in the heart. Interestingly, the differences in LV mass between mutants and WT were correlated with increased expression of (fast) MYH6. The authors conclude that miR-206 might be necessary to enforce the slow skeletal muscle phenotype and might play a role for sex-differences in striated muscles.

The data included in the manuscript conform previously published findings but also provide additional information that might be interesting to understand the role of miR-206 in skeletal muscle fiber type specification and MYH switching in the heart. Unfortunately, the study lacks any mechanistic part and the correlative observation are often incomplete. Additional evidence is needed to support the conclusions.

Reviewer 1 Comments for the Author:
The most important deficit is the lack of any mechanistic studies. Given the advanced state of the micro RNA field and research on myomirs, mechanistic studies are necessary to understand HOW miR-206 might enforce a slow muscle phenotype.

We agree that this would add further insight, but at this time we are unable to add mechanistic detail. However, we have expanded the Discussion to include not only the important miR-206 target Hdac4, which inhibits slow muscle gene expression, but the recent findings that miR-206/miR-1 KO cells have impaired mitochondrial function and miR-206 KO mice have reduced endurance exercise performance (Przanowska et al., 2020).

The potential sex-specific effects of miR-206 were not analyzed rigorously. The reduced expression of miR-206 expression in aromatase null females is interesting but it unclear whether this is simply due to a shift in fiber type composition in this model. The authors observed a male-specific increase in soleus muscle mass and changes in fiber type composition in male soleus muscles. Surprisingly, a fiber typing was not provided for female soleus muscles. Is there no change in fibers types in female soleus muscle? If yes, why?
This is a good suggestion as one would hypothesize a shift towards a faster profile if the female ArKO TAs are more “male-like” given the reduced miR-206 reported in Fig. 1B. To address this with material in hand, we performed a qPCR-based fiber typing of female WT v ArKO TA and present this in the new

Fig. 1C. While Type I/ß amplified too late to be reliably interpreted in the TA (which is expected), we found a 4-fold reduction in IIa, a 1.7-fold reduction in IIx, and no change in IIb mRNA. This can be summarized as the slower the Type II myosin (IIa is slower than IId, both slower than IIb), the greater the decrease, which supports the hypothesis.

For analysis of isoproterenol effects on hearts of miR-206 mutants a more thorough analysis was done only for male mice. Even if there is no difference in LV mass between female isoproterenol-treated miR- 206 mutants vs. WT, what happens to the expression of MHC6? Is the expression changed in females as well but without an impact on LV mass? If there is no change in MHC6 expression, why is that so? The reasons for the sex-specific differences remain in the dark.
We would like to clarify that we treated WT animals with Iso, not 206KO mice. This experiment led us to then examine whether hearts from 206KO mice had phenotypes. We feel this was clearly stated in the Results (“we treated wild-type male mice with increasing doses of the ß-adrenergic receptor agonist isoproterenol”), but to avoid confusion over genotype, we restated this in the Discussion, as well.

It would be interesting to understand why miR-206 is upregulated 3 days after initiation of voluntary running. The authors refer to previous findings describing that endurance exercise induces a shift towards a slow twitch phenotype. However, in the current study it remains unclear whether the increase of miR- 206 expression is paralleled by an increase in slow fibers.

We agree that it would be interesting to look further into this finding. Unfortunately, we were limited by sample amount and unable to perform that analysis on these animals. Fortunately, numerous publications in multiple species including some in our lab, document endurance exercise shifting fast muscles to a slower profile as we have cited in this manuscript (Allen et al., 2001; Andersen and Henriksson, 1977). We suspect that the increase in miR-206 expression precedes the increase in slow fibers, but that would not be surprising as it is an upstream regulatory molecule. However, we are unable to test this at this time.

The correlation between miR-206 expression and the slow muscle phenotype should be investigated at all time points and alternative explanations for the observed changes should be considered. Why is there no increase of miR-206 observed 7 or 14 days after initiation of running? It is necessary to include TA muscles in the analysis (see Allen 2001).

We are uncertain what the reviewer would like examined here as we did analyze the TA as stated in the text (“we examined miR-206 expression in the TAs of male WT mice during adaptation to voluntary wheel running”).

In regenerating TA after BaCl2 treatment the authors observe a robust increase of miR-206 expression, which corroborates previous findings. A new aspect, is the strong increase in pri-mir-206 after 3 days, which might be interesting and should be confirmed by northern blot analysis. Further investigations of this phenomenon are required. Is this expression peak related to

acquisition of fiber type identity?

We do not believe that a northern would improve this. We designed primers so that only the primary miRNA would be amplified (they recognize a sequence that is not present in either the pre-miRNA or the mature miRNA and the sequences are given in the Supplemental Table), and we DNase-treated the RNA to ensure genomic DNA would not be amplified.

This is the TA and so miR-206 is lower there compared to slow muscles. In addition, that early time would be when fiber type identity is lost due to damage and regeneration. To reinforce the point that miR- 206 is present when slow myosin is present, we did qPCR analysis of embryonic (a very slow myosin) and of IIx (a fast myosin). ANOVA for both shows that time post injury is a significant variable. As expected, IIx initially decreases 3-fold at D3, then increases 1.8-fold at D7, and then returns to baseline levels by D14. This would be consistent with an initial loss of fiber type identity in the injury phase with a re-establishment during the regeneration phase. Embryonic is acutely up-regulated 214-fold at D7, which is after the pri-miR-206 peak. We now present these data in Fig. S2.

The mass of soleus muscles is increased after loss of miR-206 and the authors claim to detect increased CSA of fibers. However, the changes in CSA do not seem to be statistically significant. Data showing trends that are not significant should be deleted from the manuscript. (Holds also true for the trend in the reduction of MHC7 expression)

We agree that the CSA analysis could be improved. Based on this Reviewer's concern and on a suggestion from Reviewer 3, we analyzed CSA segregated by fiber type for a finer resolution characterization. We measured fiber type-specific CSA across entire soleus cross-sections from 7 WT and 4 206KO mice (4,924 total fibers) and calculated the proportion of myofibers in 200 μm^2 bins. Heatmaps display these data in the new Fig. 3C. ANOVA indicated a significant effect from CSA bin. We observed that 206KO Type I fibers are smaller while 206KO Type IIA fibers have a broader size distribution when compared to WT. While IIX are a rarer fiber type in the SOL, there is a significant shift towards larger fibers in 206KO mice. In all fiber types, the largest size bins are only populated in the 206KO genotype.

The authors observe moderate changes in fiber type composition in the soleus after deletion of miR-206. However, these data are not fully confirmed by the molecular analysis. Loss of miR-206 induces counterintuitive changes in expression of Mef2c and Six1, but no changes in structural genes (with the exception of MHC IIa and IIx) are detected that confirm changes in fiber type identity. A more thorough molecular analysis with appropriate statistics is needed. The authors announced a discussion about the counterintuitive changes in Mef2c and Six1 expression in miR-206 mutants but only wrote rather enigmatically "…that fast oxidative fibers (Types IIa and IIx) are characterized by a stronger mitochondrial and electron transport chain signature than Type I, which is consistent with our overall change in gene expression and fiber type profiles". Why is that consistent with the unexpected increase of "slow" Mef2c? I do not get the point. Is Mef2c expressed at higher levels in type IIx compared to type I fibers? Seems not very likely.

The change in Mef2c is unexpected, but it is possible that it is higher in IIX compared to I fibers. Even if it is unlikely, if we do not know it to be the case, we cannot rule it out. The "exceptions" of IIa and IIx are important. In addition, Eya1 and linc-MYH increased in a manner consistent with fiber type switching.
We are unclear about the comment about appropriate statistics since we did apply appropriate statistics to these analyses as well.

The authors discussed that the lack of fiber type changes in the published miR-206/miR-133b double knockout mice (Boettger et al) compared to the miR-206 mutants in this study might be caused by opposing roles of miR-206/miR-133b. This is a highly exciting possibility, which should be studied further. Would a depletion of miR-133b in miR-206 mutants (e.g. by gapmers or antagomirs) prevent the fiber type shift?

We agree that that would be an exciting possibility but this is clearly beyond the scope of the current manuscript and current research environment.

Analysis of a heart phenotype in the germline miR-206 mutants is problematic in view. miR-206 is expressed at much higher levels in skeletal compared to heart muscles, raising the possibility that potential effects on the heart are secondarily mediated by changes in the skeletal musculature. To rule out the possibility and ensure cell autonomous effects, it would be necessary to specifically inactivate miR- 206 in the heart. Such an approach might also help to exclude developmental defects. Along the same line: MHC6 and MHC7 levels in the heart are mainly regulated by the thyroid hormone T3. Can the authors rule out any thyroid dysfunction? Is the effect of T3 exacerbated in miR-206 mutants?

While a conditional cardiac 206KO animal as well as a thyroid hormone functional analysis would be very interesting, they are beyond the scope of the present study.

I do have problems to appreciate the expression of miR-206 in the heart. Williams et al. and other groups convincingly reported that mir-206 is not detected in the heart (see Williams et al, figure S3), which corresponds to data shown in this study (figure S4). A four-fold increase of virtually nothing still means that expression is very low. In my view an absolute quantification of miR-206 molecules per cardiomyocyte is necessary together with appropriate controls and a comparison to other non-muscle cells (e.g. brain, smooth muscle, etc.). It is very hard to understand how extremely low levels of miR-206 exert a biological function, in particular when a highly related miRNA (i.e. miR-1) is abundantly present. As mentioned above, the authors should also include female mice when studying the acclaimed function of miR-206 in the heart. Even if there is no increase on LV mass, it would be highly informative to learn whether the same (moderate) increase of MHC6 expression occurs. Further molecular data are essential to support the suggested changes in heart structure or function after loss of miR-206.

Low expression levels cannot automatically preclude biological relevance. In addition, although miR-1 is a related family member and they do share many targets, there are instances of distinct targets as well.
This has also been observed for other myomiR families such as miR-208/499. It is possible that the set of cardiac miR-206 targets is smaller than in slow skeletal muscle and/or those targets' expression levels are themselves low and so do not require abundant levels of miR-206 to exert an inhibitory effect. These are hypotheses that remain to be tested but should not be ruled out. At this time, we are not able to quantify miR-206 molecules per individual cardiomyocyte, but we have measured miR-206 in isolated neonatal rat cardiomyocytes (NRVMs), adult rat cardiomyocytes (ARVMs), and adult rat ventricular fibroblasts (ARVFs). We found 5-fold higher miR-206 expression in ARVMs vs NRVMs and 15-fold higher miR- 206 expression in NRVMs vs NRVFs. Another publication (already cited in this manuscript; Yang et al. Circulation Research. 2015;117:891–904) has also shown miR-206 expression in NRVMs by northern blot.

We agree that a myosin expression analysis for female 206KO mice is important and we measured it in response to this review. We found that neither alpha nor beta levels change in the female LVs and now present this in the new Fig S6C.

Reviewer 2 Advance Summary and Potential Significance to Field:
miR-206 is a muscle specific miRNA that was implicated in skeletal muscle differentiation and is enriched in slow muscles. Its expression was shown to be induced in muscle pathologies including Duchene muscular dystrophy and amyotrophic lateral sclerosis. The present study is aimed to investigate whether miR-206 drives the slow muscle phenotype and to analyze miR-206 expression in physiologic and pathological conditions that promote a slow muscle phenotype. The major findings are; 1. miR-206 expression is correlated with muscle fiber type; it is low in fast (TA) and high in slow muscles (Sol). 2. Its levels are higher in female TA than in male TA muscles. Female TA muscle was shown before to contain a greater fraction of slow muscles than male TA. 3. Endurance exercise increased the levels of miR-206 in TA muscles 4. Levels of miR-206 are upregulated in fast but not in slow muscles of mdx mice compared to WT mice. Its expression is also upregulated in injured muscles. miR-206 expression is transcriptionally regulated after injury (reporter luciferase assay). 5. Analysis of miR-206 KO mice revealed, a- the mass and the cross sectional area of Soleus muscle is increases, b- the proportion of slow fibers of the soleus decreases and that of fast fibers increases, c- expression of genes associated with fast oxidative

phenotype increases. 6. In a model of pressure overload, the levels of miR-206 as well as those of slow ß MyHC (mRNA) were increased. 7. In miR-206 KO mice, different approaches indicated cardiac dilation and systolic dysfunction. Interestingly, expression of several cardiac genes associated with pathologies were not modified. The expression of fast cardiac α MyHC was slightly elevated and that of slow ß MyHC was slightly reduced relative to wild type mice. Overall, results of this study indicate that miR-206 is involved in the shift towards slow fibers occurring in both physiological and pathological conditions. This study is interesting, yet it suffers from several major weaknesses which are detailed below.

Reviewer 2 Comments for the Author: Major points:
(i) The definition of Slow and Fast muscles relates to their metabolic states and not only to MyHC isoforms. Yet, in this study, the authors investigated only the expression of myosin isoforms and did not analyze muscle metabolism. Change of slow to fast myosin isoforms should be accompanied with a parallel change of oxidative to glycolytic metabolism. Metabolic analysis will greatly improve this study.

While a metabolic study would be interesting, it is beyond the scope of this study and not currently feasible. We did not have enough samples to measure expression levels of additional metabolic genes. However, while this manuscript was under review, a paper was published that found miR-206/miR-1 KO cells have impaired mitochondrial function and miR-206 KO mice have reduced endurance exercise performance (Przanowska et al., 2020). Unfortunately, they did not examine slow muscles from their animals, but it would be an interesting future direction. We now cite this work in support of our own.

(ii) Lack of focus: This study is widespread and therefore very descriptive and lacking deep insight into the mechanism of miR-206-mediated shift towards slow fiber type. The study investigates different phenomena such as the gender effect of miR-206, its expression in different disease (mdx) and injury models, and the analysis of the effect of miR-206 expression on both skeletal muscles and cardiac muscles. The reader would benefit from a more focused and deep study. For example, a deep study of the effect of miR-206 on skeletal muscles fiber-type in endurance exercise or in disease state would be more informative.

We agree that the study is wide-ranging, but we think it is informative in its breadth. The studies suggested are interesting but would represent distinct directions.

(iii) Most of the expression analysis is based on qPCR analysis. This is especially noticeable for the cardiac myosin type (Figs 6, 7) and for the genes of slow and fast skeletal muscles (Fig 5). Conclusions of slow vs. fast fiber type should be made based on quantitative analysis of immunoblots and immunostaining of histological muscle sections.

Chemello et al. have shown that myosin composition at the RNA level is highly predictive of fiber type based on RNAseq of single myofibers of known composition (Chemello et al., Cell Rep. 2019;26, 3784– 3797.). We are uncertain why the reviewer suggests additional fiber typing based on immunostaining as that was already presented in the manuscript (now Fig. 3D, E with a representative image in Fig. 3B with the updated figure numbering).

(iv) In several cases, the results do not support the conclusions made by the authors. Does the amount of fast type IIX fibers indeed increase in soleus of miR-206 KO mice (figure 4)? The differences in this case are not statistically significant (Fig 4B). Also, expression levels of troponins and metabolic factors (Myoglobin and Serca2a) do not support the shift of slow soleus fibers toward fast oxidative fibers in miR-206 KO mice (figure 5). Can we be certain that the shift of fiber-type indeed occurs?

Although the phenotype is complicated, we do believe there is convincing evidence of fiber type shifting. This is supported by immunostaining-based fiber typing and myosin expression analysis (current Fig. 3) as well as increases in Six1, Eya1, and linc-MYH (Fig. 4). Fast oxidative fibers have a higher metabolic demand and so could be supported by increased myoglobin and Mef2c expression, although that does remain speculative at this point. We have expanded the Discussion to also include a study published during this review period that supports a fiber type shift by

looking at mitochondrial function in knockout cells as well as exercise performance in knockout mice: “In recently published work, a mouse in which miR-206 and both miR-1a loci were knocked out resulted in partial embryonic lethality, but miR-206 single KO mice and two surviving triple KO (tKO) individuals exhibited decreased physical performance, which was supported by impaired mitochondrial function in a tKO cell culture model (Przanowska et al., 2020). Although slow skeletal muscles were not examined in that study, it would be interesting to perform a metabolic analysis of slow oxidative muscles in single, double, and triple miR-1/206 family KO mice.” (Przanowska et al., FASEB J., 2020;00, 1–16).

Minor points:

1. How would one explain the results of Fig 1C? The authors should explain why the levels of miR-206 decrease back to basal after 7 and 14 days of running. Why levels of miR-206 in exercising muscles are similar to those of sedentary muscles? How can this be explained in light of the different fate of muscle fibers?

We agree that it would be interesting to look further into this finding. Unfortunately, we were limited by sample amount and are unable to perform additional analyses of these animals. We suspect that the increase in miR-206 expression precedes the increase in slow fibers, but that would not be surprising as it is an upstream regulatory molecule. It is also possible that the early increase followed by a return to basal levels is consistent with modest exercise-induced damage in the acute phase. As we know that miR-206 levels increase with muscle damage, our data would also be consistent with that hypothesis. We cannot be certain at this time whether there was evidence of damage at that stage although this was a voluntary running paradigm and not a forced exercise paradigm.

2. Figure 2E: What is the explanation for the increase in luciferase activity of miR-206 enhancer in 7 days in PBS injected muscles (Sham).

This is not statistically significant and was the result of inter-animal variability. We now present individual data points in Fig. 2E. The only statistically significant change occurs at D3 in the injured miR- 206 enhancer-injected animals.

3. Figure 2: In both Mdx mouse model and BaCl2 injury, muscle undergoes massive regeneration. Previous studies showed that miR-206 is upregulated in satellite cells following muscle injury (Liu et al , 2012 J Clin Invest.;122(6):2054-65). The authors need to show that miR-206 expression occurs in newly formed slow myofibers, and not merely in activated satellite cells.

That is an important study that we do cite. However, that study actually does not show that miR-206 increases upon SC activation. They isolate SCs that were already activated and then show that 206 increases upon differentiation of those cells in culture. This is consistent with the notion of miR-206 expression occurring in newly formed myofibers.

4. Figure 6: The increase in the expression of ß MyHC in hearts of isoproterenol-injected mice is not significant. Therefore, this result is not in line with the authors statement: “We saw a significant dose responsive increase in ß MyHC mRNA…” (page 13).

The dose-dependent increase in beta is significant by ANOVA (text: “We saw a significant dose-responsive increase in b-MyHC mRNA”. Legend: “ANOVA indicated significant treatment effects for both ß-MyHC and miR-206. Asterisks indicate post-test results. * = $p \leq 0.05$ vs. V.”). Individual post- tests of pairs were not significant for beta. To make this clearer, we added symbols to all figures with significance from ANOVA tests in addition to the text that was in the legend. We hope this clarifies these results.

Reviewer 3 Advance Summary and Potential Significance to Field:
In the manuscript the authors show that miR-206 is induced in conditions promoting a slow oxidative muscle phenotype and provide evidence for a role of miR-206 in driving a slow muscle phenotype.

Consistently, the authors find a fiber type shift towards a faster muscle phenotype in soleus muscle of miR-206 knockout male mice. Furthermore, miR-206 knockout male mice develop a dilated cardiomyopathy phenotype, which the authors hypothesize is associated with a shift in MyHC expression towards a faster phenotype. Interestingly, these changes were only observed in male mice, suggesting a role of miR-206 in sexual dimorphism. The results are interesting and significantly advances the field, also suggesting miR-206 as a potential therapeutic target.

Reviewer 3 Comments for the Author:
The manuscript is interesting and clearly written. However, before publication various issues need to be addressed as detailed below:

Major comments:

Page 9, line 11: It is mentioned that miR-206 levels were higher in female TA compared to male. Why was this checked only in TA muscle. What about SOL and GP?

We did check SOL and GP and did not find significant expression level differences between the sexes. We apologize for not having made that clear and now include a more explicit statement to that effect ("miR-206 expression was not sexually dimorphic in either the GP or SOL.")

Page 9, line 18: "There was no change in the ArKO GP and a downward trend of similar magnitude in the ArKO soleus"
Since miR-206 expression was not significantly reduced in SOL and variable, this comment should be avoided unless close to significance. Also, since the previous sentence also mentions the expression of other miRs, it is not clear to what there is a similar trend. It would be better to write: "miR-206 levels were not altered in ArKO GP and SOL", or similar.

We have amended the wording as suggested.

Page 10, line 8: "We observed this induction in human DMD vastus lateralis biopsies, a normally mixed fiber type thigh muscle"
To put more emphasis on the fact that miR-206 was induced both in mouse and human DMD, it make the sentence more clear, it be better to write "Consistently, miR-206 was induced in vastus lateralis from human DMD patients, a thigh muscle with mixed fiber type", or similar.

Thank you. We have corrected as suggested.

In Figure 2B, since there is usually a lot of variability in RNA levels in human biopsies, please show each individual measurement on the graph rather than a bar graph.

We have made this change as reflected in the new Fig. 2B.

Page 10, line 10: "Accordingly, we found a stepwise increase in miR-206 expressing during a two week regeneration time course in BaCl2-injured TA"
This statement is initially confusing, as one would expect an initial high miR-206 level, which should then decrease. It then makes sense when one reads the next sentence describing the induced pri-miR-206 level, which gradually decreases. To avoid confusion, it would be better to avoid writing "accordingly" or start the sentence by describing the pri-miR-206 level.

Thank you. We have corrected as suggested.

In Figure 3 and 4, how do you explain the increased CSA in 206KO SOL, while there was a reduction in the bigger type I fibers and increase in the smaller type IIA and possibly IIX fibers? Please measure the fiber type-specific fiber size by costainings for MHC isoforms and laminin to determine whether there is a change in fiber type specific CSA to explain this.

This is a very good suggestion and we agree that we could have done a more refined CSA analysis. Therefore, we measured fiber type-specific CSA across entire soleus cross-sections from 7 WT and

4 206KO mice (4,924 total fibers) and calculated the proportion of myofibers in 200 μm^2 bins. Heatmaps display these data in the new Fig. 3C. ANOVA indicated a significant effect from CSA bin.We observed that 206KO Type I fibers tend to be smaller while 206KO Type IIA fibers have a broader size distribution when compared to WT. While IIX are a rarer fiber type in the SOL, there is a noticeable shift towards larger fibers in 206KO mice. In all fiber types, the largest size bins are only populated in the 206KO genotype. While we cannot rule out the possibility of increased total fiber number to account for the greater normalized soleus mass in 206KO males, increased fiber size, particularly in faster IIA and IIX fiber types, could be a contributing factor.

What was the age of the mice? In the methods it is written that mice between 4-7 months of age were used, but this is a very large age span. Please write the age of the mice used for each experiment in all figures.

We have now clarified this. WT, mdx, and ArKO mice were 4 months because of phenotype-specific considerations (hormone levels, disease status) and the 206KO mice were 6-7 months old. The original publications with these mice from the Olson Lab examined them primarily at a younger age, so it could suggest that the phenotype is progressive as he did not see anything of note at baseline in the 206KOs. We now mention that as well.

It is written that there is no difference in muscle weight in female 206KO muscle. However, from Figure S3B there seems to be a trend towards to an increased muscle weight of SOL. This is based on the weight of only 3 female 206KO mice, which is not sufficient. Please increase the number of mice.

Unfortunately, this was the number of females available and we are unable to add further animal experiments at this time.

Were the measurement of muscle weight, fiber size and fiber type distribution done on littermates? Since muscle growth depends on litter sizes and other environmental factors, it is important that these experiments are done on littermates. This applies to Figure 3, 4 and S3.

Yes, we always used littermates from multiple litters to achieve the n. This is now more explicitly stated in the Methods.

Page 11, line 3 from the bottom: “Type IIX fibers are rare in the predominantly slow soleus, but we observed a striking increase in the fraction of these fast fibers in 206KO mice. While this was not statistically significant due to inter-animal variability, it is noteworthy (Fig. 4B)”
From Figure 4B it is clear that there is a huge variability in type IIX proportion, so since this observation is not statistically significant less emphasis should be put on it unless the authors will be able to get more consistent results by including additional mice and littermate controls.

We did use littermates. Unfortunately, we cannot add additional animal experiments at this time. However, we do not believe that it is overly emphasized; it is noteworthy, but it is acknowledged that it is not significant.

In the last line of Page 11 it is written that there was a modest change in ß-MyHC mRNA. However, according to Figure 4C, ß-MyHC levels were unaltered, so this is not correct.

We meant that the changes were not significant, but the wording was clearly not the right choice. Thank you for pointing this out; we have amended the text.

Figure 6: The title “miR-206 is associated with pathologic increases in slow MyHC expression in the LV” is unclear and does not correspond to the content of the figure. From the data there is no demonstration that miR-206 is directly associated with the increase in slow MyHC expression.

(With our new figure numbering, this is now Fig. 5). We have changed the figure title to read “miR-206 is associated with pathologic increases in slow MyHC expression in the LV.”

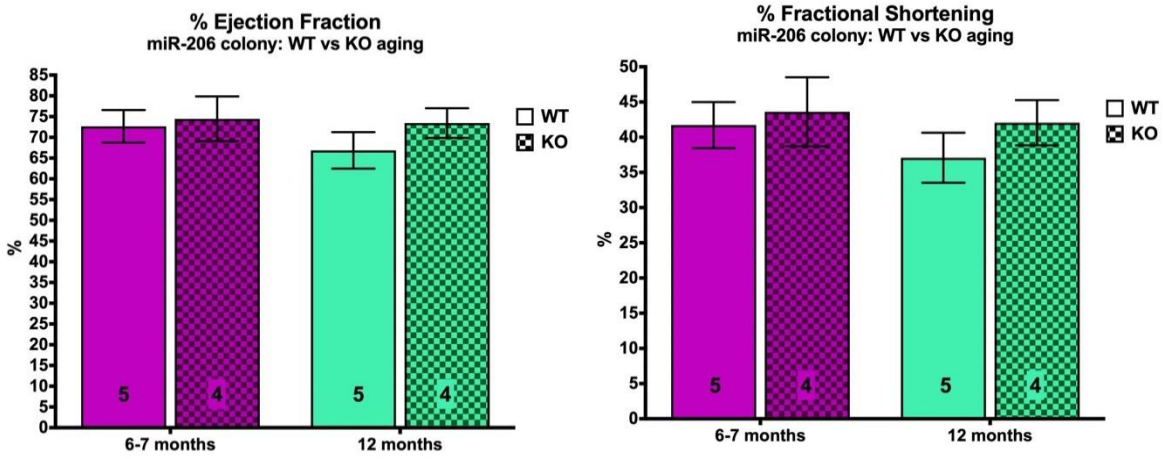
In Figure 7, the number of mice in the groups for echocardiography is to low. There should be at

least 8 mice per group. Why was anterior wall thickness rather than posterior wall thickness measured? Posterior wall thickness is a more commonly used measurement. Please write the age of the mice that were analyzed by echocardiography. Is it possible that the females may develop a phenotype at a later age?

We do not believe an n of 8 would change our findings. All of the parameters are statistically significant and consistent with each other.

While we do not believe that posterior wall is a more commonly used measurement than anterior wall, we have added those data for both male (Fig. 6B) and female (Fig. S6D). We did not observe a statistically significant change in this parameter in either sex.

We apologize for not having been clearer about the ages of the 206KO mice and have now clarified that. They were 6-7 months old. We do also have some data from 12 month-old animals. There are no significant differences at that age for the females either (please see ejection fraction and fractional shortening data below; there were no differences in any of the M-mode measurements).



To determine whether the hypothesis is correct that the cardiac phenotype of 206KO male mice is related to the shift towards the expression of the faster α-MyHC, it should be determined whether the α-MyHC/ß- MyHC ratio is unaltered in female mice.

We agree that a myosin expression analysis for female 206KO mice is important. We found that neither alpha nor beta levels change in the female LVs and now present this in the new Fig S6C.

From Figure S5C the heart rate is only little over 400 bpm, which is rather low. Usually it should be > 550 bpm, suggesting that the mice may have gotten too much isofluorane. In the methods it is mentioned that the mice got 2% isofluoreance. This is fine for making the mice sleep, but the amount should subsequently be reduced during the measurements.

This is standard procedure for our echocardiography facility. We always keep a consistent plane of anesthesia for all genotypes.

Minor comments:

Page 4, line 2: Please correct “consructs” Thank you. We have corrected as suggested.
On page 9, line 13, the abbreviations GP and SOL are introduced. However, while GP is used throughout the manuscript, soleus is not abbreviated. If using the GP abbreviation, the SOL abbreviation should be used as well.

Thank you. We have corrected as suggested.

Page 11, line 13: “This effect appears specific to lack of miR-206 as we did not observed any compensatory change in the closely related miR-1”
Please write “…in the expression of the closely related miR-1”

Thank you. We have corrected as suggested.

Page 12, line 13: “Expression of the slow-associated calcium-handling factor Serca2a did not change and there was a modest but significant increase in the oxygen-binding protein myoglobin (Mb)”
Please correct to “… but there was a modest…”

Thank you. We have corrected as suggested.

Figure S1: The description of S1B and C is inverted. Pleae correct. It is not necessary to write “We measured miRNA levels by qPCR as in Fig. 1” for each subfigure.

Thank you. We have corrected as suggested.

Figure 5 legend: Please write “N was 5-6 male mice per group”.

Thank you. We have corrected as suggested.

Page 13, line 6: Please write “we saw no significant dysregulation of other myomiRs”

Thank you. We have corrected as suggested.

Figure S2 and legend: in Figure S2B, please centralize the asterisks and daggers over the bars. There doesn't seem to be a statistical comparison between D1 and D0, which seem to be statistically different. Please add this. In the legend to Figure S2B, rather than writing “double daggers”, please write the symbol as for **.

Thank you. We have corrected as suggested.

In the legend to Figure S2E, please modify “The consensus is above the table” to “the consensus sequence is shown above the table”. Also, what does “Figure 2-figure supplement 1) mean? Does his refer to Figure 2A?

Thank you. We have corrected as suggested. We have also corrected the wording as that was a formatting error. Thank you for bringing it to our attention.

It is written that all experiments were performed in triplicate but in the methods it is written that each experiment was repeated at least twice. Please mention this also in the legend. This also applies for other legends for cellular studies.

The triplicates refer to independent wells for each thaw of cells. The “at least twice” refers to how many fresh thaws of cells were tested. In this way we ensured as much biological diversity as possible with immortalized cell lines.

Figure S5 legend second last line: Confusing sentence. What is meant with “as in Fig. 1?. Please correct to ”mRNA expression levels for cardiac stress markers in males, as indicated”, or similar.

Thank you. We have corrected as suggested.

Page 14, line 11: “The same miR-206 KO mouse studied here”
It would be better to write “The miR-206 KO mouse model studied here was previously shown to...”, or similar.

Thank you. We have corrected as suggested.

In the Materials and Methods, essentially every sentence starts with “We”. The reviewer understands that nowadays active language is generally preferred, but starting every sentence with “We” seems excessive.

We note the reviewer’s preference, but we prefer not to mix active and passive voice within a manuscript, even when it is different sections. We feel it is important to maintain consistency throughout the text.

Finally, commas are missing in many sentences.

We have thoroughly rechecked the grammar in this manuscript and thank the reviewer for a thoughtful and detailed reading.

Second decision letter

MS ID#: JOCES/2019/243162

MS TITLE: miR-206 Enforces a Slow Muscle Phenotype

AUTHORS: Kristen K Bjorkman, Martin Guess, Brooke C Harrison, Michael M Polmear, Angela K Peter, and Leslie A. Leinwand
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 recognize that part of their initial criticisms have been addressed in your revised manuscript. However, both referees still raised issues that will require amendments to your manuscript. Concerning the comments of reviewer #1, you should 1) provide a reasonable explanation why miR-206 might exert a role in cardiomyocytes despite its very low expression and 2) address the possibility (at least in discussion) that the cardiac phenotype in miR-206 mutants may be the indirect consequence of the loss of miR-206 in another organ. Concerning reviewer #2, you should address all the indicated points. If you think you can re-review the manuscript accordingly, I would be pleased to see another version of your paper.

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

In the previous review I wrote: In this study, Bjorkman (re-)investigated the expression of miR-206 in striated muscles and examined a potential function of miR-206 in enforcing a slow phenotype in muscle fibers. The authors confirm previous work from several other groups, already reporting preferential expression of miR-206 in slow myofibers under physiological and pathophysiological conditions but added some further details such as higher expression of miR-206 in female compared to male TA muscles. To understand whether miR-206 has an effect on myofiber type specification, the authors took advantage of a previously described miR-206 knockout model. Reanalysis of this model reveled male-specific increase of soleus muscle mass and a moderate shift from type I (slow oxidative) to type IIa (fast oxidative) myofibers in male mice. Furthermore the authors analyzed a potential function of miR-206 in the heart muscle and observed increased LV mass in male but not female miR-206 mutants compared to WT after isoproterenol induced hypertrophy, although miR-206 is expressed at very low levels in the heart. Interestingly, the differences in LV mass between mutants and WT were correlated with increased expression of (fast) MYH6. The authors conclude that miR-206 might be necessary to enforce the slow skeletal muscle phenotype and might play a role for sex-differences in striated muscles.

The data included in the manuscript conform previously published findings but also provide additional information that might be interesting to understand the role of miR-206 in skeletal muscle fiber type specification and MYH switching in the heart. Unfortunately, the study lacks any mechanistic part and the correlative observation are often incomplete. Additional evidence is needed to support the conclusions.

My previous assessment still applies. Now, Bjorkman et al have revised the manuscript, responding to the comments of three reviewers. However, the quality of the study has only improved marginally. Unfortunately, I do not see major improvements of the revised manuscript compared to the previously reviewed version.

Comments for the author

The authors agree with this reviewer about the lack of any mechanistic insights but argue that “at this time” they are unable to add further data. I am afraid that, given the advanced state of the microRNA field, I have to insist that mechanistic studies are a prerequisite for publication in a recognized journal. The authors still do not provide a convincing explanation for the described molecular changes (Mef2c, Six1, Eya1 and linc-MYH).
In the present state, the transcriptional changes are still anecdotic findings, without any further context and understanding. The results are not suitable to explain the described subtle changes in myosin expression.
Similarly, the authors do not give any insights into why miR-206 seems to be upregulated in the initial phase of voluntary running.

The authors still do not explain why loss of a molecule that is barely detectable might cause a phenotype in the heart. No convincing additional data are provided by the authors to demonstrate meaningful expression of miR-206 in the heart. In the manuscript the authors now refer to rat cardiomyocyte data (not shown - which is not acceptable as well), but are unable to demonstrate expression in mouse cardiomyocytes.

I do not question the findings indicating that miR-206 mutants show a heart phenotype but it needs to carefully analyzed whether this is due to absence of miR-206 in cardiomyocytes or in a different organ that might have an effect on heart. Convincing demonstration of the function of miR-206 in the heart probably requires a conditional KO of miR-206, but alternatively a knock-down of miR-206 in isolated cardiomyocytes might be appropriate to support the findings.

My main three points of criticisms are still not addressed: (i) mechanistic studies to understand how miR-206 works and how its absence might cause the observed phenotype; (ii) a reasonable explanation why miR-206 might exert a role in cardiomyocytes despite its very low expression; (iii)

a clear demonstration that miR-206 exerts a cell-autonomous function in cardiomyocytes, ruling out that the cardiac phenotype in miR-206 mutants is the indirect consequence of the loss of miR-206 in another organ. In my view these requests are not inappropriate but reflect the current status of the field.

Reviewer 3

Advance summary and potential significance to field

In the manuscript the authors show that miR-206 is induced in conditions promoting a slow oxidative muscle phenotype and provide evidence for a role of miR-206 in driving a slow muscle phenotype. Consistently, the authors find a fiber type shift towards a faster muscle phenotype in soleus muscle of miR-206 knockout male mice. Furthermore, miR-206 knockout male mice develop a dilated cardiomyopathy phenotype, which the authors hypothesize is associated with a shift in MyHC expression towards a faster phenotype. Interestingly, these changes were only observed in male mice, suggesting a role of miR-206 in sexual dimorphism. The results are interesting and significantly advances the field, also suggesting miR-206 as a therapeutic target.

Comments for the author

The authors have appropriately responded to most of my comments. However, there are still some issues that need to be addressed as detailed below:

Considering the rather surprising result that miR-206 KO males have a cardiac phenotype despite of the low expression level of miR-206 in the heart, I do not agree that a sufficient number of mice were analyzed by echocardiography. 4 WTs and 5 KO is really a low number compared to normal standards.

In Figure 1C, it is shown a reduction in MyHC IIa and IIx mRNA level, while MyHC IIb mRNA is unaltered in the TA of female ArKO mice compared to WT.
How do the authors explain that? If there are less type IIa and IIx fibers, there should be an increase in another fiber type.

Line 460, page 16: “and mdx4cv (a kind gift from Dr. Bradley Olwin, University of Colorado Boulder) were months old” The age of the mice is missing.

To my comment regarding the title “miR-206 is associated with pathologic increases in slow MyHC expression in the LV” the authors responded that they changed the title, but the title has not been changed.

In Figure S3C, the asterisks are not centralized over the last bar and the text on the x-axis is too close to the axis compared to Figure S3B. Also the graphs in Figure S3B and S3C are not aligned vertically. It would make sense to keep the bars equally wide in S3B and S3C in the subfigures (S3B, S3C and S3F).
The statistical comparison between D1 and D0 in Figure S3B is still missing.

Second revision

Author response to reviewers' comments

MS ID#: JOCES/2019/243162
MS TITLE: miR-206 Enforces a Slow Muscle Phenotype
AUTHORS: Kristen K Bjorkman, Martin Guess, Brooke C Harrison, Michael M Polmear, Angela K Peter, and Leslie A. Leinwand
ARTICLE TYPE: Research Article

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The authors agree with this reviewer about the lack of any mechanistic insights but argue that “at this time” they are unable to add further data. I am afraid that, given the advanced state of the microRNA field, I have to insist that mechanistic studies are a prerequisite for publication in a recognized journal. The authors still do not provide a convincing explanation for the described molecular changes (Mef2c, Six1, Eya1 and linc-MYH). In the present state, the transcriptional changes are still anecdotic findings, without any further context and understanding. The results are not suitable to explain the described subtle changes in myosin expression. Similarly, the authors do not give any insights into why miR-206 seems to be upregulated in the initial phase of voluntary running.

The authors still do not explain why loss of a molecule that is barely detectable might cause a phenotype in the heart. No convincing additional data are provided by the authors to demonstrate meaningful expression of miR-206 in the heart. In the manuscript the authors now refer to rat cardiomyocyte data (not shown - which is not acceptable as well), but are unable to demonstrate expression in mouse cardiomyocytes.

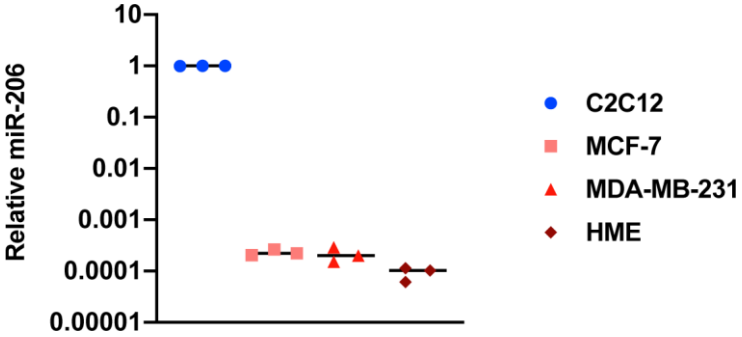
I do not question the findings indicating that miR-206 mutants show a heart phenotype but it needs to carefully analyzed whether this is due to absence of miR-206 in cardiomyocytes or in a different organ that might have an effect on heart. Convincing demonstration of the function of miR-206 in the heart probably requires a conditional KO of miR-206, but alternatively a knock-down of miR-206 in isolated cardiomyocytes might be appropriate to support the findings.

My main three points of criticisms are still not addressed: (i) mechanistic studies to understand how miR- 206 works and how its absence might cause the observed phenotype; (ii) a reasonable explanation why miR-206 might exert a role in cardiomyocytes despite its very low expression; (iii) a clear demonstration that miR-206 exerts a cell-autonomous function in cardiomyocytes, ruling out that the cardiac phenotype in miR-206 mutants is the indirect consequence of the loss of miR-

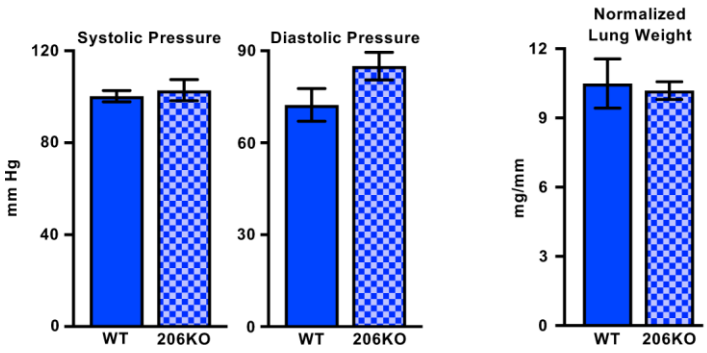
206 in another organ. In my view these requests are not inappropriate but reflect the current status of the field.

(i) Further mechanistic studies are of interest but beyond the scope of the current manuscript. Fortunately, amongst the already-identified mRNA targets of miR-206, such as Hdac4, there are some that shed some light on how it may be exerting its effects to control the slow muscle phenotype. We do highlight this in the Discussion and have also updated it to include the need for further detailed mechanistic analyses (lines 367-9).

(ii) We do not agree that low expression levels of a regulatory molecule, particularly one that is part of an enzymatic complex with turnover properties such as miRISC, must necessarily negate a potential functional role. Many molecules with low expression levels have widely accepted biological roles. There are several phenomena that can explain this, including, but not limited to, the following. First, there can be restricted regional or developmentally regulated expression within a tissue. As cited in the Discussion, Krenz *et al*. demonstrated that, despite apparent low □-MyHC expression when examining the adult mouse heart as a whole, there are distinct regions where this myosin isoform actually predominates over α-MyHC. In addition, a single missense mutation in the MYH7 gene (encoding β-MyHC) is sufficient to cause embryonic lethality by day E10 due to heart failure in homozygous mice (Gifford *et al*. Science 31 May 2019:Vol. 364, Issue 6443, pp. 865-870). As β-MyHC is expressed at higher levels in the embryonic mouse heart compared to the adult heart, it would be interesting to examine embryonic cardiac miR-206 expression in the future. Finally, certain key biomolecules display broader expression but play very specific subcellular roles (for example, telomere binding proteins or certain sequence-specific transcription factors with limited numbers of important gene targets). In this vein, miR-206 has an established tumor suppressor role in multiple cancers (>200 publications examine miR-206 in the context of various cancers, where loss of miR-206 often results in poor prognosis and more aggressive tumor behaviors), but, as shown below, we have measured levels in 2 human breast cancer cells lines in which miR-206 has been extensively studied (MCF-7 and MDA-MB-231) and 1 normal immortalized human mammary epithelial cell line and found that miR-206 expression is >3 orders of magnitude lower than in C2C12 mouse myoblasts. Even so, miR-206 anti-cancer activity is widely accepted due at least in part to targeting cell cycle and metabolic genes. At this time, we are unable to measure miR-206 in isolated mouse cardiomyocytes as we did for rat cardiomyocytes, but we do now include in the Discussion that future work should include *in situ* analysis of miR-206 expression to assess cardiac distribution (lines 372-7).



(iii) We acknowledge that it is possible that the miR-206 null cardiac phenotype could be at least partially secondary to loss of expression in another organ. We have updated the Discussion to present this possibility (lines 384-5). We have also examined the possibility of hypertension accounting for the cardiac phenotype, but we found no difference in either systolic or diastolic blood pressure or in normalized lung weight even in aged, 12-month-old mice (shown below).



Reviewer 3 Advance Summary and Potential Significance to Field:
In the manuscript the authors show that miR-206 is induced in conditions promoting a slow oxidative muscle phenotype and provide evidence for a role of miR-206 in driving a slow muscle phenotype.
Consistently, the authors find a fiber type shift towards a faster muscle phenotype in soleus muscle of miR-206 knockout male mice. Furthermore, miR-206 knockout male mice develop a dilated cardiomyopathy phenotype, which the authors hypothesize is associated with a shift in MyHC expression towards a faster phenotype. Interestingly, these changes were only observed in male mice, suggesting a role of miR-206 in sexual dimorphism. The results are interesting and significantly advances the field, also suggesting miR-206 as a therapeutic target.

Reviewer 3 Comments for the Author:
The authors have appropriately responded to most of my comments. However, there are still some issues that need to be addressed as detailed below:

Considering the rather surprising result that miR-206 KO males have a cardiac phenotype despite of the low expression level of miR-206 in the heart, I do not agree that a sufficient number of mice were analyzed by echocardiography. 4 WTs and 5 KO is really a low number compared to normal standards.

NIH vertebrate research animal guidelines state that investigators should “limit animal involvement by using the minimum number required to obtain valid results.” https://www.niaid.nih.gov/grants- contracts/research-vertebrate-animals We performed an *a priori* power analysis with G*Power. Moreover, the echocardiographic data are consistent within groups and corroborated by organ weight (Fig. 6A).

In Figure 1C, it is shown a reduction in MyHC IIa and IIx mRNA level, while MyHC IIb mRNA is unaltered in the TA of female ArKO mice compared to WT. How do the authors explain that? If there are less type IIa and IIx fibers, there should be an increase in another fiber type.

Although we did not observe an increase in IIb mRNA to compensate for the decrease in IIa and IIx expression, it is possible that fiber type composition shifted to a higher proportion of pure IIB and/or IIX fibers and away from mixed fiber types, yielding a faster phenotype overall. This might not be reflected in a change in IIb mRNA expression. This would be an interesting area of future investigation.

Line 460, page 16: “and mdx4cv (a kind gift from Dr. Bradley Olwin, University of Colorado Boulder) were months old”
The age of the mice is missing.

We apologize for the oversight; the mice were 4 months old and we have amended the text accordingly.

To my comment regarding the title “miR-206 is associated with pathologic increases in slow MyHC expression in the LV” the authors responded that they changed the title, but the title has not been

changed.

That was our mistake in preparing the highlighted revised submission. We have changed the title to: “Increased miR-206 expression correlates with pathologic increases in slow MyHC expression in the LV.”

In Figure S3C, the asterisks are not centralized over the last bar and the text on the x-axis is too close to the axis compared to Figure S3B. Also the graphs in Figure S3B and S3C are not aligned vertically. It would make sense to keep the bars equally wide in S3B and S3C in the subfigures (S3B, S3C and S3F). The statistical comparison between D1 and D0 in Figure S3B is still missing.

We have fixed the graph sizes and asterisk placement. The statistical comparison is not missing: the 2- way ANOVA was significant (P = 0.0007), but the D0 v D1 post-test is not while the D0 v D4 and D1 v D4 post-tests were significant. This is reflected by both the symbols and the legend.

Third decision letter

MS ID#: JOCES/2019/243162

MS TITLE: miR-206 Enforces a Slow Muscle Phenotype

AUTHORS: Kristen K Bjorkman, Martin Guess, Brooke C Harrison, Michael M Polmear, Angela K Peter, and LESLIE A. LEINWAND
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.