Apoptosis inhibitors and mini-agrin have additive benefits in congenital muscular dystrophy mice

Sarina Meinen, Shuo Lin, Raphael Thurnheer, Michael Erb, Thomas Meier and Markus A. Rüegg

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Review timeline:

Submission date: 23 December 2010
Editorial Decision: 15 February 2011
Revision received: 29 April 2011
Accepted: 17 May 2011

Transaction Report:

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

1st Editorial Decision 15 February 2011

Thank you for the submission of your manuscript "Apoptosis inhibitors and mini-agrin have additive benefits in congenital muscular dystrophy mice" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #1 highlights that it is crucial to investigate possible differential effects of mini-agrin and Bcl2 on the different cell types they are expressed in. This will permit unequivocal interpretation of the presented findings. This reviewer also points to the apparent discrepancy (increased fibrosis and fiber number, but same cross-sectional area) between Fig 1D and 2E, which should be clarified. Importantly, reviewer #3 notes that diaphragm function should be investigated.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine
REFEREE REPORTS:

Referee #1 - Comments on Novelty/Model System:

The authors used two different transgenic mouse models expressing either BCL2 or mini-agrin under two different promoters: MyoD which drives Bcl2 expression and MCK that drives mini-agrin expression. This can generate confusion in the interpretation of the data. The activity of Bcl2 and mini-agrin can be directed on different muscle compartments: Bcl2 on satellite cells (considering that MyoD is expressed by activated and proliferating satellite cells), whereas MCK is a marker of a more mature muscle phenotype. The authors should address whether there is a differential activity of Bcl2 and mini-agrin on both satellite cells and muscle fibers.

Referee #1 - Other Comments:

In the manuscript, "Apoptosis inhibitors and mini-agrin have additive benefits in congenital muscular dystrophy mice", Meinen et al. tested whether mini-agrin and antiapoptotic agents exert additive benefits in a congenital muscular dystrophy (MDC1A) mouse models, in which mutation in the laminin-2 gene causes severe muscle degeneration. This observation is of interest both to our understanding of the pathogenetic mechanisms of MDC1A, and may have relevance to the development of treatment strategies for the disease. Nevertheless, several points remain to be elucidated and also the lack of conclusive and convincing data weak the reported results.

Here are the major points:

1) The authors used two different transgenic mice expressing either BCL2 or mini-agrin under two different promoters: MyoD which drives Bcl2 expression and MCK that drives mini-agrin expression. This can generate confusion in the data interpretation. In fact, the activity of both Bcl2 and mini-agrin can be directed on different muscle compartments: Bcl2 on satellite cells (considering that MyoD is expressed by activated and proliferating satellite cells), whereas MCK on mature muscle fibers. The authors should address whether there is a differential activity of Bcl2 and mini-agrin on both satellite cells and muscle fibers.

2) The authors reported that Bcl2 induces a "strong increase in the number of macrophages in triceps of dyW/Bcl mice". What is the mechanisms by which Bcl2 increases the recruitment of macrophages? Does Bcl2 affect the emigration of macrophages from the injured tissue? In addition, the immunofluorescence analysis, reported in Figure S2, is a qualitative approach and the authors should use more quantitative methods (i.e. RT-PCR, western blot, FACS) to quantify the percentage of macrophage and the expression of relevant markers of the inflammatory cells within the entire muscle. Moreover, does Bcl2 inhibit the apoptosis of inflammatory cells? This can justify the evidence that inflammation is higher in dyW/Bcl mice. There have been several reports suggesting the involvement of members of the Bcl-2 family in the regulation of neutrophil apoptosis.

3) The results showed in Figure 1D and 2E are apparently in contrast with the data showed in Figure 1C and 2B. The authors reported that expression of Bcl2 increased the number of fibers in dyW/Bcl and dyW/Bcl/mag mice compared to dyW/dyW and dyW/mag mice", suggesting the activation of regenerative mechanisms. Nevertheless, the authors also reported that concomitantly "Bcl2 expression increases fibrosis, a finding that was confirmed by quantification of the relative area covered by fibrosis (Fig. 1C)". Additionally, they reported that overexpression of Bcl2 did not significant increase the cross-sectional area of triceps and soleus muscle.

How do the authors justify this discrepancy (more fibers, more fibrosis, same cross sectional area of the control dyW/dyW mice)? The increase in the fibrotic tissue would suggest that the contractile muscle fibers are replaced by the fibrotic component.
4) The authors reported that "although the muscle of dyW/Bcl mice shows early signs of a successful regeneration, late steps in this process are not completed". Does Bcl2 affects the maturation process and therefore the completion of the regenerative mechanism? To address this point the authors should induce muscle injury in the control MyoD/Bcl2 transgenic mouse and analyse, in a time course experiment, the different stages of muscle regeneration.

5) The potential role of apoptosis in the loss of fibers in dyW/dyW mice is not sufficiently addressed. The TUNNEL staining should be supported by the expression of relevant markers of the apoptotic pathways and proteolytic system.

6) Figure 5: the survival analysis should be performed using the Kaplan Meir method.

7) The study lacks of a conclusive and clear molecular mechanism and signalling underlying the additive or synergistic effect of Bcl2 and mini-agrin.

Referee #2 - Other Comments:

This is an excellent manuscript with very little to criticise and much to be enthusiastic about. If I were reviewing it I would recommend that it be accepted with the following comments:

The authors describe a detailed study of expression of an agrin mini-gene plus either genetic or pharmacological inhibition of apoptosis on the phenotype of dy/dy, the mouse model for MDC1A. The experiments are described in detail and fully justify the conclusion that apoptosis inhibition potentiates mini-agrin therapy and should be considered as an adjunct. In general terms the paper is well written with clear figures and an interesting message. Although both mini-agrin and apoptosis inhibition have been tested in this model before, the combination is novel and the results therefore of considerable interest both mechanistically and as a pre-clinical study. I have two minor comments to make.

1. The number of animals in each experiment should be clearly stated either in the figure legend, materials and methods, results section or on the figure itself. This is essential.

2. The discussion is extensive and interesting, but does not adequately explore the mechanistic insights which the combination therapy explores. The second to last paragraph of the discussion should be expanded upon to discuss how the different pathways which are perturbed in dy/dy animals interact and are corrected by the single and combination therapies.

In addition:

Page 2, abstract line 3 - I would substitute "artifical" for "designed"
Page 2, abstract line 14 - I would add and "and" after "treatment" and replace "suggesting" with "suggest"
Page 18, line 9 - "outmost" should read "utmost"
Page 19, line 3 - "omigail" should read "omigapil"
Page 19, line 4 - I would replace "our" with "this"
Page 20, title - "Henatoxylin" should read "Hematoxylin"
Page 22, line 2 - confirm that the % acrylamide is correct (I would have thought the other way around)
Page 22, line 7 - delete product code in brackets
Page 23, line 1 - move statistical test description into the relevant section further down the page

Referee #3 - Comments on Novelty/Model System:

This is an interesting and well written paper that provides evidence for the potential combined use of drugs that improve muscle cell adhesion and muscle cell survival as treatment options for MDC1A. A major concern is the lack of study of the diaphragm function or histology in the control and treated mouse models of MDC1A.

Referee #3 - Other Comments:
Previous studies by these authors have shown that mini-agrin and omigapil can individually improve outcomes of mouse models of MDC1A. This paper investigates if both mini-agrin and apoptosis inhibitors have combined activities and further reduce muscle disease. The authors produced transgenic mice that overexpress both mini-agrin and Bcl2 to determine if there are improved benefits of combined therapy to further prevent muscle disease in the dyw/dyw mouse model of MDC1A. The authors then treated transgenic mice that overexpressed mini-agrin with the anti-apoptotic drug omigapil. Analyzing muscle pathology, survival, activity and muscle function the authors conclude that combined mini-agrin and apoptosis inhibitors act additively with beneficial effects larger that with each treatment alone.

This is an interesting and well written paper that provides evidence for the potential combined use of drugs that improve muscle cell adhesion and muscle cell survival as treatment options for MDC1A. The following concerns need to be addressed:

**Major Concerns**

1. The authors should report the genetic backgrounds of the mice used in this study. Are these mice on a mixed genetic background or are they inbred strains?
2. Figure 1: It would be appropriate to quantify fibrosis in the muscle using Sirius red or a hydroxyproline assay.
3. Figure 7: The survival curves for dyw/dyw mice should be included as a control.
4. Figure 7: Combined mini-agrin and omigapil did not change the survival of dyw/mag treated mice. Can the authors provide an explanation for why dyw/mag transgenic mice treated with omigapil showed no improvement in longevity compared to dyw/mag transgenic mice alone? Was death due to diaphragm pathology and pulmonary failure?
5. Respiratory insufficiency is the cause of death in the majority of MDC1A patients. It is therefore surprising that no examination of diaphragm pathology or function was undertaken in this study. It would be of significant interest to report at least diaphragm pathology in these mice.
6. The authors performed statistical analysis using pair-wise Student t-tests. Since there are at least 5 experimental groups in this study (wild-type, dyw, dy/Bcl, dw/Bcl/mag) ANOVA would be the most appropriate statistical test so that conclusions and comparisons can then be drawn across all groups.

**Minor Concerns:**

2. In the Discussion section there are references to the specific figures (Fig. 1 etc) already presented in the Results section. These should be removed from the Discussion to improve flow.

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The authors used two different transgenic mouse models expressing either BCL2 or mini-agrin under two different promoters: MyoD which drives Bcl2 expression and MCK that drives mini-agrin expression. This can generate confusion in the interpretation of the data.

The activity of Bcl2 and mini-agrin can be directed on different muscle compartments: Bcl2 on satellite cells (considering that MyoD is expressed by activated and proliferating satellite cells), whereas MCK is a marker of a more mature muscle phenotype. The authors should address whether there is a differential activity of Bcl2 and mini-agrin on both satellite cells and muscle fibers.

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This observation is of interest both to our understanding of the pathogenetic mechanisms of MDC1A, and may have relevance to the development of treatment strategies for the disease.

Nevertheless, several points remain to be elucidated and also the lack of conclusive and convincing data weak the reported results.

Here are the major points:

1) The authors used two different transgenic mice expressing either BCL2 or mini-agrin under two different promoters: MyoD which drives Bcl2 expression and MCK that drives mini-agrin expression. This can generate confusion in the data interpretation.

   In fact, the activity of both Bcl2 and mini-agrin can be directed on different muscle compartments: Bcl2 on satellite cells (considering that MyoD is expressed by activated and proliferating satellite cells), whereas MCK on mature muscle fibers.

   The authors should address whether there is a differential activity of Bcl2 and mini-agrin on both satellite cells and muscle fibers.

   We appreciate the concern of the referee but we do not share it as argued below:

   We have documented previously (Meinen et al, 2007; Moll et al, 2001) that the mini-agrin transgenic mice express the protein in all muscles fibers and that the protein is stably incorporated into the muscle basement membrane.

   - Transgenic mice expressing Bcl2 under the control of the MyoD promoter were obtained from Dr. Janice Dominov who has used the mice to show that Bcl ameliorates the dystrophic phenotype of dy/dy mice (Dominov et al, 2005; Girgenrath et al, 2004). As described in Girgenrath et al., 2004, Bcl2 is driven by an approximately 7kb fragment of the mouse MyoD promoter. The authors write in their paper:

     ... “Western blotting and immunohistochemistry with the human Bcl-2–specific mAbs 6C8 (BD Biosciences — Pharmingen) or Ab-1 (Oncogene Research Products) was used to confirm that the transgene (pMyoD-hBcl-2) was expressed only in skeletal muscle and that the transgenes were expressed in all myofibers (Figure 2B) and J.A. Dominov, unpublished observation)”...

   Thus, Dominov et al have controlled for the expression of Bcl2 in muscle fibers.

   - In addition, the paper by (Charge et al, 2008) also shows that a 6kb promoter fragment of MyoD is sufficient to drive expression of LacZ in adult myofibers (see for example their Figure 4).

   - Finally, we have now also included Western Blot analysis for Bcl2 in the transgenic mice on a wild-type background (Fig. S1B). In those mice, the muscles are healthy and thus the protein detected must be expressed in adult muscle fibers.

   In summary, those data show that Bcl2 is also expressed in adult muscle fibers and they indicate that there is no differential activity between mini-agrin and Bcl2 in muscle fibers. We have added some sentences to explain this in the Materials and Methods section.

2) The authors reported that Bcl2 induces a "strong increase in the number of macrophages in triceps of dyW/Bcl mice". What is the mechanisms by which Bcl2 increases the recruitment of macrophages? Does Bcl2 affect the emigration of macrophages from the injured tissue?
We observed that Bcl2 expression in dy\(^w\)/dy\(^w\) alone increases fibrosis (Fig. S2). We have now also quantified the fibrosis by hydroxyproline measurements (Fig. S2A, B). As a consequence of the increased fibrosis, many more macrophages infiltrate the fibrotic tissue (Fig. S2C, D). Our findings demonstrate that dy\(^w\)/Bcl/mag mice have significantly less fibrosis (Fig. S2A) and also fewer infiltrating macrophages (Fig. S2C, D) than dy\(^w\)/Bcl mice. This in fact shows that the extent of fibrosis correlates with the number of macrophages and not with the expression of Bcl2. Thus, the high number of macrophages is not dependent on the expression of Bcl2 (as is suggested by the reviewer).

In addition, the immunofluorescence analysis, reported in Figure S2, is a qualitative approach and the authors should use more quantitative methods (i.e. RTPCR, western blot, FACS) to quantify the percentage of macrophage and the expression of relevant markers of the inflammatory cells within the entire muscle.

Maybe we did not describe the method we used in enough detail but we do not agree with the reviewer's comment that our data on the macrophages are not quantitative. In fact, they are highly quantitative as we counted the number of F4-80 positive cells in muscle cross-sections from mice of all genotypes. We have then set the number of F4-80 positive cells in control animals to 1 and have compared those numbers with those obtained in the other genotypes. As shown in Fig. S2C, there are more than 200 times more F4-80 positive macrophages in dy\(^w\)/dy\(^w\)/Bcl mice than in controls.

Moreover, does Bcl2 inhibit the apoptosis of inflammatory cells? This can justify the evidence that inflammation is higher in dyW/Bcl mice. There have been several reports suggesting the involvement of members of the Bcl-2 family in the regulation of neutrophil apoptosis.

Because expression of Bcl2 is under the control of the MyoD promoter, it is unlikely that it is expressed in macrophages. Moreover, Figure 3A shows several cases of TUNEL-positive nuclei that lie outside of muscle fibers. These may (besides other cells) also include macrophages.

3) The results showed in Figure 1D and 2E are apparently in contrast with the data showed in Figure 1C and 2B. The authors reported that expression of Bcl2 increased the number of fibers in dyW/Bcl and dyW/Bcl/mag mice compared to dyW/dyW and dyW/mag mice, suggesting the activation of regenerative mechanisms. Nevertheless, the authors also reported that concomitantly "Bcl2 expression increases fibrosis, a finding that was confirmed by quantification of the relative area covered by fibrosis (Fig. 1C)". Additionally, they reported that overexpression of Bcl2 did not significant increase the cross-sectional area of triceps and soleus muscle.

How do the authors justify this discrepancy (more fibers, more fibrosis, same cross sectional area of the control dyW/dyW mice)? The increase in the fibrotic tissue would suggest that the contractile muscle fibers are replaced by the fibrotic component.

It was not easy for us to understand what the reviewer meant but indeed, dy\(^w\)/Bcl muscles have more fibers, more fibrosis and a similar cross-sectional area as muscles from dy\(^w\)/dy\(^w\) mice. Because the fibers in dy\(^w\)/Bcl are, however, significantly smaller in diameter than the fibers in dy\(^w\)/dy\(^w\) muscles (Fig. 1D, F and Fig 2C), there is no discrepancy. To avoid misunderstanding, we now tried to clarify this in the Discussion.

4) The authors reported that "although the muscle of dyW/Bcl mice shows early signs of a successful regeneration, late steps in this process are not completed". Does Bcl2 affect the maturation process and therefore the completion of the regenerative mechanism? To address this point the authors should induce muscle injury in the control MyoD/Bcl2 transgenic mouse and analyse, in a time course experiment, the different stages of muscle regeneration.

There is no evidence that Bcl2 itself impedes the regeneration process of muscles. In fact, we conducted the experiment suggested by the reviewer already and injured a muscle from a mouse expressing Bcl2 on a wild-type background with notexin. The muscle fully regenerated, similar to control, wild-type muscles. We have not repeated those experiments enough to see minute differences so that we do not show those data. In our view, a more likely explanation for the failed regeneration in dy\(^w\)/Bcl mice is that the anti-apoptotic effect of Bcl2 is just not sufficient to complete
Muscle regeneration, probably because Bcl2 does not provide for mechanical stability. This concept is explained in detail in the Discussion, under “Mini-agrin and Bcl2 potentiate their effect on muscle regeneration in dy^W/dy^W mice” (last paragraph).

5) The potential role of apoptosis in the loss of fibers in dyW/dyW mice is not sufficiently addressed. The TUNNEL staining should be supported by the expression of relevant markers of the apoptotic pathways and proteolytic system.

The scope of this study was to examine the potential of combining two different therapy approaches and not to study the role of apoptosis in dy^W/dy^W mice. This question has been addressed by many laboratories before and we refer to those papers in the introduction.

6) Figure 5: the survival analysis should be performed using the Kaplan Meir method

Kaplan-Meier analysis has been performed and included in Fig. 5A.

7) The study lacks of a conclusive and clear molecular mechanism and signalling underlying the additive or synergistic effect of Bcl2 and mini-agrin.

In previous work, we have provided several data sets to show that mini-agrin exerts its positive effect on dy^W/dy^W mice by reconnecting the basement membrane with the plasma membrane of the skeletal muscle fiber. This function both stabilizes muscle fibers during contraction and improves regeneration of injured muscle fibers (Bentzinger et al, 2005; Meinen et al, 2007; Moll et al, 2001). There is also ample evidence that inhibition of apoptosis has some ameliorative effect in dy^W/dy^W mice. Because we now show that a combination of both treatments has additive effects, one can argue that the two pathways act in parallel. Therefore, it is difficult to define a common molecular pathway for both treatments and we doubt that there is such a “conclusive and clear molecular mechanism and signaling underlying the additive or synergistic effect of Bcl2 and mini-agrin” as implied by the referee.

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This is an excellent manuscript with very little to criticise and much to be enthusiastic about. If I were reviewing it I would recommend that it be accepted with the following comments:

The authors describe a detailed study of expression of an agrin mini-gene plus either genetic or pharmacological inhibition of apoptosis on the phenotype of dy/dy, the mouse model for MDC1A. The experiments are described in detail and fully justify the conclusion that apoptosis inhibition potentiates mini-agrin therapy and should be considered as an adjunct. In general terms the paper is well written with clear figures and an interesting message. Although both mini-agrin and apoptosis inhibition have been tested in this model before, the combination is novel and the results therefore of considerable interest both mechanistically and as a pre-clinical study. I have two minor comments to make.

1. The number of animals in each experiment should be clearly stated either in the figure legend, materials and methods, results section or on the figure itself. This is essential.

2. The discussion is extensive and interesting, but does not adequately explore the mechanistic insights which the combination therapy explores. The second to last paragraph of the discussion should be expanded upon to discuss how the different pathways which are perturbed in dy/dy animals interact and are corrected by the single and combination therapies.
As explained in our response to referee #1, we think that the two treatments affect independent pathways. As we have already discussed the potential mechanisms involved in the ameliorative effect of mini-agrin and anti-apoptosis treatment in previous papers, we refrained from re-iterating those aspects again (see for example the discussion in Meinen et al, 2007). As we can see the argument of this reviewer to get some more insights into mechanisms, we have now added two sentences to the discussion in the second to last paragraph.

In addition:
Page 2, abstract line 3 - I would substitute "artifical" for "designed"
we cannot find the word artificial in the text, we already used "designed"
Page 2, abstract line 14 - I would add and "and" after "treatment" and replace "suggesting" with "suggest"
DONE
Page 18, line 9 - "outmost" should read "utmost"
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DONE
Page 22, line 2 - confirm that the % acrylamide is correct (I would have thought the other way around)
DONE
Page 22, line 7 - delete product code in brackets
DONE
Page 23, line 1 - move statistical test description into the relevant section further down the page
DONE

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This is an interesting and well written paper that provides evidence for the potential combined use of drugs that improve muscle cell adhesion and muscle cell survival as treatment options for MDC1A. The following concerns need to be addressed:

Major Concerns

1. The authors should report the genetic backgrounds of the mice used in this study. Are these mice on a mixed genetic background or are they inbred strains?

   All the transgenic and knockout mice (dy<sup>W</sup>/dy<sup>W</sup> mice: (Kuang et al, 1998); chick mini-agrin mice: (Moll et al, 2001); pMyoD-hBcl-2 mice: (Girgenrath et al, 2004)) have been back-crossed to C57BL/6J mice for many generations. However, we do not know exactly how often they have been back-crossed (at least 10 times). To avoid phenotypical variations due to genetic background we used littermates as controls (whenever possible). In Materials and Methods (under “Mice”), we have included a sentence to make this clear.

2. Figure 1: It would be appropriate to quantify fibrosis in the muscle using Sirius red or a hydroxyproline assay.

   We have now included hydroxyproline assays from triceps, soleus and diaphragm muscles. Results are shown in Fig. S2A, B and S3B, and are mentioned in the results section. The method is described in supplementary information.

3. Figure 7: The survival curves for dyw/dyw mice should be included as a control.

   We now include the survival probability of dy<sup>W</sup>/dy<sup>W</sup> mice in Fig. 7J.

4. Figure 7: Combined mini-agrin and omigapil did not change the survival of dyw/mag treated mice. Can the authors provide an explanation for why dyw/mag transgenic mice treated with omigapil showed no improvement in longevity compared to dyw/mag transgenic mice alone? Was death due to diaphragm pathology and pulmonary failure?

   We do not have a good explanation of why survival of dy<sup>W</sup>/mag mice was not prolonged by Bcl2 expression or by omigapil treatment. One issue here is that the mini-agrin transgene has already such a strong effect on survival of the dy<sup>W</sup>/dy<sup>W</sup> mice that some moderate further prolongation could not be seen with the number of mice studied. The local animal ethics committees do not allow us to further increase the number of mice for such studies. We do not know the cause of death in the mice but think it is likely that they die of respiratory failure.

5. Respiratory insufficiency is the cause of death in the majority of MDC1A patients. It is therefore surprising that no examination of diaphragm pathology or function was undertaken in this study. It would be of significant interest to report at least diaphragm pathology in these mice.

   We now have performed histopathology and a hydroxyproline assay (assessment of fibrosis) of the diaphragm (see Fig S3). We present those results in the result section under “Bcl2 and mini-agrin affect different parameters in muscle of dy<sup>W</sup>/dy<sup>W</sup> mice”.

6. The authors performed statistical analysis using pair-wise Student t-tests. Since there are at least 5 experimental groups in this study (wild-type, dyw, dy/Bcl, dw/Bcl/mag) ANOVA would be the most appropriate statistical test so that conclusions and comparisons can then be drawn across all groups.

   ANOVA was performed in cases where the 5 experimental groups were tested in at least two different conditions (e.g. different ages). We have discussed our statistical evaluation also with an expert and explained to us that statistics on experimental groups with only one condition is done with a t-test and not with ANOVA.
Minor Concerns:

   DONE

2. In the Discussion section there are references to the specific figures (Fig. 1 etc) already presented in the Results section. These should be removed from the Discussion to improve flow.

   We understand the point but were told by many others that they actually like the re-mentioning of the figures in the discussion. Because this is a question of style, we left it as it is and leave it to the discretion of the editor to change this.

References:


Please find enclosed the final reports on your manuscript. We are pleased to inform you that your manuscript is accepted for publication and will be sent to our publisher to be included in the next available issue of EMBO Molecular Medicine if or once we have received your licenses.

Congratulations on your interesting work.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:
Referee #1 - Comments on Novelty/Model System:

The authors sufficiently addressed the points I raised.
Referee #1 - Other Remarks:

The authors sufficiently addressed the points I raised. The novel part of this study, compared to previous already published works, is that the dual treatment (mini-agrin with either transgenic Bcl2 expression or oral omigapil application) results in a marked increase in muscle force. And this might be relevant to develop more appropriate therapy for human patients.

Referee #3 - Other Remarks:

The authors have adequately address all concerns.

A typo was noted on page 4, line 15 (link is spelled linkt).