

Porcine myogenesis in cloned wildtype and MYF5/MYOD/MYF6-null porcine embryo

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Dear authors,

I have reviewed the manuscript entitled "Molecular mechanisms that govern porcine myogenesis in the cloned wildtype and the MYF5/MYOD/MYF6-null porcine embryo". The study examined MYF5/MYOD/MYF6-null embryos produced by cloning MYF5/MYOD/MYF6-deleted fibroblasts using methods of gross anatomical, morphological, histological, molecular biological analyzes, bulk-RNA sequencing, and magnetic resonance imaging. The study is interesting. However, there are some questions and several areas required improvement. I hope my comments can help improve your work. The comments are listed as follows:

Title:

Actually, the manuscript is not deeply studied the molecular mechanisms that govern porcine myogenesis. Therefore, I recommend remove the words of molecular mechanisms and reorganization of the title.

Abstract:

The more methods or process were being introduced, which should present more results.

Introduction:

In the first paragraph, the more evidence of muscle loss should be introduced.

What's the research gap or scientific question?

Why produced the MYF5/MYOD/MYF6-null embryos? It means that why not the MYF5-null, MYOD-null or MYF6-null embryos?

Why chosen the stages of E41, E62, and E90 days? Cite ref or explanation.

If authors can introduce more meanings of MYF5/MYOD/MYF6-null embryos for medical research or some else?

Results:

In fact, "Developmental dynamics of muscle and internal organ systems in cloned WT porcine embryos" and "The myofiber development in WT porcine embryos was dynamic" were being studied. What's the new finding of your results?

Line 253-258: Except for phenotypes, if authors examined the molecular to grantee the success production of MYF5/MYOD/MYF6-null embryos?

Line 288: Why studied the myogenic stem cell and myogenic markers should explain in the results as a story.

Line 349: Why studied the neuromuscular junction should explain in the results as a story.

Figure 5a and 6a, I am curious that the expression of MYF5/MYOD/MYF6 in the MYF5/MYOD/MYF6-null embryos is 0?

Discussion

The results are not represented in Discussion again. In addition, the current content of Discussion is too much. Authors

should pay attention on several key points to discuss.
If possible, please add the future application of the current study.

Methods:

Bulk RNA-sequencing analysis: which version of gtf annotation file was used?

Reviewer #3

(Remarks to the Author)

This study explores the myogenesis of WT pigs and embryos lacking MYF5, MYOD, and MYF6 during development, which has significant biological relevance and application potential. The authors provide an in-depth understanding of muscle development in pigs, especially in the context of cloning technology. This research not only fills a knowledge gap regarding muscle development in pig models but also advances the field of regenerative medicine and gene therapy.

Major Review Comments

1. In the introduction of MYF5, MYOD, and MYF6, further emphasis on the roles of these factors in muscle development and the systemic effects of their absence would enhance clarity. It is recommended to include more citations from existing literature to support your arguments and provide a comprehensive background.

While the potential of pig models in regenerative medicine is mentioned, there is a lack of discussion on how these findings could impact related fields (e.g., disease models, gene therapy, or regenerative medicine). Adding this information in the introduction or discussion would strengthen the manuscript.

2. The current experimental design needs a more detailed description, particularly regarding the use of SCNT (somatic cell nuclear transfer) technology. Providing more information on the cell lines used, experimental procedures, and sample sizes will enhance the reliability and reproducibility of the results.

Detailed explanations of the statistical methods and software used, including the specific types of statistical tests applied and their appropriateness, are required to ensure the statistical significance of the results is properly validated.

3. While the phenotypes and gene expression changes in MYF5/MYOD/MYF6-null embryos are described, there is a lack of thorough biological interpretation of these results. It is recommended to clearly identify which key gene changes may directly lead to the observed phenotypic abnormalities and to provide possible mechanisms.

A more systematic comparative analysis of the developmental outcomes in mice and pigs is needed to highlight the advantages and uniqueness of the pig model. Consider referencing related literature to illustrate similar phenomena observed in mouse models and discuss the differences.

4. All figures should include detailed legends and descriptions that clearly indicate experimental conditions and observed results. Particularly in describing the comparisons of different developmental stages, it is essential to specify the time points and their significance.

In the results section, it is suggested to incorporate more quantitative data and statistical analysis results to strengthen the support for observed phenomena. For example, the cross-sectional area and number of muscle fibers should be presented in figure form, with statistical analysis included.

5. The current conclusions section lacks a comprehensive summary of the study's findings and a deep reflection on their biological significance. It is recommended to reiterate the important discoveries in the conclusion and discuss the implications for future research directions.

When discussing future research, more specific recommendations should be provided, such as further mechanistic studies or the potential for validating these findings in other animal models.

Minor Review Comments

1. Please carefully proofread the article for grammatical errors and unclear expressions, ensuring the use of precise, professional terminology. Some sentence structures are complex; simplifying them will improve readability.

2. It is necessary to explicitly state whether all experiments comply with ethical standards, including whether the use of SCNT technology received approval from relevant ethics committees.

This study provides important insights into the muscle development of pigs and the effects of gene deletion on this process, but comprehensive revisions are needed regarding experimental design, result analysis, and discussion depth. I look forward to seeing how you address these suggestions to enhance the quality and academic value of the article.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Dear authors,

I have rereviewed the manuscript entitled "Porcine myogenesis in cloned wildtype and MYF5/MYOD/MYF6-null porcine embryo". According to my previous comments, the major questions have been addressed. However, there are minor questions required improvement. The comments are listed as follows:

As responses in the rebuttal letter, "Ensembl's biomaRt database" was used to annotate genes, which should be introduced in the revised manuscript.

The significance of the histogram should be noted in all Figures, some figures were lacking.

The styles of all references should be checked carefully.

Reviewer #3

(Remarks to the Author)

This is an interesting study about the abnormal myogenesis of MYF5/MYOD/MYF6-null embryos in pig, and provides a valuable platform for myogenesis research of xeno- or exogeneic organs. In order to make the paper better published, authors should pay more attention to the concise theme, the cohesion of the story, the comparison between MYF5/MYOD/MYF6 alone and combined deletion with WT pigs, highlight the innovation of the paper, and further verify the mechanism on myogenesis of the muscle-specific transcription factors MYF5/MYOD/MYF6.

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We are grateful for the reviewers' insightful comments with regard to our manuscript (Manuscript ID: **COMMSBIO-24-4425-T**). We responded to each query and highlighted additional information added to the manuscript. Our point by point responses are detailed below.

Reviewer #1

Reviewer's summary: Dear authors, I have reviewed the manuscript entitled "Molecular mechanisms that govern porcine myogenesis in the cloned wildtype and the MYF5/MYOD/MYF6-null porcine embryo". The study examined MYF5/MYOD/MYF6-null embryos produced by cloning MYF5/MYOD/MYF6-deleted fibroblasts using methods of gross anatomical, morphological, histological, molecular biological analyzes, bulk-RNA sequencing, and magnetic resonance imaging. The study is interesting. However, there are some questions and several areas required improvement. I hope my comments can help improve your work. The comments are listed as follows:

Comment 1. Title: Actually, the manuscript is not deeply studied the molecular mechanisms that govern porcine myogenesis. Therefore, I recommend remove the words of molecular mechanisms and reorganization of the title.

Response 1: We have modified the title to read: "Porcine myogenesis in cloned wildtype and MYF5/MYOD/MYF6-null porcine embryo"

Comment 2. Abstract: The more methods or process were being introduced, which should present more results.

Response 2: We thank the reviewer for this suggestion. We were initially limited by the number of words for the manuscript format of *Communications Biology* but we agree with reviewer and the more results of this study are now included in the revised manuscript.

Comment 3. Introduction: In the first paragraph, the more evidence of muscle loss should be introduced. What's the research gap or scientific question?

Response 3: We thank the reviewer for this suggestion. As recommend by the reviewer, the revised manuscript, particularly in the section of Introduction, includes the more information regarding muscle loss as well as a description of research gaps.

Comment 4. Introduction: Why produced the MYF5/MYOD/MYF6-null embryos? It means that why not the MYF5-null, MYOD-null or MYF6-null embryos?

Response 4: We thank for reviewer's insightful comment. The selection of the triple knockout (*MYF5/MYOD/MYF6*) model was informed by findings from previous mouse genetic studies, which highlighted the compensatory interactions among myogenic regulatory factors (MRFs) and their hierarchical roles in skeletal muscle development.

Studies demonstrated that mice carrying single mutations in *Myf5*, *Myod*, or *Myf6* (*Mrf4*) exhibit normal skeletal muscle formation with typical patterning (**PMID: 1423602, 1330322, 7797078**). This is attributed to the compensatory mechanisms within the MRF family, where the absence of one factor is compensated by the upregulation or activity of another. Double mutations in both *Myod* and *Myf5* genes resulted in the complete absence of myoblasts and skeletal myofibers (**PMID: 8269513**). While *Myod* and *Myf5* double mutants lack myoblasts and skeletal myofibers, Kassam-Duchossoy *et al.* (**PMID: 15386014**) demonstrated that *Myf6* also plays additional determination factor in myogenesis. They identified that mice with *Myf5/Myod* double-null mutations were capable of forming skeletal muscle only when *Myf6* expression remained intact, revealing the role of *Myf6* as a myogenic determination gene.

Based on this evidence, we selected the triple knockout model targeting *MYF5*, *MYOD*, and *MYF6* to eliminate all compensatory mechanisms and comprehensively investigate the consequences of disrupting the myogenic lineage.

Thank you for allowing us to clarify this critical aspect of our study design. In response to your suggestion and the comments of reviewer #2 (regarding the need for emphasizing the roles of these factors and the systemic effects of their absence), we have revised the Introduction.

Comment 5. Introduction: Why chosen the stages of E41, E62, and E90 days? Cite ref or explanation.

Response 5: Thank you for reviewer's insightful comment. The stages E41, E62, and E90 were selected because they represent key time points in pig skeletal muscle development. The initial phase of skeletal muscle development, known as primary myogenesis, occurs between gestational days 30–60, during which myoblasts fuse to form primary myotubes (**PMID: 6630038**). This is followed by secondary myogenesis, which takes place from days 54–90, where myoblasts continue to fuse, using the primary myotubes as scaffolds to form secondary myotubes (**PMID: 6630038**). These stages provide a systematic framework for exploring pig myogenesis.

We appreciate your suggestion and have revised the manuscript accordingly. The explanation of these stages, originally part of the Discussion section, has been relocated to the Introduction to improve clarity and conciseness, aligning with reviewer's additional comment that "the current content of discussion is too much."

Comment 6. Introduction: If authors can introduce more meanings of MYF5/MYOD/MYF6-null embryos for medical research or some else?

Response 6: Thank you for reviewer's valuable feedback. Based on this reviewer's comments and those from another reviewer, we have revised the conclusion section in the Discussion to address these points. Future applications for medical research have been added to the manuscript, emphasizing the potential of the *MYF5/MYOD/MYF6*-null pig model as a platform for exogeneic donor muscle generation. This model creates a vacant niche for interspecific cell proliferation, allowing the production of human skeletal muscle as a promising therapeutic approach for volumetric muscle loss (VML). Furthermore, it supports the isolation of human myogenic stem cells, providing a critical resource for cell transplantation therapies and advancing the field of regenerative medicine.

Comment 7. Results: In fact, "Developmental dynamics of muscle and internal organ systems in cloned WT porcine embryos" and "The myofiber development in WT porcine embryos was dynamic" were being studied. What's the new finding of your results?

Response 7: Thank you for reviewer's thoughtful question. The specific section highlighted by the reviewer, "Developmental dynamics of muscle and internal organ systems in cloned WT porcine embryos" and "The myofiber development in WT porcine embryos was dynamic" focuses on our findings related to myogenesis in the cloned pig model (Figure. 1, Figure. 2, and related Supplementary Figures).

Our study utilized pigs produced through somatic cell nuclear transfer (SCNT) technology, a critical model for biomedical research and particularly valuable for advancing interspecies chimera production. Given the increasing importance of cloned pigs in these field, a detailed analysis and understanding of their key developmental stages are essential. Notably, there has been no prior research focusing on muscle development in cloned pigs, especially at the embryonic stages. Our study provides a unique and valuable contribution by investigating key myogenesis stages during embryonic development, presenting findings that fill a significant gap in the field.

In line with the primary objectives of this study, we concentrated on skeletal muscle development, presenting critical findings related to myogenesis. Additionally, the analysis of internal organ development offers important foundational data, which has

substantial value for future research. The implications and significance of these findings are further elaborated in the Discussion section, where we provide a comprehensive interpretation of their relevance. We hope this addresses your concern and highlights the novel contributions of our work.

Comment 8. Line 253-258: Except for phenotypes, if authors examined the molecular to grantee the success production of MYF5/MYOD/MYF6-null embryos?

Response 8: Thank you for reviewer’s question. In our previous study (**Maeng et al. PMID: 33782573**), we reported the PCR genotyping results confirming the successful production of *MYF5/MYOD/MYF6*-null embryos (See below). Furthermore, as addressed in our response to reviewer’s other comment, our Bulk RNA-seq data from the current study demonstrates that the expression levels of *MYF5*, *MYOD*, and *MYF6* in E41 and E62 *MYF5/MYOD/MYF6*-null embryos are indeed zero. These data provide robust molecular evidence supporting the successful generation of *MYF5/MYOD/MYF6*-null embryos.

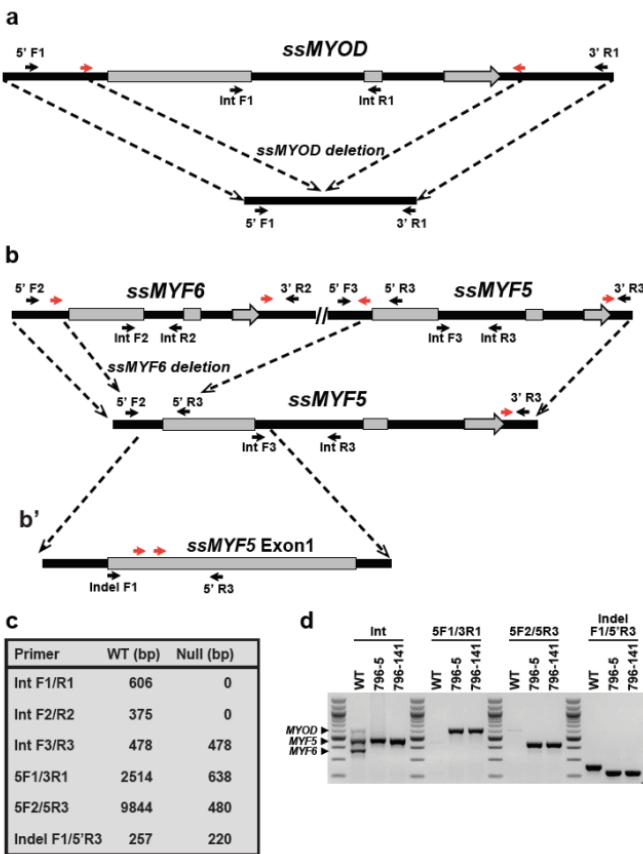


Figure in Maeng et al. (PMID: 33782573). Strategy and confirmation of MYF5/MYOD/MYF6 deletion in porcine fibroblasts. a,b Schematic genomic loci of the pig *MYOD*, *MYF5* and *MYF6* (aka MRF4) genes. *MYF5* and *MYF6* are adjacent genes in the same chromosome (**b**). Shaded rectangles: coding exons. Red arrows: gRNA targets. Black arrows: forward and reverse screening primers. Each gene was targeted with two gRNAs to delete the entire gene.

Only *MYOD* and *MYF6* genes were initially deleted. The *MYF5* gene was then sequentially targeted with two gRNAs after the start codon to generate a deletion as illustrated in b' which led to an in-frame stop codon for *MYF5*. c, Expected PCR products from the wild-type (WT) and null gene loci from each of the genes. d, PCR genotyping results of two clones having complete deletion of *MYOD*, *MYF6* and a deletion in *MYF5*.

Comment 9. Line 288: Why studied the myogenic stem cell and myogenic markers should explain in the results as a story.

Response 9: Thank you for reviewer's comment regarding the need to explain why we studied myogenic stem cells in *MYF5/MYOD/MYF6*-null porcine embryos.

Satellite cells, marked by Pax7 expression, are the primary stem cell population responsible for muscle regeneration and development. Myogenic regulatory factors (MRFs) are essential for the early stages of muscle fiber formation and growth, as they regulate the proliferation and differentiation of myogenic precursor cells, including satellite cells. In the absence of MRFs, satellite cells fail to commit to the myogenic lineage, leading to impaired differentiation. Therefore, it is critical to analyze the existence and impairment of satellite cells in the newly developed *MYF5/MYOD/MYF6*-null porcine model to understand the full extent of these regulatory disruptions

Previous studies in mice have shown that the deletion of MRFs like *Myf5* and *Myod* drastically altered the developmental trajectory of satellite cells during embryogenesis. For example, in the lack of *Myod*, satellite cells are unable to properly commit to the myogenic lineage, leading to impaired activation and proliferation (**PMID: 20544915**). In *Myf5*-null, muscle progenitor cells undergo normal epithelial-mesenchymal transition but exhibit defective migration, disrupting proper muscle development (**PMID: 8918877**). Additionally, in *Myod/Myf5* double KO, myogenic programming of satellite cell is disrupted (**PMID: 29478898**). These findings highlight the critical role of MRFs not only in maintaining the population size of satellite cells but also in ensuring their normal progression through the myogenic program.

However, despite these findings, there has been no study examining the fate of satellite cells in a triple knockout model, where all compensatory mechanisms among *MYF5*, *MYOD*, and *MYF6* are eliminated. Given this gap in the literature, we specifically aimed to investigate the impact of *MYF5/MYOD/MYF6* deletion on satellite cells and their differentiation in pigs.

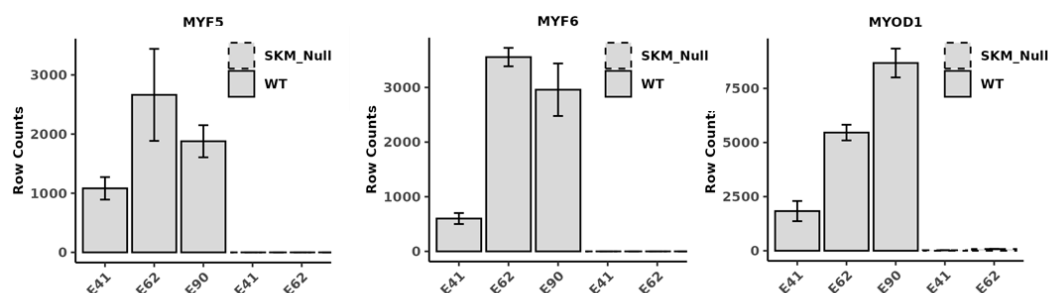
We have revised the manuscript to address this rationale explicitly in the Results. In addition, we have revised the Discussion to include a comparative analysis with existing mouse studies. We appreciate reviewer's valuable feedback, which has helped strengthen the scientific rationale and overall clarity of our manuscript.

Comment 10. Line 349: Why studied the neuromuscular junction should explain in the results as a story.

Response 10: We further explained and justified within the Results and Discussion the need to directly evaluate the neuromuscular junction (NMJ). As the synapse connecting the nervous system to the skeletal muscle system, and the site of innervation for each myofiber, the development of the NMJ during myogenesis is of key importance. The data highlight the post-synaptic limitations in NMJ formation following *MYF5/MYOD/MYF6* deletion (i.e., null embryo), and the expected patterning of the cloned WT embryos. For the long-term development and expansion of the platform for donor skeletal muscle generation this information is imperative.

Comment 11. Figure 5a and 6a, I am curious that the expression of *MYF5/MYOD/MYF6* in the *MYF5/MYOD/MYF6*-null embryos is 0?

Response 11: We have created bar plots showing the raw counts of *MYF5*, *MYOD*, and *MYF6* in each group (See below). Additionally, we have listed the mean and standard deviation of their raw counts. As expected, the expression of *MYF5*, *MYOD*, and *MYF6* in the *MYF5/MYOD/MYF6*-null embryos is indeed 0.



gene	condition	time	sd_count	mean_count
<chr>	<chr>	<chr>	<dbl>	<dbl>
MYF5	SKM_Null	E41	0.00	0.00
MYF5	SKM_Null	E62	0.00	0.00
MYF6	SKM_Null	E41	0.00	0.00
MYF6	SKM_Null	E62	0.00	0.00
MYOD1	SKM_Null	E41	5.66	24.00
MYOD1	SKM_Null	E62	7.78	82.50
MYF5	WT	E41	190.21	1082.50
MYF5	WT	E62	777.82	2663.00
MYF5	WT	E90	271.53	1878.50
MYF6	WT	E41	100.41	601.00
MYF6	WT	E62	169.00	3555.50
MYF6	WT	E90	480.46	2958.50
MYOD1	WT	E41	465.28	1832.00
MYOD1	WT	E62	362.04	5459.00
MYOD1	WT	E90	666.34	8672.75

Comment 12. Discussion: The results are not represented in Discussion again. In addition, the current content of Discussion is too much. Authors should pay attention on several key points to discuss. If possible, please add the future application of the current study.

Response 12: For the content of Discussion section, we agree to some extent with reviewer's observation regarding the length and focus. However, the revised manuscript reflects not only this reviewer's suggestion but also feedback from another reviewer, which required additional context and explanations. We believe that these revisions significantly enhance the manuscript by providing a thorough discussion of our findings and their implications. Given the comprehensive nature of our study, we feel that a detailed Discussion is necessary to adequately explain and interpret the results, as well as to highlight their significance in the broader context of porcine myogenesis and regenerative medicine. We have made every effort to ensure that the discussion remains focused and relevant, addressing all key findings and their biological and translational significance.

For future application, based on this reviewer's comments (comment #6) and those from another reviewer, we have revised the conclusion section in the Discussion to address these points. Future applications have been added to the manuscript, emphasizing the potential of the *MYF5/MYOD/MYF6*-null pig model as a platform for exogeneic donor muscle generation and the potential source for human myogenic stem cells.

Comment 13. Methods: Bulk RNA-sequencing analysis: which version of gtf annotation file was used?

Response 13: In this bulk RNA-seq analysis pipeline, we first used tximport to prepare the input for DESeq2. The transcriptome index was generated from the Ensembl release 108 FASTA file for *Sus scrofa* (Sscrofa11.1 genome build). Although a GTF file was not directly used in the pipeline, the transcript-to-gene mapping required for downstream analysis was obtained from Ensembl's biomaRt database, aligned with the Ensembl release 108 transcript sequences used to build the index.

Reviewer #2

Reviewer's summary: This study explores the myogenesis of WT pigs and embryos lacking MYF5, MYOD, and MYF6 during development, which has significant biological relevance and application potential. The authors provide an in-depth understanding of muscle development in pigs, especially in the context of cloning technology. This research not only fills a knowledge gap regarding muscle development in pig models but also advances the field of regenerative medicine and gene therapy.

Major Review Comments

Comment 1. In the introduction of MYF5, MYOD, and MYF6, further emphasis on the roles of these factors in muscle development and the systemic effects of their absence would enhance clarity. It is recommended to include more citations from existing literature to support your arguments and provide a comprehensive background. While the potential of pig models in regenerative medicine is mentioned, there is a lack of discussion on how these findings could impact related fields (e.g., disease models, gene therapy, or regenerative medicine). Adding this information in the introduction or discussion would strengthen the manuscript.

Response 1: Thank you for reviewer's insightful comment regarding the need for further emphasis on the roles of *MYF5*, *MYOD*, and *MYF6* in muscle development and the systemic effects of their absence. We have revised the Introduction to provide a more comprehensive background on the distinct roles of these myogenic regulatory factors (MRFs).

In response to reviewer's suggestion, we have included additional references from existing literature to highlight the compensatory mechanisms among *MYF5*, *MYOD*, and *MYF6* (*MRF4*) observed in mouse models. Furthermore, we emphasized the systemic consequences of their simultaneous absence, such as the lack of skeletal myoblasts and myofibers in *Myf5/Myod* double mutants and the critical role of *Myf6* as an additional myogenic determination factor. These revisions enhance the clarity and depth of the Introduction, addressing the roles of these factors in muscle development.

In addition, as reviewer's comment, we have revised the conclusion section of Discussion. Future applications have been added to the manuscript, highlighting the *MYF5/MYOD/MYF6*-null pig model as a platform for exogeneic donor muscle generation. This model creates a vacant niche for interspecific cell proliferation, enabling the production of human skeletal muscle as a potential therapy for volumetric muscle loss (VML). Additionally, it facilitates the isolation of human myogenic stem cells, offering a valuable resource for cell transplantation therapies and advancing regenerative medicine.

We appreciate reviewer's valuable feedback, which has helped strengthen the scientific rationale and overall clarity of our manuscript.

Comment 2. The current experimental design needs a more detailed description, particularly regarding the use of SCNT (somatic cell nuclear transfer) technology. Providing more information on the cell lines used, experimental procedures, and sample sizes will enhance the reliability and reproducibility of the results.

Detailed explanations of the statistical methods and software used, including the specific types of statistical tests applied and their appropriateness, are required to ensure the statistical significance of the results is properly validated.

Response 2: Thank you for reviewer's comment regarding the need for a more detailed description of the experimental design, particularly the use of SCNT technology. In response, we have provided detailed information regarding the production of *MYF5/MYOD/MYF6*-null porcine embryos. Specifically, we included data on the cell lines used, the number of cloned embryos transferred, the number of gilts used for embryo transfer, the number of confirmed pregnancies, and the number of embryos harvested at each developmental stage. This information has been compiled into a table, which we have included as a **Supplementary Table 2** in the revised manuscript (See below).

Regarding the need for detailed explanations of the statistical methods and software used. We have carefully revisited and thoroughly reviewed the statistical analyses and software used in the manuscript.

We believe that these revisions enhance the clarity, reliability, and reproducibility of our experimental procedures. We appreciate reviewer's feedback, which has strengthened the methodological transparency of our study

Supplementary Table 2. Summary of the production for *MYF5/MYOD/MYF6*-null porcine embryo

Donor cell line for SCNT	Transferred embryos	Transferred gilts	Pregnant gilts	E-age at harvest	Harvested embryos
<i>MYF5/MYOD/MYF6</i> -null	694	5	1	E42	10*
<i>MYF5/MYOD/MYF6</i> -null	520	4	1	E62	6
<i>MYF5/MYOD/MYF6</i> -null	348	3	3	E90	4**

*One was identified as a resorbing embryo

**All four embryos harvested were identified as resorbing embryos

Comment 3. While the phenotypes and gene expression changes in MYF5/MYOD/MYF6-null embryos are described, there is a lack of thorough biological interpretation of these results. It is recommended to clearly identify which key gene changes may directly lead to the observed phenotypic abnormalities and to provide possible mechanisms.

A more systematic comparative analysis of the developmental outcomes in mice and pigs is needed to highlight the advantages and uniqueness of the pig model. Consider referencing related literature to illustrate similar phenomena observed in mouse models and discuss the differences.

Response 3: Thank you for reviewer's valuable comment. To address reviewer's feedback, we have significantly revised the Discussion section to include a detailed comparison between our findings in pigs and the well-characterized phenotypes of MRF-disrupted mouse models. Specifically, we incorporated references to mouse studies that describe abnormal phenotypes, such as spinal curvature, limb deficiencies, and edema, in *Myf5/Myod* double mutants and *Myog*-null mice, and compared these with the analogous phenotypes observed in our MYF5/MYOD/MYF6-null porcine embryos. Furthermore, we discussed unique features of the pig model, including the severity of rib and sternal defects and their potential implications for respiratory failure, which were not observed in mice. This comparison highlights the translational value of the pig model in understanding the systemic effects of skeletal muscle loss.

We also expanded on the interpretation of gene expression changes by referencing studies that detail the roles of MRFs in regulating satellite cell populations and differentiation. These comparisons strengthen the biological interpretation of our results and link the observed phenotypes to specific gene regulatory mechanisms.

By integrating these revisions, we have provided a more comprehensive analysis of our findings, clarified the biological significance of the gene expression changes, and emphasized the advantages of the pig model for studying myogenesis. Thank you for reviewer's constructive feedback, which has greatly enhanced the clarity and depth of our manuscript.

Comment 4. All figures should include detailed legends and descriptions that clearly indicate experimental conditions and observed results. Particularly in describing the comparisons of different developmental stages, it is essential to specify the time points and their significance.

In the results section, it is suggested to incorporate more quantitative data and statistical analysis results to strengthen the support for observed phenomena. For example, the cross-sectional area and number of muscle fibers should be presented in figure form, with statistical analysis included.

Response 4: Thank you for reviewer's comment regarding the need for detailed descriptions to clarify the experimental conditions and observed results, particularly concerning the developmental time points. In response, we have revised the Introduction section to address another reviewer's comment, "*Why chosen the stages of E41, E62, and E90 days?*". This revision provides a clear explanation of the developmental time points and their significance. These changes will help readers better understand the rationale behind the selected time points and their importance in the study. We appreciate reviewer's feedback, which has enhanced the clarity and comprehensibility of our manuscript.

Regarding the comment on the cross-sectional area and muscle fiber results, the average cross-sectional area at each developmental time point is displayed in **Fig. 2h**; additionally, the distribution of fiber sizes for each embryo is shown in **Supplementary Fig. 3g-h**. A one-way ANOVA across the average CSA is not different between the embryo stages.

Comment 5. The current conclusions section lacks a comprehensive summary of the study's findings and a deep reflection on their biological significance. It is recommended to reiterate the important discoveries in the conclusion and discuss the implications for future research directions.

When discussing future research, more specific recommendations should be provided, such as further mechanistic studies or the potential for validating these findings in other animal models.

Response 5: Thank you for reviewer's valuable feedback. As reviewer suggested, we have revised the conclusion section to include a concise summary of the study's key findings and their biological significance. Additionally, in response to reviewers' other comment recommendation, we have expanded the conclusion section to address future applications of our findings,

Minor Review Comments

Comment 1. Please carefully proofread the article for grammatical errors and unclear expressions, ensuring the use of precise, professional terminology. Some sentence structures are complex; simplifying them will improve readability.

Response 1: Thank you for reviewer's valuable suggestion. We have carefully proofread the manuscript to address grammatical errors and unclear expressions, ensuring the use of precise and professional terminology throughout the text. Additionally, we simplified complex sentence structures where necessary to enhance readability and improve clarity.

Comment 2. It is necessary to explicitly state whether all experiments comply with ethical standards, including whether the use of SCNT technology received approval from relevant ethics committees.

Response 2: Thank you for reviewer's comment regarding ethical compliance. In our original manuscript, the Methods section (subtitle: *Animal Assurance*) states that our study received approval from the relevant ethics committees, including Institutional Review Board (IRB), Institutional Animal Care and Use Committee (IACUC), and Stem Cell Research Oversight (SCRO). Specifically, all experiments involving the use of SCNT technology were conducted in accordance with the ethical standards approved by these committees. Thank you for reviewer's attention to this important matter.

Response to Reviewers:

We are pleased that our manuscript was viewed favorably. We wish to respond to the remaining comments of Reviewers 2 and 3. All changes are demarcated in red in the manuscript.

Reviewer 2:

1. As responses in the rebuttal letter, “Ensembl’s biomaRt database” was used to annotate genes, which should be introduced in the revised manuscript.
We have added the text to include the use of Ensembl biomaRt database to the text of the manuscript.
2. The significance of the histogram should be noted in all Figures, some figures were lacking.
Statistical significance has now been added to all the figures. We apologize for this oversight on our part.
3. The styles of all references should be checked carefully.
We have corrected the reference styles to match that of the Nature Journals.

Reviewer 3:

1. In order to better understand the main findings of this paper, reorganization of the title is needed.
In response to reviewer 1 of the initial review, we modified the title. Can Reviewer 3 offer some guidance as to the request for further modification?
2. The abstract is too long, summarization of the main idea of this paper is needed.
We have modified the abstract to make it more succinct.
3. The sentence of “These studies provide an important platform for engineering humanized muscle in gene-edited pigs” is exaggerated.
We have modified and clarified this sentence.
4. The part of “Introduction” did not well elicit relevant knowledge about the differences between species in the muscle-specific transcription factors MYF5/MYOD/MYF6, and why these transcription factors are selected.
We have added a clarifying statement to the introduction.
5. The presentation of the results suggests a focus on comparing the developmental differences in skeletal muscle development between MYF5/MYOD/MYF6 alone and combined deletion with WT pigs.
To clarify, our study focuses on comparing the developmental differences in skeletal muscle between MYF5/MYOD/MYF6-null pigs (triple knockout) and WT pigs. We did not analyze individual deletions (MYF5, MYOD, or MYF6 alone) in pigs; these comparisons are drawn from previous mouse studies, as referenced.
6. Why don't authors combine multiple omics data to analyze the dynamic changes in myofibers, such as articles “Yang Y, Fan X, Yan J, et al. A comprehensive epigenome

atlas reveals DNA methylation regulating skeletal muscle development. Nucleic Acids Res. 2021". If there is a difference between the results?

If the reviewer is suggesting that we perform multiomics, we feel that this request is problematic as 1) it was not mentioned in the first round of review, 2) this will delay the manuscripts by several months, and 3) it is beyond the scope of this manuscript. Furthermore, any direct comparison between our results and Yang et al., should occur at age matched dates in development and our dates do not match those of Yang et al.

7. The relationship between the neuromuscular junction development and MYF5/MYOD/MYF6 transcription factors is not well elaborated.

We have added the rationale for this evaluation in the Introduction.

8. Connectivity and paragraph writing need to be further modified.

We have made modifications to create smoother transitions between sentences and paragraphs.

Comments:

MYF5, MYOD and MYF6 are important transcription factors for skeletal muscle development. This is an interesting study about the abnormal myogenesis of MYF5/MYOD/MYF6-null embryos in pig, and provides a valuable platform for myogenesis research of xeno- or exogeneic organs. To make the manuscript more rigorous, I suggest the authors to improve as below:

1. In order to better understand the main findings of this paper, reorganization of the title is needed.
2. The abstract is too long, summarization of the main idea of this paper is needed.
3. The sentence of “These studies provide an important platform for engineering humanized muscle in gene-edited pigs” is exaggerated.
4. The part of “Introduction” did not well elicit relevant knowledge about the differences between species in the muscle-specific transcription factors MYF5/MYOD/MYF6, and why these transcription factors are selected.
5. The presentation of the results suggests a focus on comparing the developmental differences in skeletal muscle development between MYF5/MYOD/MYF6 alone and combined deletion with WT pigs.
6. Why don't authors combine multiple omics data to analyze the dynamic changes in myofibers, such as articles “Yang Y, Fan X, Yan J, et al. A comprehensive epigenome atlas reveals DNA methylation regulating

skeletal muscle development. Nucleic Acids Res. 2021”. If there is a difference between the results?

7. The relationship between the neuromuscular junction development and MYF5/MYOD/MYF6 transcription factors is not well elaborated.

8. Connectivity and paragraph writing need to be further modified.