Myosin Heavy Chain-embryonic regulates skeletal muscle differentiation during mammalian development.

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AUTHORS: Akashi Sharma, Megha Agarwal, Amit Kumar, Pankaj Kumar, Masum Saini, Gabrielle Kardon, and Sam J. Mathew

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

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Sharma and colleagues investigate the skeletal muscle phenotype of a newly generated mouse strain in which the expression of embryonic myosin heavy chain was genetically ablated.
This is a careful work that discloses and describes a muscle phenotype during embryonic and fetal development. Importantly, although the severity of the developmental defect appears relatively mild, it is resulting in an adult phenotype that recapitulates certain aspects (i.e. scoliosis) occurring in patients affected by congenital contracture syndromes. This observation is giving to these studies a translational relevance.
In the second part of this study the authors investigate some mechanistic aspects involving a deregulation of the FGF signaling pathway in absence of embryonic myosin heavy chain and propose a dual action relying both on cell autonomous and paracrine mechanisms.

Comments for the author

Overall, I think the bulk of the data presented here supports authors' main claim that embryonic myosin heavy chain is contributing to proper muscle development. In my opinion the mechanistic aspects are incompletely developed, but I think that they could be object for more in depth future studies. I therefore think that this paper is an interesting contribution to the field.

Told that, in my opinion a few points may be addressed to improve the present manuscript:
1) It is unclear the cause of in utero death reported for the “generalized” MyHC-emb KO strain. Is possibly the reported death (lines 125-126) occurring at birth for respiratory (possibly ivolving the diaphragm) defects? Embryos completely devoid of differentiated muscle, such as for example the Myogenin, MyoD, MRF4 triple KOs are still born at mendelian ratio, although they survive only shortly after birth (Valdez et al 2000)… Are pups for the pax3 or pax7-driven MyHC-emb KO strains born at expected mendelian ratios?
2) MyHC-emb knockdown is reducing the secretion of different FGF ligands (Fig. 5F). Nevertheless the results of the in vivo gene expression studies are highlighting a decrease in activated forms of FGF pathway members but not directly a change in FGF ligands production (fig.5 E). Although I acknowledge that this information is suggesting that altered expression of FGF ligands may occur also in vivo, a formal demonstration is currently missing.
3) It is confusing the report of a reduced fusion index in MyHC-emb siRNA treated cells compared to control cells (Fig 4K), when MyHC-emb siRNA cells conditioned media is reportedly inducing an increase in fusion index (Fig.5C). The authors should clarify. Moreover, I would suggest to evaluate also the fusion index in the in vitro rescue experiments with FGF, for completeness and to further support the idea that exogenously added FGF is able to rescue the differentiation defects of MyHC emb siRNA treated cells.

Minor points:
1) The changes in MyHC-slow expression seem to be inconsistent in different experiments, such as those reported in Fig.2 I, Fig1K and Fig3A. Which muscle has been analyzed in Fig. 1K and Fig3A? The authors should clarify.
2) The physiological relevance of the quantification of the reserve cells in the in vitro cell cultures is unclear. The authors should discuss it more extensively on the light of the reported in vivo defects in MyHC-emb KOs.
3) A representative image should be added for the quantification of PHH3+ cells in Figure S3E.
4) Line 155 there is an unnecessary comma after the word “diaphragm”.

Reviewer 2

Advance summary and potential significance to field

The role of developmental myosins is unknown, indeed this is a major open issue in muscle cell biology. Therefore the first report of embryonic myosin knockout is very important.

Comments for the author

The role of developmental myosins is still unknown, thus the first paper to report the specific knockout of Myh3, the gene coding for embryonic myosin heavy chain, is welcome.
This study reports interesting results on the effect of Myh3 loss, including the finding that Myh3 loss affects myogenic progenitors in a non-cell autonomous manner. However, some parts of the study are incomplete and a number of points should be addressed.

Major points
1. A major result of this study, not stressed by the authors, is that MyHC-emb is not required for muscle differentiation, as myogenesis can take place almost normally in the absence of Myh3. At birth, the overall size of leg muscles, as seen in leg cross-section (Fig.1C,D), appears to be similar in K0 and WT mice, fiber number is unchanged in SOL and even increased in EDL, fiber size is unchanged in EDL and shows only an increase of small fibers in SOL. On the other hand, postnatal muscle development is clearly affected, leading to severe scoliosis by 4-6 weeks in Myh3 KO mice. The authors should analyze the muscle phenotype, including muscle weight, fiber size and type, at P15 and P30. This is crucial period when muscle growth is first accompanied by proliferation and fusion of satellite cells (first 3 weeks), then by myofiber hypertrophy (see White … Zammit, Dev Biol 2010).
2. The effect of Myh3 loss in vitro should be examined using primary cultures from fetal or neonatal WT and Myh3 KO muscles rather than in C2C12 cells treated with Myh3 siRNAs.
3. The nomenclature of myosins should be correct and consistent throughout the paper both in text and figures: use Myh3, not MyH3, for mouse gene, and MYH3 for human gene (see line 325); MyHC-emb should be used only for the protein product (e.g. 218, 414, etc), same for the other MYHs (e.g. 194-196, Fig. 2I,J, etc); the protein product of the Myh1 gene should be always indicated as MyHC-IIx, not in some case MyHC-IId (see 75 vs 79).
4. Inspection of whole leg muscle cross sections (Fig. 1C,D, Fig. S2A,B) shows that the size of soleus is much larger and that of gastro much smaller in K0 compared to WT samples. However, this could be due to the fact that the WT leg was sectioned at the mid belly of the muscles whereas the KO leg was sectioned towards the distal part of the leg. If this is true and these sections were used for muscle morphometry, unreliable data of fiber number and fiber size in soleus would be generated. It is imperative to compare muscles sectioned at the same level.
5. Some relevant papers and reviews should be cited in the Introduction: Whalen et al (Nature 1981) first reported clear evidence for the existence of developmental myosins; Condon et al (Dev Biol 1990) described the pattern of expression of emb and neonatal/perinatal MYHs during development of mouse muscles; Schiaffino et al (Skelet Muscle 2015) recently reviewed the expression and function of developmental myosins.
6. The legends of the figures are difficult to read and should be rewritten following the standard format used in Development: (A) …. . (B) … etc; or (A,B) … (A) …. (B).

Other points
1. How to explain the fact that about half of the K0 mice die in utero? This is surprising because even complete loss of muscles , e.g. in myogenin KO and even myogenin+MyoD double KO, does not lead to embryonic death. It would be of interest to determine at what stage embryos die. Is cardiac muscle involved? Rutland et al (Development 2011) reported that a probe specific for human MYH3 reacts with human and mouse developing heart. To my knowledge this is the only report on the presence of MyHC-emb in mammalian cardiac muscle and it would be important to verify this finding.
2. An interesting question to be addressed is whether muscle regeneration is affected by Myh3 loss.
3. MYH accumulation could be examined by WB as a marker of muscle cell differentiation to validate the effect of FGF supplementation.
4. Inspection of whole leg muscle cross sections (Fig. 1C,D, Fig. S2A,B) shows that the size of soleus is much larger and that of gastro much smaller in K0 compared to WT samples. However, this could be due to the fact that the WT leg was sectioned at the mid belly of the muscles whereas the KO leg was sectioned towards the distal part of the leg. If this is true and these sections were used for muscle morphometry, unreliable data of fiber number and fiber size in soleus would be generated. It is imperative to compare muscles sectioned at the same level.
5. The finding that diaphragm shows a distinct response to Myh3 K0 compared to leg muscle (173-181) could result not only from his anatomical and functional differences, as pointed out by the

authors, but also from the fact that its degree of maturation is more advanced since it starts working immediately at birth with the onset of respiration, whereas ambulatory movements are gradually acquired several days after birth. In the rat, this leads to more rapid downregulation of Myh3, with transcripts almost undetectable at P0 in diaphragm but still present in leg muscles, and more precocious upregulation of Myh2 (IIa), which is already present at P0 in diaphragm but not in gastro (see Kelly et al, J Neurosci 1991).
6. The relevance of the mouse model to human disease should be better discussed. The Freeman-Sheldon syndrome, the most severe of the syndromes due to MYH3 mutations, is characterized by distal arthrogryposis, with congenital contractures of hands, feet and orofacial muscles, while scoliosis is only seen in the most severe cases. Are signs of distal arthrogryposis in forelimbs and hindlimbs seen in the mouse mutants?
7. The comments about human disease in the Results (325-330) should be transferred to the Discussion.
8. Line 203: the term “drastic changes” does not seem appropriate (see major point 1).

Reviewer 3

Advance summary and potential significance to field

In this study Sharma et al study several inducible and non-inducible models of Myh3 mutant mice. Mutations of this gene in human are responsible for Freeman-Sheldon and Sheldon-Hall syndromes, characterized by congenital contracture. The authors show that in absence of embryonic Myosin heavy chain (Myh3, expressed in post mitotic myogenic cells), half of animals die in utero. Half of the mutants survive at birth. These surviving newborns have been explored in the present study that shows that expression of Myh3 during development is required for harmonious muscle growth. Absence of its expression leads to several muscle defects that seem to depend upon specific muscle. Complementary transcriptomic experiments, cell culture experiments to knock down Myh3 by siRNA, and proteomic studies to identify secreted proteins by wt and mutant myotubes have been conducted, that demonstrate deficient FGF signaling in the mutant myotubes. This impaired FGF signaling is suggested to contribute to the mutant phenotype observed in the mouse model. Most of the experiments are well done, and the presented results are of major interest.

Comments for the author

Major concerns
1
- In the Figure 1C, the name of some muscles used in the study should be indicated, particularly the Sol and the EDL. From figures 1C (wt) and 1D(mutant) it seems that at P0 the mutant Soleus is larger and contains many more myofibers than its wt counterpart. It is surprising that Figure 1H does not reflect this observation.
- Since the EDL is studied it would be more interesting to present EDL in Figure 1C’’ and 1D’’ rather than an undefined (peroneus?) muscle.
- From the data shown in Figures 1C and 1D, it cannot be concluded that MyHC-emb (Myh3) regulates myofiber number in the fast EDL, because the muscle showing the most obvious increase in size is the slow Soleus.
- It is also difficult to conclude (lines 139-140) that myofiber cross sectional area (CSA) is decreased only in slow muscles such as the soleus, since no zoom of the fast EDL is provided and myofiber CSA seems reduced in the fast muscle of the mutant Figures 1C’’ and D’’.

2 It is important to indicate the percentage of genes downregulated in C2C12 cells after Myh3 knockdown that are downregulated in the four muscles studied (or in some of them) and the percentage of genes downregulated both in vivo and ex vivo. This information is relevant considering the effects of innervation or other cell/cell interactions that do not occur ex vivo myotubes/myofibers.

3 The quality of Figures 3J and 3K must be improved. Furthermore, immunohistochemistry with activated Caspase3 antibodies would reveal if the increased cell death observed at P0 in Myh3-/- new born is specific of the mutant myofibers and or myoblasts (or both), a point that is not addressed by Western blot.

4 Line 245: since MyhC-embryonic is not expressed in myogenic progenitors or myoblasts, perhaps the authors could had supplementary data showing the absence of MyhC-emb protein in those cell populations (immunohistochemistry with MyhC-emb +/- MyoD or +/- Pax7 at E13. 5 or E14.5) or give a reference, since it is a very important aspect of this work and readers should be guided.

5
- I have a major problem with the fusion index methodology. This index, which is different from the differentiation index (MyHC+ cells), corresponds to cells with two or more nuclei. From the methods it seems that MyhC+ cells with a single nucleus have been counted.

6 It is commonly admitted that Myogenin+ cells do not proliferate and are named myocyte. MyoD+ Myogenin- cells on the other hand are proliferating myoblasts. Caution must be taken to keep this nomenclature in the text and Figure 6I.

7 It is not explained why the conditioned medium from mutant cells (which present a reduced fusion index, Figure 4K) added to wt myogenic cells favor their fusion (Figure 5C). Addition of FGF2 on C2 cells inhibits the expression level of MyoD and of Myogenin, thus FGF2 impairs their differentiation and favors their reserve cells behavior. It is not clear why the authors used FGF2 since it not presented as a target of Myh3 in Figure 5F. This must be explained. At the opposite use of FGF6 (or a cocktail of identified misregulated FGF) which seems the most downregulated FGF in mutant cells would have more sense to challenge the consequences of its presence on myogenic cells properties.

8 The quantity and timing of Tamoxifen injection in pregnant Pax7iCRE animals must be indicated. Without this information it is impossible to interpret Figure 3. Have the authors verified that only secondary myofibers are deleted for Myh3 in this model, while all primary myofibers are not?

9
- Pax7iCRE Myh3-/fl animals present a 50% reduction in the number of Pax7+ and a 60% reduction of MyoD+ cells at E16.5. Is this also the case in the total Myh3-/- fetuses? How is this result explained, considering the increased number of myofibers present in P0 animals, Figure1?
- While Pax7+ cells are depleted in mutant Pax7iCRE Myh3-/fl animals at E16.5 (line 242), their number is not altered at P0 in Myh3-/- animals. It would be important to indicate the number of Pax7+ cells and of MyoD + cells in Myh3-/- animals present at E16.5, to reconcile conflicting data arising probably from the use of distinct genetic models.

10 Figure 6I must be improved since it is misleading in the present form. The authors do not show that FGF promotes the differentiation of myogenic cells, rather that FGF produced by Myh3+ myofibers may control the balance between Pax7+ reserve/stem cells and their differentiation, while FGF blocks their differentiation.

First revision

Author response to reviewers' comments

Formatted PDF of Response to Reviewer Comments uploaded as a Supplementary Information file.

We thank the Reviewers for their insightful comments and have responded to them inline, marked in red font color.

Reviewer 1 Advance summary and potential significance to field
In this manuscript, Sharma and colleagues investigate the skeletal muscle phenotype of a newly generated mouse strain in which the expression of embryonic myosin heavy chain was genetically ablated.

This is a careful work that discloses and describes a muscle phenotype during embryonic and fetal development. Importantly, although the severity of the developmental defect appears relatively mild, it is resulting in an adult phenotype that recapitulates certain aspects (i.e. scoliosis) occurring in patients affected by congenital contracture syndromes. This observation is giving to these studies a translational relevance.
In the second part of this study the authors investigate some mechanistic aspects involving a deregulation of the FGF signaling pathway in absence of embryonic myosin heavy chain and propose a dual action relying both on cell autonomous and paracrine mechanisms.

Reviewer1 Comments for the author
Overall, I think the bulk of the data presented here supports authors' main claim that embryonic myosin heavy chain is contributing to proper muscle development. In my opinion the mechanistic aspects are incompletely developed, but I think that they could be object for more in depth future studies. I therefore think that this paper is an interesting contribution to the field.

Told that, in my opinion a few points may be addressed to improve the present manuscript:
1) It is unclear the cause of in utero death reported for the “generalized” MyHC-emb KO strain. Is possibly the reported death (lines 125-126) occurring at birth for respiratory (possibly ivolving the diaphragm) defects?
The MyHC-emb KO pups that are born generally survive [although they are smaller, weigh less (Fig. 2A), and at times die] and do not exhibit overt respiratory defects. Therefore, we believe death occurs during embryonic or fetal development. While we cannot rule out respiratory defects involving the diaphragm, as indicated by Reviewer 2 (Other Points, comment 1), it is possible that MyHC-emb is important for heart development and the in-utero death might be due to this. We have checked MyHC-emb expression in the embryonic heart and do find it to be expressed at E16.5, which has been added to the Supplementary Data (Fig. S4D) and to the results (lines 127-128).
Embryos completely devoid of differentiated muscle, such as for example the Myogenin, MyoD, MRF4 triple KOs are still born at mendelian ratio, although they survive only shortly after birth (Valdez et al 2000)…Are pups for the pax3 or pax7-driven MyHC-emb KO strains born at expected mendelian ratios?
This is an interesting suggestion and we have tried to obtain neonates from the Pax3Cre and Pax7iCre mediated loss of MyHC-emb. We could never obtain any live pups of the genotypes Pax3Cre; Myh3delta/fl or Pax7iCre; Myh3delta/fl. Pax3Cre is a knock in knock out allele (Engleka et al Dev Biol 2005) and Pax7iCre has Cre inserted in the 3’UTR of Pax7, which in our hands rarely produces homozygous Pax7iCre/iCre animals (Keller et al Genes Dev 2004), suggesting that in both cases the Cre insertion interferes with the respective genes. Both Pax3Cre and Pax7iCre drivers are likely to drive Cre expression in the heart (Conway et al Cardiovasc Res 1997; Engleka et al Dev Biol 2005; Olaopa et al Dev Biol 2011; Murdoch et al PLoS One 2012), where MyHC-emb might have essential roles as detailed in response to the first part of Comment 1. Also, heterozygosity of Pax3 (in the case of Pax3Cre) and insertion of Cre in the 3’UTR of Pax7 (in the case of Pax7iCre) might affect viability and be a contributing factor.

2) MyHC-emb knockdown is reducing the secretion of different FGF ligands (Fig. 5F). Nevertheless the results of the in vivo gene expression studies are highlighting a decrease in activated forms of FGF pathway members but not directly a change in FGF ligands production (fig.5 E). Although I acknowledge that this information is suggesting that altered expression of FGF ligands may occur also in vivo, a formal demonstration is currently missing.
We thank the Reviewer for this suggestion and have carried out qPCR to quantify the levels of FGFs that are known to bind FGFR4, using cDNA derived from E16.5 Pax7iCre; Myh3+l+ (control) and Pax7iCre; Myh3delta/fl (fetal myogenesis specific knockout of MyHC-emb) embryo hind limbs. We find that FGF1 and FGF2 expression are significantly reduced, FGF4 expression is significantly increased and FGF5, FGF6 and FGF8 expression are unaffected in Pax7iCre; Myh3delta/fl compared to controls. This data has been added to the Supplementary data (Fig. S4A) and the results (lines 356-360).

3) It is confusing the report of a reduced fusion index in MyHC-emb siRNA treated cells compared to control cells (Fig 4K), when MyHC-emb siRNA cells conditioned media is reportedly inducing an increase in fusion index (Fig.5C). The authors should clarify. Moreover, I would suggest to evaluate also the fusion index in the in vitro rescue experiments with FGF, for completeness and to further

support the idea that exogenously added FGF is able to rescue the differentiation defects of MyHC emb siRNA treated cells.
The reduced fusion index is observed when C2C12 cells are treated with Myh3 siRNA (which leads to lower FGF levels in the media), and allowed to differentiate. Decrease in FGF levels leads to accelerated differentiation and depletion of the progenitor cell pool [as seen in vivo – Fig. 4A-C (compare E13.5 and E16.5 blots) and in the C2C12 experiment where higher MyoD levels are seen early in differentiation following knockdown of MyHC-emb – Fig. 5G, H]. However, in the conditioned media experiment, the cells are normal with respect to MyHC-emb, but the media has lower FGF levels. We believe this promotes differentiation compared to when the cells have depleted Myh3. Thus, while the Myh3 siRNA treatment results in both non-cell autonomous and cell autonomous effects, the conditioned media experiment tests only for non-cell autonomous effects (lines 329-334).
We have confirmed the reduced fusion index upon loss of MyHC-emb by culturing and differentiating primary myoblasts from Myh3Δ/Δ mice (Fig. 5K and Fig. S5C-D), as was the observed in vitro in C2C12 cells (previously Fig. 4K; currently Fig. S5E). As suggested by the Reviewer, we have exogenously added FGF to the media in Myh3 siRNA treated cells and observed a significant increase in the fusion index, which has been incorporated in the manuscript (Fig. 7D and lines 367-369).

Minor points:
1) The changes in MyHC-slow expression seem to be inconsistent in different experiments, such as those reported in Fig.2 I, Fig1K and Fig3A. Which muscle has been analyzed in Fig. 1K and Fig3A? The authors should clarify.
This is an interesting observation that we see consistently; RNA levels of MyHC-slow decreases upon loss of Myh3 whereas protein levels increase. This effect is consistent in C2C12 cells also, where MyHC-slow protein levels increase upon Myh3 knockdown (Fig. 5A, C) but the transcript levels decrease (data not shown). This inverse correlation suggests that MyHC-slow is not simply regulated at the transcriptional level, but other post-transcriptional, translational, or post-translational mechanisms might also play a role. Although there have been previous studies which also reported an inverse correlation between MyHC transcript and protein levels, the precise mechanism behind this has not been investigated, and could be related to changes in mRNA or protein stability, or rate of translation (Lyons et al J Cell Biol 1990; Cox et al Dev Biol 1991).
In Fig 1K, we have analyzed the number of MyHC-slow+ fibers over the entire shank region cross section and not of a single type of muscle which has now been mentioned clearly (lines 146 and 903-904). Similarly, for Fig. 4A (previously Fig. 3A), MyHC-slow levels is quantified using protein lysates from the entire hind limb (line 982).

2) The physiological relevance of the quantification of the reserve cells in the in vitro cell cultures is unclear. The authors should discuss it more extensively on the light of the reported in vivo defects in MyHC-emb KOs.
Reserve cells are the pool of undifferentiated cells in C2C12 cultures and are the equivalent of the satellite cells. We have now mentioned this explicitly, citing references, in the discussion (lines 409-411).

3) A representative image should be added for the quantification of PHH3+ cells in Figure S3E.
Representative images for quantification of PHH3+ cells added (Fig. S3E, E’ and F and line 302).

4) Line 155 there is an unnecessary comma after the word “diaphragm”.
Removed the comma.

Reviewer 2 Advance summary and potential significance to field
The role of developmental myosins is unknown, indeed this is a major open issue in muscle cell biology. Therefore the first report of embryonic myosin knockout is very important.

Reviewer 2 Comments for the author
The role of developmental myosins is still unknown, thus the first paper to report the specific knockout of Myh3, the gene coding for embryonic myosin heavy chain, is welcome.
This study reports interesting results on the effect of Myh3 loss, including the finding that Myh3 loss affects myogenic progenitors in a non-cell autonomous manner. However, some parts of the study are incomplete and a number of points should be addressed.

Major points
1. A major result of this study, not stressed by the authors, is that MyHC-emb is not required for muscle differentiation, as myogenesis can take place almost normally in the absence of Myh3. We have now mentioned that MyHC-emb is not absolutely required for muscle differentiation (lines 389-390). At birth, the overall size of leg muscles, as seen in leg cross-section (Fig.1C,D), appears to be similar in K0 and WT mice, fiber number is unchanged in SOL and even increased in EDL, fiber size is unchanged in EDL and shows only an increase of small fibers in SOL. On the other hand, postnatal muscle development is clearly affected, leading to severe scoliosis by 4-6 weeks in Myh3 KO mice. The authors should analyze the muscle phenotype, including muscle weight, fiber size and type, at P15 and P30. This is crucial period when muscle growth is first accompanied by proliferation and fusion of satellite cells (first 3 weeks), then by myofiber hypertrophy (see White … Zammit, Dev Biol 2010). As suggested by the Reviewer, we have carried out characterization of the muscle phenotypes at postnatal time points P15 and P30, which has been included in the manuscript as a new figure (Fig. 2). We have included whole body weight comparison between control and Myh3Δ/Δ mice at P0, and comparisons of TA, gastrocnemius and quadriceps muscle weights between control and Myh3Δ/Δ mice at P15 and P30. The fiber size analysis and MyHC-slow+ fiber type proportion have also been documented in control and Myh3Δ/Δ mice at P15 and P30 for the TA, EDL and soleus muscles. We have described these in detail in the Results (lines 153-181) and Discussion (lines 395-397).
2. The effect of Myh3 loss in vitro should be examined using primary cultures from fetal or neonatal WT and Myh3 KO muscles rather than in C2C12 cells treated with Myh3 siRNAs.
We thank the Reviewer for this suggestion and have carried out primary myoblast differentiation experiments using neonatal myoblasts from Myh3Δ/Δ and control pups. We observe a significant reduction in fusion index in the Myh3Δ/Δ samples, which has been included in the manuscript (Fig. 5K and lines 316-318).
3. The nomenclature of myosins should be correct and consistent throughout the paper, both in text and figures: use Myh3, not MyH3, for mouse gene, and MYH3 for human gene (see line 325); MyHC-emb should be used only for the protein product (e.g. 218, 414, etc), same for the other MYHs (e.g. 194-196, Fig. 2I,J, etc); the protein product of the Myh1gene should be always indicated as MyHC-IIx, not in some case MyHC-IId (see 75 vs 79). We thank the Reviewer for this correction and have incorporated these changes to the whole manuscript.

4. Inspection of whole leg muscle cross sections (Fig. 1C,D, Fig. S2A,B) shows that the size of soleus is much larger and that of gastro much smaller in K0 compared to WT samples. However, this could be due to the fact that the WT leg was sectioned at the mid belly of the muscles whereas the KO leg was sectioned towards the distal part of the leg. If this is true and these sections were used for muscle morphometry, unreliable data of fiber number and fiber size in soleus would be generated. It is imperative to compare muscles sectioned at the same level.
We thank the Reviewer for this comment. We have now carried out these experiments with additional control and knockout replicates, ensuring that the sections taken are from the middle of the limb. We sectioned the whole limb from knee to ankle and identified the sections towards the middle of the limb, which were used for analysis. We find that the number of myofibers per unit area is higher in the Myh3Δ/Δ mice compared to controls in both EDL and soleus, which has been incorporated in the manuscript (Fig. 1G-H and line 135).

5. Some relevant papers and reviews should be cited in the Introduction: Whalen et al (Nature 1981) first reported clear evidence for the existence of developmental myosins; Condon et al (Dev Biol 1990) described the pattern of expression of emb and neonatal/perinatal MYHs during development of mouse muscles; Schiaffino et al (Skelet Muscle 2015) recently reviewed the expression and function of developmental myosins.
We have included the suggested references (lines 84-85).

6. The legends of the figures are difficult to read and should be rewritten following the standard format used in Development: (A) …. . (B) … etc; or (A,B) … (A) …. (B).
We thank the Reviewer for pointing this out and have now corrected the figure legends as suggested.

Other points
1. How to explain the fact that about half of the K0 mice die in utero? This is surprising because even complete loss of muscles , e.g. in myogenin KO and even myogenin+MyoD double KO, does not lead to embryonic death. It would be of interest to determine at what stage embryos die. Is cardiac muscle involved? Rutland et al (Development 2011) reported that a probe specific for human MYH3 reacts with human and mouse developing heart. To my knowledge this is the only report on the presence of MyHC-emb in mammalian cardiac muscle and it would be important to verify this finding.
This is an interesting point and we agree with the Reviewer that it is unlikely the observed muscle defects lead to about half of the knockout mice dying in utero. It is likely due to cardiac expression of MyHC-emb although detailed characterization would require a cardiac specific Cre driver and stage specific characterization of cardiac phenotypes that is beyond the scope of the current manuscript. We have however checked MyHC-emb protein expression in the embryonic heart and do find it to be expressed at E16.5 (and absent in E16.5 Myh3Δ/Δ hearts), which has been added to the Supplementary Data (Fig. S4D) and results (lines 127-128). During our analysis, we found that most knockout embryos survive up to E13.5, and death happens thereafter, although the precise developmental stage at which they die is not clear.

2. An interesting question to be addressed is whether muscle regeneration is affected by Myh3 loss.
This is a very interesting point and will involve detailed analysis of adult myosin isoforms and differentiation markers over multiple time points of regeneration. Since the current manuscript is on embryonic, fetal and neonatal development, and since the regeneration aspect involves a large amount of additional work, we would like to present this data as a separate manuscript.

3. MYH accumulation could be examined by WB as a marker of muscle cell differentiation to validate the effect of FGF supplementation.
We have carried out this experiment and find that MyHC-slow levels are increased upon FGF supplementation at days 5 and 7, validating our hypothesis. This data has been incorporated in the manuscript (Fig. S4C and lines 374-375).

4. Inspection of whole leg muscle cross sections (Fig. 1C,D, Fig. S2A,B) shows that the size of soleus is much larger and that of gastro much smaller in K0 compared to WT samples. However, this could be due to the fact that the WT leg was sectioned at the mid belly of the muscles whereas the KO leg was sectioned towards the distal part of the leg. If this is true and these sections were used for muscle morphometry, unreliable data of fiber number and fiber size in soleus would be generated. It is imperative to compare muscles sectioned at the same level.
This comment is a repetition of major comment 4, which has been addressed.

5. The finding that diaphragm shows a distinct response to Myh3 K0 compared to leg muscle (173-181) could result not only from his anatomical and functional differences, as pointed out by the authors, but also from the fact that its degree of maturation is more advanced since it starts working immediately at birth with the onset of respiration, whereas ambulatory movements are gradually acquired several days after birth. In the rat, this leads to more rapid downregulation of Myh3, with transcripts almost undetectable at P0 in diaphragm but still present in leg muscles, and more precocious upregulation of Myh2 (IIa), which is already present at P0 in diaphragm but not in gastro (see Kelly et al, J Neurosci1991).
We have added a sentence to this effect (lines 207-209).

6. The relevance of the mouse model to human disease should be better discussed. The Freeman-Sheldon syndrome, the most severe of the syndromes due to MYH3 mutations, is characterized by distal arthrogryposis, with congenital contractures of hands, feet and orofacial muscles, while scoliosis is only seen in the most severe cases. Are signs of distal arthrogryposis in forelimbs and hindlimbs seen in the mouse mutants?
As suggested by the Reviewer, we have now included a paragraph discussing the Myh3Δ/Δ mouse model and its relevance to FSS in the discussion (lines 432-440). A study by Stevenson et al 2006 (cited in the manuscript), suggests that scoliosis is observed in 85% of FSS cases. The scoliosis phenotype is observed at ~6 weeks of age onwards in Myh3Δ/Δ mice and has been 100% penetrant. We do not see obvious signs of arthrogryposis in the Myh3Δ/Δ mice, although a detailed analysis needs to be carried out to confirm this and will be presented separately.

7. The comments about human disease in the Results (325-330) should be transferred to the Discussion.
We have transferred the comments about human disease to the discussion (lines 441-444).

8. Line 203: the term “drastic changes” does not seem appropriate (see major point 1).
We have replaced “drastic” with “led to” (line 237).

Reviewer 3 Advance summary and potential significance to field
In this study Sharma et al study several inducible and non-inducible models of Myh3 mutant mice. Mutations of this gene in human are responsible for Freeman-Sheldon and Sheldon-Hall syndromes, characterized by congenital contracture. The authors show that in absence of embryonic Myosin heavy chain (Myh3, expressed in post mitotic myogenic cells), half of animals die in utero. Half of the mutants survive at birth. These surviving newborns have been explored in the present study that shows that expression of Myh3 during development is required for harmonious muscle growth. Absence of its expression leads to several muscle defects that seem to depend upon specific muscle. Complementary transcriptomic experiments, cell culture experiments to knock down Myh3 by siRNA, and proteomic studies to identify secreted proteins by wt and mutant myotubes have been conducted, that demonstrate deficient FGF signaling in the mutant myotubes. This impaired FGF signaling is suggested to contribute to the mutant phenotype observed in the mouse model. Most of the experiments are well done, and the presented results are of major interest.

Reviewer 3 Comments for the author

Major concerns
1. - In the Figure 1C, the name of some muscles used in the study should be indicated, particularly the Sol and the EDL. From figures 1C (wt) and 1D(mutant) it seems that at P0 the mutant Soleus is larger and contains many more myofibers than its wt counterpart. It is surprising that Figure 1H does not reflect this observation.
We thank the Reviewer for these suggestions and have now labeled the soleus and EDL muscles with ‘S’ and ‘E’ respectively in Figure 1C’ and D’, as suggested by the Reviewer. We have now included additional control and knockout replicates and find that the number of myofibers per unit area is higher in the Myh3Δ/Δ mice compared to controls in both EDL and soleus, which has been incorporated in the manuscript (Fig. 1G-H and line 135).

- Since the EDL is studied it would be more interesting to present EDL in Figure 1C’’ and 1D’’ rather than an undefined (peroneus?) muscle.
We find that although MyHC-slow+ fiber number is trending towards an increase in the EDL, the increase is not significant (data not shown). Therefore, we have shown magnification of the flexor digitorum longus (FDL) muscle (line 898) where the difference in MyHC-slow+ fibers is clearly apparent.

- From the data shown in Figures 1C and 1D, it cannot be concluded that MyHC-emb (Myh3) regulates myofiber number in the fast EDL, because the muscle showing the most obvious increase in size is the slow Soleus.
As mentioned in part 1 of this comment, we have now included additional replicates and find that the number of myofibers per unit area is higher in the Myh3Δ/Δ mice compared to controls in both EDL and soleus (Fig. 1G-H and line 135).

- It is also difficult to conclude (lines 139-140) that myofiber cross sectional area (CSA) is decreased only in slow muscles such as the soleus, since no zoom of the fast EDL is provided and myofiber CSA seems reduced in the fast muscle of the mutant Figures 1C’’ and D’’.
We have now included magnified images of control and Myh3Δ/Δ EDL muscles from P0 pups (Fig. S2D, E and line 137).

2. It is important to indicate the percentage of genes downregulated in C2C12 cells after Myh3 knockdown that are downregulated in the four muscles studied (or in some of them) and the percentage of genes downregulated both in vivo and ex vivo. This information is relevant considering the effects of innervation or other cell/cell interactions that do not occur ex vivo myotubes/myofibers.

We did not carry out RNA-seq for C2C12 cells following Myh3 knockdown. Therefore, we do not have a list of genes that are mis-regulated across the 4 muscles in vivo compared to C2C12 cells in vitro. What we have presented is a validation of some of the mis-regulated genes in the 4 muscles in vivo, using C2C12 cells. It is possible that innervation or other cell-cell interactions are affected in Myh3 knockouts, possibly because of paracrine effects of loss of MyHC-emb from the muscle fibers. We feel adult muscle would be a better model to study this and plan to address this in the future.

3. The quality of Figures 3J and 3K must be improved. Furthermore, immunohistochemistry with activated Caspase3 antibodies would reveal if the increased cell death observed at P0 in Myh3-/- new born is specific of the mutant myofibers and or myoblasts (or both), a point that is not addressed by Western blot.
We thank the Reviewer for this suggestion and have incorporated better quality images for the MyoD immunofluorescence (Fig. 4J, K). We have carried out Caspase3 immunofluorescence and find that the increased cell death in P0 Myh3Δ/Δ muscles occurs in the myofibers. A representative image has been included (Fig. S5A-B' and line 265).

4. Line 245: since MyhC-embryonic is not expressed in myogenic progenitors or myoblasts, perhaps the authors could had supplementary data showing the absence of MyhC-emb protein in those cell populations (immunohistochemistry with MyhC-emb +/- MyoD or +/- Pax7 at E13. 5 or E14.5) or give a reference, since it is a very important aspect of this work and readers should be guided.
We agree that this is a crucial point and thank the Reviewer for this suggestion. To address this point, we isolated muscle stem cells by FACS (Pax7+ cells) and generated cDNA from these, along with cDNA from whole muscle, and carried out semi-quantitative PCR to detect levels of Myh3 transcripts, compared to positive control GAPDH (Fig. S4B). We find that Myh3 is not expressed in the muscle stem cell-derived cDNA compared to whole muscle cDNA, indicating that muscle progenitors do not express Myh3 (Fig. S4B). We have also added this in the results (lines 283-285). The following reference states that muscle stem cells do not express myosin heavy chains, which has also been included (Yoshida et al J Cell Sci 1998).

5. - I have a major problem with the fusion index methodology. This index, which is different from the differentiation index (MyHC+ cells), corresponds to cells with two or more nuclei. From the methods it seems that MyhC+ cells with a single nucleus have been counted.
We have included only those myofibers with two or more nuclei in all of our fusion index analyses. We agree that this was not explicitly mentioned in the methods and have now incorporated this (line 639).

6. It is commonly admitted that Myogenin+ cells do not proliferate and are named myocyte. MyoD+ Myogenin- cells on the other hand are proliferating myoblasts. Caution must be taken to keep this nomenclature in the text and Figure 6I.
We thank the Reviewer for the correction. We have removed Myogenin+ from the legend of the model (7J).

7. It is not explained why the conditioned medium from mutant cells (which present a reduced fusion index, Figure 4K) added to wt myogenic cells favor their fusion (Figure 5C). Addition of FGF2 on C2 cells inhibits the expression level of MyoD and of Myogenin, thus FGF2 impairs their differentiation and favors their reserve cells behavior. It is not clear why the authors used FGF2 since it not presented as a target of Myh3 in Figure 5F. This must be explained. At the opposite use of FGF6 (or a cocktail of identified misregulated FGF) which seems the most downregulated FGF in mutant cells would have more sense to challenge the consequences of its presence on myogenic cells properties.
The first part of this comment was raised by Reviewer 1 also (major point 3). We believe the difference is because in the Myh3 siRNA experiment, both non-cell autonomous and cell autonomous effects occur, whereas the conditioned media experiment tests only for non-cell autonomous effects (since the conditioned media experiment is carried out on normal C2C12 cells) (lines 329-334). We have confirmed the reduced fusion index upon loss of MyHC-emb by culturing and differentiating primary myoblasts from Myh3Δ/Δ mice, where we observe a reduction in fusion index (Fig. 5K and Fig. S5C-D).
With respect to the second part of the comment, FGF ligands are known to bind multiple FGF receptors to activate downstream signaling. Both FGF2 and FGF6 are expressed by the limb muscle

mass, with FGF2 expressed widely in the limb bud during development (Marics et al, Development 2002). Rather than try to identify the specific FGF or FGFs which might be involved in binding and activating FGFR4, our intention was to show proof of principle that the paracrine effect of loss of MyHC-emb on muscle progenitors is mediated by FGF signaling.
Since a related query was raised by Reviewer 1 (major point 2), we have now quantified the levels of FGFs that are known to bind FGFR4, using cDNA derived from E16.5 Pax7iCre; Myh3+l+ (control) and Pax7iCre; Myh3delta/fl (fetal myogenesis specific knockout of MyHC-emb) embryo hind limbs. We find that FGF1 and FGF2 expression are significantly reduced, FGF4 expression is significantly increased and FGF5, FGF6 and FGF8 expression are unaffected in Pax7iCre; Myh3delta/fl compared to controls. This data has been added in the Supplementary data (Fig. S4A) and the results (lines 356-360). Thus, FGF2 is one of two FGFs whose levels are downregulated upon loss of MyHC-emb function in vivo in the E16.5 limb.

8. The quantity and timing of Tamoxifen injection in pregnant Pax7iCRE animals must be indicated. Without this information it is impossible to interpret Figure 3. Have the authors verified that only secondary myofibers are deleted for Myh3 in this model, while all primary myofibers are not? Pax7iCre is not a tamoxifen inducible driver line (Keller et al, Genes and Dev 2004). This driver has been characterized in detail by Hutcheson et al, Genes and Dev 2008, where it has been clearly shown that Cre expression is driven in fetal myoblasts by this driver (Figures 2 and 3 in Hutcheson et al, Genes and Dev 2008).

9. - Pax7iCRE Myh3-/fl animals present a 50% reduction in the number of Pax7+ and a 60% reduction of MyoD+ cells at E16.5. Is this also the case in the total Myh3-/- fetuses? How is this result explained, considering the increased number of myofibers present in P0 animals, Figure1?
We thank the Reviewer for this suggestion and have carried out experiments to quantify Pax7+ progenitor numbers and MyoD levels at E16.5 in Myh3Δ/Δ and control embryos. We observe a reduction in the number of Pax7+ muscle progenitors and levels of MyoD in Myh3Δ/Δ embryos (Fig. S5G-H and S5F respectively and lines 272-275). These results validate the embryonic and fetal specific loss of MyHC-emb function. While we do observe an increase in myofibers at P0, the proportion of fibers with smaller area are increased in some muscles such as soleus (Fig. 1J). In addition, compensatory upregulation of other MyHCs (MyHC-IIx and MyHC-IIa are shown in Quad and Diaphragm in Figure 3I, J), which we observe at P0 could also be making up for loss of MyHC-emb function.

- While Pax7+ cells are depleted in mutant Pax7iCRE Myh3-/fl animals at E16.5 (line 242), their number is not altered at P0 in Myh3-/- animals. It would be important to indicate the number of Pax7+ cells and of MyoD + cells in Myh3-/- animals present at E16.5, to reconcile conflicting data arising probably from the use of distinct genetic models.
We have validated these as explained in the first part of Comment 9 above (Fig. S5G-H and S5F respectively and lines 272-275).

10. Figure 6I must be improved since it is misleading in the present form. The authors do not show that FGF promotes the differentiation of myogenic cells, rather that FGF produced by Myh3+ myofibers may control the balance between Pax7+ reserve/stem cells and their differentiation, while FGF blocks their differentiation.
We thank the Reviewer for this comment and have now included a paragraph explaining the model in detail (lines 445-453). We explicitly state that FGF controls the rate of differentiation of myogenic progenitors and myoblasts into myofibers (lines 448-449 and 1081-1082).

Second decision letter

MS ID#: DEVELOP/2019/184507

MS TITLE: Myosin Heavy Chain-embryonic regulates skeletal muscle differentiation during mammalian development.

AUTHORS: Megha Agarwal, Akashi Sharma, Pankaj Kumar, Amit Kumar, Anushree Bharadwaj, Masum Saini, Gabrielle Kardon, and Sam J. Mathew
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

I think this is a good quality study addressing for the first time with a genetic ablation approach the role of embryonic Myosin heavy chain during mouse development.

Comments for the author

In my opinion, the data presented by the authors are convincing and worth publication.

As a minor remaining issue, I would suggest adding one sentence of clarification to Reviewer 1's "minor point-1" highlighting the RNA vs protein discrepancy for MyHC-7. Line 234 of the present manuscript seems to be an appropriate location to insert such sentence. Obviously this is unnecessary if Reviewer's comments and Author's responses will be published along the manuscript...

Reviewer 2

Advance summary and potential significance to field

The first report of the effect of knockout of a developmental myosin is an important contribution to muscle biology.

Comments for the author

The points I made were addressed in the revised version. I agree that the analysis of muscle regeneration can be the onject of a future work.

Reviewer 3

Advance summary and potential significance to field

In this study Sharma et al study several inducible and non-inducible models of Myh3 mutant mice. Mutations of this gene in human are responsible for Freeman-Sheldon and Sheldon-Hall syndromes characterized by congenital contracture syndromes. They show that in absence of embryonic Myosin heavy chain (Myh3, expressed in post mitotic myogenic cells), half of mutant animals die in utero. Half of the mutants survive at birth, and many presented experiments have been performed with these newborn. This study shows that expression of Myh3 during development is required for harmonious muscle growth. Absence of its expression leads to several muscle defects that seem specific for each studied muscle. Complementary transcriptomic experiments, cell culture experiments to knock down Myh3 by siRNA, proteomic studies to identify secreted proteins by wt and mutant myotubes characterizing that FGF signaling is deficient in mutant myotubes have been conducted. This impaired FGF signaling is suggested to participate for the mutant phenotype observed in the mouse model. Most of the experiments are well done, and the presented results are of major interest.

Comments for the author

The manuscript has been greatly improved, and the authors answered all concerns.
Two minor points may be addressed before publication.

1- Line 269 of the manuscript : To be more precise concerning the observed phenotype, the sentence ".....increased cell death was evident at P0 in Myh3-/- muscles (Fig...” could be changed by: "increased cell death was evident at P0 in Myh3-/- myofibers (Fig...”

2- After reading the revised manuscript I was less convinced by the explanations concerning the cell autonomous effects observed in mutant myofibers. The authors could better explain these cell autonomous effects. The phenotypes observed are the consequences of diseased mutant myofibers. These mutant myofibers may be responsible for up-regulating other Myh genes to compensate Myh3 deficiency through paracrine effects (among which FGF and other molecules). The authors did not show that the mutant Myh3 allele was no more transcribed. If the mutant allele is still transcribed, it is difficult to argue that the transcription machinery of the myonucleus activates the nearest Myh2 gene to compensate Myh3 loss, without specific transcriptional reprogramming, that may be under the control of paracrine signals. Accordingly, Figure 7J still needs to be improved, for example the direct arrows linking Myh and FGF are misleading; impaired myofiber contraction due to Myh3 loss may intervene to imbalance of key signaling pathways - as suggested in the text- through mechanical signals that not only influence the surrounding myogenic stem cells but also the myofiber itself. This should be better explained.