# Formal proof of the requirement of MESP1 and MESP2 in mesoderm specification and the transcriptional control via specific enhancers in mice

Rieko Ajima, Yuko Sakakibara, Noriko Sakurai-Yamatani, Masafumi Muraoka and Yumiko Saga
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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/194613

MS TITLE: Formal proof of the requirement of MESP1 and MESP2 in mesoderm specification and control via the Wnt signaling pathway in mice

AUTHORS: Rieko Ajima, Yuko Sakakibara, Noriko Sakurai-Yamatani, and Yumiko Saga

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

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

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

In this study Ajima et al re-evaluate and extended understanding of mouse Mesp1 and Mesp2 functions using genome editing. The work is carefully performed and clearly documented and clarifies a number of questions important for understanding Mesp gene function and mesoderm patterning during gastrulation, as well as issues concerning gene mutation studies more generally. However, the following points should be addressed.

*Comments for the author*

1. Concerning the CRISPR-Cas9 technology, please elaborate on the increased numbers of Type 1 embryos over other types in the F0 analysis (Table 1). Is Mesp1 targeting favored when both genes are targeted? Were the genes also targeted singly? Does the proximity of the genes impact on the Crispr mutations? Finally, please note if any large deletions between the two genes were generated and evaluate or comment on the risk of off-target mutations.

2. The mechanism underlying the differences in penetrance of the various Mesp1 alleles is unresolved, though as the authors discuss likely concerns regulation of the neighboring Mesp2 gene. It seems important to confirm this - can the authors add any data on Mesp1 expression to show Mesp2 activation or upregulation with the new alleles, that may fail when PGK-Neo is present?

3. Please discuss the paper by Chiapparo et al (PMID: 27185833) and the suggestion that some genes may be exclusive Mesp1 targets. This would suggest that defects in cardiac progenitor cell migration might be anticipated even on more precise deletion of Mesp1, yet no cardiac phenotype was apparent.

4. If the EME is required for maintenance rather than initiation of Mesp1 transcription, is it correct to say (as stated in the abstract) that Wnt signaling is important for activation of Mesp1? At least, "transcriptional activation" should be replaced by "transcription".

5. Do the authors consider that Wnt signaling also regulates Mesp2? Or that such regulation may be indirect via Mesp1-mediated repression?

6. The structure of the mutant embryo in Fig 1C is not very clear.

Reviewer 2

*Advance summary and potential significance to field*

The authors re-investigated the requirements for Mesp1 and Mesp2 in the mesoderm specification by utilizing CRISPR/Cas9 genome editing. Since previously reported Mesp mutant mice contain the PGK-neo cassette, it cannot be excluded that this cassette may affect gene expression and phenotype in Mesp mutant mice. Inconsistent with their previous reports, newly generated Mesp1-single null mutant looks obviously normal. Because Mesp2 expression was upregulated in this Mesp1-single null mutant, this enhanced Mesp2 expression probably compensate loss of Mesp1 function in this mutant. On the other hand, the newly established Mesp1/Mesp2 dKO mutants showed similar phenotype to that of previously reported dKO. In addition to these analyses of Mesp mutant embryos, the authors showed that Mesp1 expression was lost in Wnt3 KO embryos and that canonical Wnt signaling regulates Mesp1 expression via a TCF/LEF binding site on the early mesoderm enhancer of Mesp1. Based on these results, the authors concluded that spatiotemporal transcriptional activation of Mesp regulated by canonical Wnt signaling is required for mesoderm identification.

As mentioned above, the authors reexamined the phenotypes of previously generated MespP1 and Mesp2 KO mutants by utilizing modern genome editing technique. This approach is very important, as it is not excluded that some additional sequences integrated into the genome close to the Mesp loci can have unpredicted effect on expression of Mesp genes. Especially, I would like to emphasize here that the authors' efforts to thoroughly elucidate the gene function are worthy of respect. The data reported in the paper basically well supported the conclusions drawn although some points should be clarified. Unfortunately, however, the results shown by the newly generated mutants did not have a strong impact on the field. The advance made in the latter part of this paper, activation of Mesp1 expression by Wnt signaling, is important but the biological significance of this finding should be further explored.

*Comments for the author*

Specific comments:
1) Figure 1b: it is difficult to judge whether somite morphology is impaired or not. I would like to ask the authors to improve the quality of the image or to add in situ hybridization data. In addition, it would be better to show cardiac morphology in Mesp1 null mutant established by genome-editing.
Since F0 embryos may have mosaicism that cause variable severity of the phenotype, it seems difficult to compare the phenotypes of Mesp1 and/or Mesp2 mutants with or without the PGK-neo cassette. One of the ways to more accurately confirm the phenotype of Mesp mutants, CRISPR/Cas9 genome editing should be performed on the established Mesp1 and/or Mesp2 KO mouse strains.

2) Fig.3A, B; Accumulation of mesodermal cells around the PS is not clear. Could the authors add cross section analysis as shown in Kitajima et al., 2000?

3) Fig.4; the author generated Mesp1Cre?neo mice in which Cre was inserted at the Mesp1 locus but PGK-neo cassette was removed. This mutant developed normally and showed up-regulated Mesp2 expression. From these experiments, they concluded ?prolonged Mesp2 expression rescued the phenotypes caused by Mesp1 null, but the PGK-neo cassette insertion prevented this rescue mechanism.? (line185,186). However, some points are still unclear.
First, in order to evaluate the variable phenotype of the Mesp1 mutants in the targeting strategy, it is important to compare directly the Mesp1 mutant phenotypes with and without the PGK-neo cassette. The authors described this point in the text but should show image data.
Second, in the previous paper, Mesp1 KO embryos with the PGK-neo cassette inserted showed abnormal heart morphogenesis although Mesp2 expression was upregulated (Kitajima et al., 2000). The authors should cite "Kitajima et al., 2000" in this section of the manuscript and discuss the relationship between the phenotype of Mesp1 mutants and Mesp2 expression more carefully.
In addition, since they cannot exclude other possibilities such as inserted PGK-neo cassette affect another gene expression and cause defect in heart morphogenesis, they should be discussed more carefully in this point.

4) Figure 5: They hypothesized that the expression of Mesp1 and Mesp2 might be regulated by Wnt and Nodal signaling. However, they analyzed only Mesp1 expression but not Mesp2. Since down-regulation of Mesp1 can result in up-regulation of the Mesp2 gene, the authors should analyze Mesp2 expression in Wnt3 and Lefty2 mutant mice. Furthermore, to analyze the requirement for continued activation of Wnt signaling and suppression of Nodal signaling in the mesoderm specification, it had better to check expression of Wnt and Nodal signaling target genes in Wnt3 and Lefty2 mutants, respectively.

5) To evaluate the possibility that Lefty2 expression regulated by Mesp1 and Mesp2 is essential for mesoderm differentiation, the authors generated Mesp1Lefty2 / Lefty2. Mesp2 null mice lacking the Mesp gene in Lefty2 knocked in at the Mesp1 locus. They conclude that "Lefty2 expression cannot rescue the loss of MESP1/2 in mesoderm differentiation". (Rows 221,212). However, it is difficult to judge from Fig. 5G-L whether mesoderm differentiation is occurring. To reveal the mesoderm status of cells, it is necessary to analyze expression of marker genes, including Eomes, Wnt3, Wnt3a, in Mesp1/2 dKO (Fig. 3).

6) Figure 6; The authors concluded that "Mesp1 expression in early mesoderm is regulated by canonical Wnt signaling." (Line 214). However, in FIG. 6B, the embryonic stage was not described

and it seems relatively later stage at which Mesp1 is not expressed. Does this reporter recapitulate Mesp1 expression? Furthermore to reveal the transcriptional regulation of Mesp1 in early mesoderm, it is necessary to perform the Mesp1 reporter assay at an early stage.
Also, what is "Ex-2" in Figure 6D? Is this a previously identified TCF/LEF binding site?

7) Figure 7: What is the significance of Wnt-regulated Mesp1 expression after the LB stage? The author has already discussed this point at lines 338-346. However, analysis of markers involved in mesoderm specification or epithelial-mesenchymal transition in the Mesp1EME?1 mutant is informative.

Minor comments:
1) Figures 2A-C, 6A and S3A are not referenced in the main part of the manuscript.
2) Please indicate size of the scale bars in Figures 1 and 6B.
3) Lines 225, 226: Incorrect number in figure.
4) Figures 6C, D: Which statistical test did the authors use? What does the error bar indicate?

Reviewer 3

*Advance summary and potential significance to field*

The manuscript by Ajima, Saga and colleagues re-explores the role of Mesp1 and Mesp2 in mesoderm formation in mice based on findings in zebrafish that double KO of Mesp1/2 affects somite formation but not early mesoderm formation (as previously reported in double KO mice). The authors remake the Mesp1 and Mesp1 KOs using Crispr technology and these lack the PGK-neo cassette used to make the original KOs. The findings indicate that the new double KO does replicate the phenotype reported earlier with the difference that the single Mesp1 KO does not show the heart phenotype described previously, thought to be because Mesp2 is up-regulated in the primitive streak of Mesp1 mutants. Removal of the PGK-neo cassette from one of the earlier Mesp1 KO strains (a Cre insertion) then resulted in upregulation of Mesp2. The study goes forward by considering how Mesp1 might be regulated by Wnt3 (expressed transiently in the primitive streak) and whether one of the key downstream effectors of Mesp genes might be the nodal inhibitor Lefty2 (severely downregulated in Mesp1/2 KOs). Indeed, Mesp1 is downregulated in Wnt3 KOs and the authors showed that several Tcf transcription factor binding sites present in and around a documented upstream Mesp1 enhancer were essential for maintenance of Mesp1 expression in vivo and in vitro, and that Tcf factors occupy these sites even in the absence of induced canonical Wnt signaling. However, a knockin of the Lefty 2 gene into the Mesp1 locus, when crossed to Mesp2 KOs, did not rescue the double KO phenotype.

*Comments for the author*

Whereas the embryology and in situs are exquisite, the paper fails to constitute a compelling story or make major advances. The clarification of the Mesp1 phenotype and confirmation of the double KO phenotype are interesting details that would be better suited to another journal. The additional data on the Lefty2 knockin (which offered no advance) and clarification that Wnt signaling through Tcf is likely to be important for cis-regulation of Mesp1 is an interesting but incremental advance, and a more detailed functional analysis of this enhance is still pending. Under these circumstances, I cannot support publication.

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**First revision**

Author response to reviewers' comments

Our responses to the reviewers’ comments are as follows:

Reviewer 1 Comments for the author

1. Concerning the CRISPR-Cas9 technology, please elaborate on the increased numbers of Type 1 embryos over other types in the F0 analysis (Table 1). Is Mesp1 targeting favored when both genes are targeted? Were the genes also targeted singly? Does the proximity of the genes impact on the Crispr mutations? Finally, please note if any large deletions between the two genes were generated and evaluate or comment on the risk of off-target mutations.

The number of Type 1 embryos was high because of the low double-strand break efficiency of the sgRNA for Mesp2. To evaluate if there is a preference for targeting between the neighboring Mesp1 and Mesp2 genes, we conducted single injections and added the results in Table S2. The efficiency of one of the sgRNAs for Mesp2 (Mesp2-5) was low, but the other (Mesp2-2) was comparable to that of the sgRNA for Mesp1. We considered the efficiency to depend on each sgRNA, and there to be no preference between Mesp1 and Mesp2 genes. The reason why the overall efficiency increased this time is not clear, but it may have been because we purchased a new injector for microinjection between the previous experiment and experiment for the revised manuscript.
We added a column for the observation of large deletions between the two genes in Table 1 and Table S2. We back-crossed the mouse lines established by the CRISPR/Cas9 technique with wildtype mice to eliminate the risk of off-target mutations, and added the details in the Materials and Methods section on p28 line 595.

2. The mechanism underlying the differences in penetrance of the various Mesp1 alleles is unresolved, though as the authors discuss likely concerns regulation of the neighboring Mesp2 gene. It seems important to confirm this - can the authors add any data on Mesp1 expression to show Mesp2 activation or upregulation with the new alleles, that may fail when PGK-Neo is present?

In order to reveal the mechanism underlying the difference in phenotypes of different Mesp1 alleles more directly, we established a new Mesp1 null allele (Mesp1ΔNeo) by removing the PGK-neo cassette from the previously established Mesp1Neo mice and compared between them, as shown in Figure 4. Mesp1ΔNeo/ΔNeo mice developed normally and were fertile, suggesting that the phenotypes observed in the previous Mesp1 null mice lines were due to PGK-neo cassette insertion. Furthermore, the phenotype was likely due to the difference in the expression level of Mesp2

3. Please discuss the paper by Chiapparo et al (PMID: 27185833) and the suggestion that some genes may be exclusive Mesp1 targets. This would suggest that defects in cardiac progenitor cell migration might be anticipated even on more precise deletion of Mesp1, yet no cardiac phenotype was apparent.

As mentioned above, Mesp1ΔNeo/ΔNeo mice developed normally and were fertile, suggesting that MESP2 can fully rescue MESP1 function in vivo. There are several possibilities, such as exclusive MESP1 target genes being indespensable for mesoderm formation, or the up-regulation of MESP2 being sufficient to rescue the expression of the genes directly or indirectly. We added further discussion and cited the paper by Chiapparo et al. on p17 line 357.

4. If the EME is required for maintenance rather than initiation of Mesp1 transcription, is it correct to say (as stated in the abstract) that Wnt signaling is important for activation of Mesp1? At least, "transcriptional activation" should be replaced by "transcription".

We agree with this comment. The sentence was removed from the abstract.

5. Do the authors consider that Wnt signaling also regulates Mesp2? Or that such regulation may be indirect via Mesp1-mediated repression?

In order to address such a possibility, we examined Mesp2 expression in Mesp1EMEΔ1/Δ1 embryos and found that the expression was unchanged by deletion of the EME that contained TCF/Lef1 binding sites (Figure 8I-N). However, simultaneous deletion of the EME and HCR2 resulted in down-regulation of Mesp2 expression (Figure 9D, F), suggesting that Mesp2 expression is regulated by Wnt signaling through the EME. We discussed this on page 20 line 420-435.

6. The structure of the mutant embryo in Fig 1C is not very clear.

We replaced the image in Figure 1C.

Reviewer 2 Comments for the author

Specific comments:
1) Figure 1b: it is difficult to judge whether somite morphology is impaired or not. I would like to ask the authors to improve the quality of the image or to add in situ hybridization data. In addition, it would be better to show cardiac morphology in Mesp1 null mutant established by genome-editing.

We conducted in situ hybridization of Type II mutants obtained from F0 assay with a caudal somite marker, Uncx4.1, and PSM marker, T (Figure S2). We added images of the cardiac morphology of Mesp1Neo/Neo and Mesp1ΔNeo/ΔNeo embryos (Figure 4A-C).

Since F0 embryos may have mosaicism that cause variable severity of the phenotype, it seems difficult to compare the phenotypes of Mesp1 and/or Mesp2 mutants with or without the PGK-neo cassette. One of the ways to more accurately confirm the phenotype of Mesp mutants, CRISPR/Cas9 genome editing should be performed on the established Mesp1 and/or Mesp2 KO mouse strains.

We agree with this comment and established a new Mesp1 null allele (Mesp1ΔNeo) by removing the PGK-neo cassette from previously established Mesp1Neo mice and compared them, as shown in Figure 4. Mesp1ΔNeo/ΔNeo mice developed normally and were fertile, demonstrating that the phenotypes observed in previously established Mesp1 null mice lines were due to PGK-neo cassette insertion.

2) Fig.3A, B; Accumulation of mesodermal cells around the PS is not clear. Could the authors add cross section analysis as shown in Kitajima et al., 2000?

We added cross-section analysis of Mesp1/2 DKO embryos (Figure 3A, B).

3) Fig.4; the author generated Mesp1CreDneo mice in which Cre was inserted at the Mesp1 locus but PGK-neo cassette was removed. This mutant developed normally and showed up-regulated Mesp2 expression. From these experiments, they concluded ?prolonged Mesp2 expression rescued the phenotypes caused by Mesp1 null, but the PGK-neo cassette insertion prevented this rescue mechanism.? (line185,186). However, some points are still unclear.
First, in order to evaluate the variable phenotype of the Mesp1 mutants in the targeting strategy, it is important to compare directly the Mesp1 mutant phenotypes with and without the PGK-neo cassette. The authors described this point in the text but should show image data. Second, in the previous paper, Mesp1 KO embryos with the PGK-neo cassette inserted showed abnormal heart morphogenesis although Mesp2 expression was upregulated (Kitajima et al., 2000). The authors should cite "Kitajima et al., 2000" in this section of the manuscript and discuss the relationship between the phenotype of Mesp1 mutants and Mesp2 expression more carefully. In addition, since they cannot exclude other possibilities such as inserted PGK-neo cassette affect another gene expression and cause defect in heart morphogenesis, they should be discussed more carefully in this point.

As mentioned above, we established the Mesp1ΔNeo allele, and directly compared Mesp1Neo/Neo and Mesp1ΔNeo/ΔNeo embryos carefully (Figure 4). We observed up-regulation of Mesp2 gene expression in Mesp1Neo/Neo embryos (Figure 4D, F, G, I) compared with controls. However, instead of Mesp2 gene expression being up-regulated in Mesp1ΔNeo/ΔNeo embryos, it was maintained from the early bud stage until the late bud stage. When we compared late bud stage embryos, Mesp1ΔNeo/ΔNeo embryos had higher expression level than controls (Figure 4G, H). The morphological abnormality of Mesp1ΔNeo/ΔNeo embryos started around the late bud stage, which was likely caused by mesoderm specification defects and later caused bifurcation of the cardiac primordia. Retained Mesp2 expression may be due to a delay in mesoderm specification in Mesp1ΔNeo/ΔNeo embryos. We added these observations to the Results section on p10 to 11, and added discussion on p17 and 21.

4) Figure 5: They hypothesized that the expression of Mesp1 and Mesp2 might be regulated by Wnt and Nodal signaling. However, they analyzed only Mesp1 expression but not Mesp2. Since down-

regulation of Mesp1 can result in up-regulation of the Mesp2 gene, the authors should analyze Mesp2 expression in Wnt3 and Lefty2 mutant mice. Furthermore, to analyze the requirement for continued activation of Wnt signaling and suppression of Nodal signaling in the mesoderm specification, it had better to check expression of Wnt and Nodal signaling target genes in Wnt3 and Lefty2 mutants, respectively.

We analyzed Mesp2 expression in Wnt3 mutants (Figure 5C, D) and Lefty2 mutant embryos (Figure 5I, J). Similar to Mesp1 expression, Mesp2 expression was not observed in Wnt3 mutants and was unchanged in Lefty2 mutants. We also examined the expression of T as a Wnt signaling target and Eomes as a Nodal signaling target in Wnt3 and Lefty2 mutants (Figure S5). As expected, T was not expressed in Wnt3 mutants and Eomes was up-regulated by the loss of LEFTY2 as an inhibitor of Nodal signaling.

5) To evaluate the possibility that Lefty2 expression regulated by Mesp1 and Mesp2 is essential for mesoderm differentiation, the authors generated Mesp1Lefty2 / Lefty2. Mesp2 null mice lacking the Mesp gene in Lefty2 knocked in at the Mesp1 locus. They conclude that "Lefty2 expression cannot rescue the loss of MESP1/2 in mesoderm differentiation". (Rows 221,212). However, it is difficult to judge from Fig. 5G-L whether mesoderm differentiation is occurring. To reveal the mesoderm status of cells, it is necessary to analyze expression of marker genes, including Eomes, Wnt3, Wnt3a, in Mesp1/2 dKO (Fig. 3).

We analyzed mesoderm marker expression in Mesp1Lefty2/Lefty2; Mesp2-null embryos, including Eomes, Wnt3, and Wnt3a (Figure 6), and obtained similar results to those of Mesp1/2 dKO embryos.

6) Figure 6; The authors concluded that "Mesp1 expression in early mesoderm is regulated by canonical Wnt signaling." (Line 214). However, in FIG. 6B, the embryonic stage was not described and it seems relatively later stage at which Mesp1 is not expressed. Does this reporter recapitulate Mesp1 expression? Furthermore, to reveal the transcriptional regulation of Mesp1 in early mesoderm, it is necessary to perform the Mesp1 reporter assay at an early stage. Also, what is "Ex-2" in Figure 6D? Is this a previously identified TCF/LEF binding site?

We conducted the transient transgenic assay again and dissected the embryos earlier than in previous experiments. The reporter expression was delayed but it recapitulated the pattern of endogenous Mesp1 expression (Figure 7B).
The “Ex-2” primer sets were designed in the 3’UTR of exon2 of the Mesp1 gene as a negative control. We added the discription in the Figure Legend for Figure 7H. ChIP assay demonstrated weak interaction with the Ex-2 primer sets, possibly because the primer sets were close to a previouly identified TCF/LEF binding site (T/LBS-P; Li et al. 2013).

7) Figure 7: What is the significance of Wnt-regulated Mesp1 expression after the LB stage? The author has already discussed this point at lines 338-346. However, analysis of markers involved in mesoderm specification or epithelial-mesenchymal transition in the Mesp1EMEΔ1 mutant is informative.

As Mesp1EMEΔ1/Δ1 embryos did not exhibit developmental defects, we assumed that the mesoderm specification markers were unchanged. While performing experiments for the revision of this paper, we found that Mesp1EMEΔ1Δ2/Δ1Δ2 embryos displayed early mesoderm formation defects.

Minor comments:
1) Figures 2A-C, 6A and S3A are not referenced in the main part of the manuscript.

We referred to all figures accordingly in the revised manuscript.

2) Please indicate size of the scale bars in Figures 1 and 6B.

We added the size of each scale bar in each Figure legend.

3) Lines 225, 226: Incorrect number in figure.

We corrected this accordingly.

4) Figures 6C, D: Which statistical test did the authors use? What does the error bar indicate?

We used the Student's t-test for the statistical analysis and the error bar indicates the standard deviation. We added the description in each Figure Legend.

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Second decision letter

Dear Dr. Ajima,

I have now received all the referees' reports on the above manuscript, and have reached a decision. I am sorry to say that the outcome is not a positive one. The referees' comments are appended below, or you can access them online: please go to Development's submission site and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, Reviewer 2 and previously Reviewer 1 have raised concern of the merit and novelty of the findings of this study (please also see Editor's Note appended below). Having considered their view, I have to decline this submission.

I do realise this is disappointing news, but Development receives many more papers than we can publish, and we can only accept manuscripts that receive strong support from referees.

I do hope you find the comments of the referees helpful, and that this decision will not dissuade you from considering Development for publication of your future work. Many thanks for sending your manuscript to Development.

Reviewer 1

*Advance summary and potential significance to field*

This paper uses state of the art approaches to very carefully address the roles of neighboring Mesp1 and Mesp2 genes, with important implications for understanding early events in anterior mesoderm and cardiovascular development. Moreover the authors identify mechanisms activating cooperative enhancers that regulate Mesp gene expression.

*Comments for the author*

The authors have significantly modified their manuscript and addressed my previous concerns.

Minor points:
1. Please check spelling carefully, for example line 360 MESP1
2. Title: should it read "...their transcriptional control..."

Reviewer 2

*Advance summary and potential significance to field*

The authors responded appropriately to all the specific points I had raised. As a result, I appreciate that the revised manuscript has been greatly improved. However, despite these improvements, the impact of this paper on the field still seems not so strong.

As I, as well as another reviewer, mentioned previously, the results shown by the newly generated mutants themselves seems not match with broad interest to the developmental biology. Thus, whether other part of this paper makes a significant and novel contribution to our understanding of developmental mechanisms is an important point for consideration of suitability of this paper for publication in Development, I feel. In this revised version, the authors present new data showing the importance of the enhancer elements of Mesp genes by generating a large deletion of enhancer

elements, including EME and HCR2. In this mutant expression of Mesp1 and Mesp2 is modestly down-regulated, and cardiac morphogenesis and early embryogenesis are impaired, indicating biological significance of the regulation of Mesp genes through these enhancers. As the authors discussed, it seems plausible that decrease of the total amount of MESP1 and MESP2 in embryos causes these defects, but still speculative. In addition, the regulatory machinery associated with HCR2 is still unclear. Therefore, it is unlikely that this revised version fully satisfies the criteria for acceptance for publication.

*Comments for the author*

Personally, I feel that one of the interesting aspects of the newly generated mutant phenotype is that the upregulation of Mesp2 compensates for the loss of Mesp1 in cardiac development. This point could be studied in more depth by linking it to the mechanism of regulation of Mesp1 and Mesp2 expression.

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Rebuttal letter

MS ID#: DEVELOP/2020/194613

MS TITLE: Formal proof of the requirement of MESP1 and MESP2 in mesoderm specification and the transcriptional control via specific enhancers in mice

Dear Development Staff, and Editor Dr. Patrick Tam,

Thank you very much for taking the time to evaluate our manuscript.
We apologize for taking your time again, but we must ask whether this was a fair decision following the standards of *Development*.

We understand that manuscripts cannot be accepted without support from the referees. However, even though Reviewer 3 was not supportive, we believed that Reviewer 1 and 2 were supportive in the beginning. Reviewer 2 (previously Reviewer 1) initially commented that “The work is carefully performed and clearly documented and clarifies a number of questions important for understanding Mesp gene function and mesoderm patterning during gastrulation, as well as issues concerning gene mutation studies more generally.” They expressed no concerns as to the merit or novelty of this manuscript. We believe that if the reviewer was this concerned, they should have mentioned it from the start.

We spent so much time and effort to address all of the reviewer’s comments because you mentioned in the initial decision letter that “Provided you are able to fully address the referees’ comments, we are positive about publication of your paper (we accept over 95% of revision submissions).”. We requested the editor to provide a generous extension for this revision, and we are glad to hear that both Reviewer 1 and 2 stated that we responded appropriately to all of the specific points and that the revised manuscript was significantly improved. After such supposed improvement, we do not understand why our manuscript is now considered to be in the lower 5% and unacceptable.
We greatly appreciated your and the Reviewer’s comments. However, we would like to make clear the impact of *Mesp2* expression on *Mesp1* KO. The important point is that *Mesp1ΔNeo/ΔNeo* mice developed normally and were fertile, which means that MESP2 was able to fully rescue the original *Mesp1* KO phenotype in *Mesp1ΔNeo/ΔNeo* mice. As the Reviewers stated in their initial comments, we considered making and evaluating *Mesp1ΔNeo/ΔNeo* mice to be the most direct evidence that insertion of the PGK-neo cassette was the cause of the cardiac morphological defects observed in the *Mesp1* KO mice with PGK-neo cassette including *Mesp1Neo/Neo* mice. Therefore, we spent the time to establish the *Mesp1ΔNeo* line and analyzed it. We found that *Mesp2* was up-regulated in *Mesp1ΔNeo/ΔNeo* embryos, and *Mesp1ΔNeo/ΔNeo* mice developed normally. In the Editor’s Note, you asked “Is it known that the “upregulated” expression of *Mesp2* in the mutant replicates the “normal” pattern of expression in the wild type?”. It is difficult to demonstrate whether the up-regulation of *Mesp2* can directly compensate for MESP1 function. However,

because *Mesp1ΔNeo/ΔNeo* mice developed normally, we considered that “the mutant replicates the “normal” pattern of expression in the wild type”.

You mentioned in the Editor’s Note that “The assertion that “retained Mesp2 expression may be due to a delay in mesoderm specification in Mesp1ΔNeo/ΔNeo embryos” suggests that Mesp2 activity may not have fully reverted/rescued the mutant phenotype, or the extended Mesp2 activity may be counterproductive.”. This description was about the *Mesp1Neo/Neo* embryos (not about the *Mesp1ΔNeo/ΔNeo* embryos) which displayed cardiac development defects. We agree that we cannot exclude the possibility that the retained *Mesp2* at the LB stage is simply a result of a delay in mesoderm specification, as described in the manuscript, or that extended Mesp2 activity is counterproductive. In either case, however, the phenotype was caused by the insertion of the PGK-neo cassette and its removal abolished the phenotype.

We agree that we have not clarified the regulatory mechanism of *Mesp2* expression through the EME and HCR2 enhancers, which you and Reviewer 2 mentioned in the comments. However, we believe that this is beyond what we were asked to address for this revision stage. It is, of course, a highly important and interesting point, and we would like to address the mechanism in a future study. We still think it worthwhile to include our data regarding the regulation of *Mesp1/2* expression through the EME and HCR2 enhancers (Figure 9) in this paper because *Mesp1EME*Δ1Δ2/Δ1Δ 2 embryos displayed very similar phenotypes to *Mesp1Neo/Neo* embryos, and down-regulation of both *Mesp1* and *Mesp2* expression was observed. Importantly, this phenotype was not observed in *Mesp1*-KO without the PGK-neo cassette, *Mesp1EMEΔ1/Δ1*, or *Mesp1EMEΔ2/Δ 2* embryos. These genetic data support that the effects of the PGK-neo cassette on another neighboring gene were not the cause of the cardiac phenotype because *Mesp1EMEΔ1Δ2* mice do not have the PGK-neo cassette. Moreover, they strengthened the hypothesis that both MESP1 and MESP2 are important for the early mesoderm formation, and that a sufficient amount of both proteins is required for proper cardiac formation.

Overall, this is why we feel that your decision is not fair and we hope that there is room for discussion.

We are looking forward to hearing from you soon.

Sincerely yours,

Rieko Ajima
Yumiko Saga

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Editor's response to author's rebuttal letter

MS ID#: DEVELOP/2020/194613

MS TITLE: Formal proof of the requirement of MESP1 and MESP2 in mesoderm specification and the transcriptional control via specific enhancers in mice

AUTHORS: Rieko Ajima, Yuko Sakakibara, Noriko Sakurai-Yamatani, and Yumiko Saga
Thank you for your appeal on your recently rejected manuscript. I do understand your disappointment, but given the opinions expressed by the reviewers of the previous two rounds, I am not able to accept the revised version of the paper in this instance. However, we are always willing to give authors the chance to defend their manuscripts, and I do recognise that you make some valid comments in your rebuttal. Therefore we are prepared to reconsider a revised version of your manuscript that deals, to your best ability, with the points raised in the Editor's note appended to this letter. Upon resubmission, please provide a detailed response to the comments and highlighting particularly any points that have not been addressed in the revised manuscript.

The revised manuscript will be taken through an editorial review. If the response and revision are appropriate, then we would be able to consider the manuscript for publication.

To submit a revision, please go to your Author Area and click on the 'Submit a Revision' link.

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Editor's note:

The choice of enhancer for this study was founded on the findings that the conserved HCR1 region contains the P1-PSME with TCF/LEF sites that is required for maintaining (but not the onset of) Mesp1 expression and that another enhancer region (HCR2) may regulate Mesp2 expression during early mesoderm formation. Deletion of the EME and EME + upstream HCR1 both affect Mesp1 expression at advanced stage of mesoderm development, while deletion of EME+HCR2 affects Mesp2 expression in early mesoderm but not in the lateral mesoderm. In addition to the mesoderm defect, EME+HCR2 mutants displayed heart defects. The complexity of the role of enhancers in initiating/maintaining Mesp1/2 expression begs the question of how these enhancers are regulated and act on the target genes (Reviewer 2). For example, what are the critical target sites for upstream factors (e.g., TCF/LEF, T-box/Eomes/T) in EME, HCR1 and HCR2 that independently and cooperatively drive the ‘dosage-dependent’▫ expression of Mesp1 and Mesp2? It was inferred that the TCF/LEF1 enhancer in EME is essential for Mesp1 activation, and the T-box enhancers in HCR1 and HCR2 are ‘strongest candidates’▫ for activation of both Mesp1 and Mesp2. Is the E-box in EME has any role in transcriptional regulation by the two bHLH factors? Given the dual requirement of Mesp1 and Mesp2 activity in a relatively strict spatio-temporal context in vivo, It would be useful to find out if the activation of Mesp1 and Mesp2 requires the cooperativity of all enhancer components or only subsets of enhancers contained within the EME/HCR1/HCR2. This information may be gleaned from studies of luciferase reporter in cell lines, as previously performed for EME and HCR1. Whether they are distinctly from the putative ‘another enhancer’▫ for somitogenesis is not a critical issue.

As the first round Reviewer 3 and second round reviewer 2 indicated, “the results shown by the newly generated mutants themselves seems not match with broad interest to the developmental biology". The more interesting finding is that the upregulation of Mesp2 compensates for the loss of Mesp1 in mesoderm development. The phenotypic outcome of the single and compound mutants points positively to the phenomenon of genetic rescue but provides no information of the underpinning molecular activity ( re: your response - ‘Mesp1ΔNeo/ΔNeo mice developed normally, we considered that ‘the mutant replicates the ‘normal’▫ pattern of expression in the wild type’▫). The expression pattern of Mesp2 in the Mesp1-delneo/delneo deviates somewhat from the wild-type pattern, but appeared to have rescued much of the Mesp1/2-null phenotype though not the delay in mesoderm differentiation. It is reasonable to take the view that it is neither informative nor productive to examine why/how the NeoR cassette has disrupted the heart development in the MEsp1-neo mutants. However, it would be imperative to understand the impact of Mesp2 activity (up-regulated or otherwise) on restoring/compensating the molecular/gene activity that are disrupted by the Mesp1-delneo alleles. In essence, what gene/molecular activity is carried by Mesp2 up-regulation? This molecular activity that are restored by Mesp2 up-regulation in the Mesp1-del neo mutant versus those not restored in the Mesp1:Mesp2-dko mutant, would provide the evidence of the separate contribution of Mesp1 and Mesp2 to the molecular mechanism regulating early mesoderm differentiation and the persisting delayed differentiation. This information would add to the claim of ‘formal proof’▫ of the dual requirement of these two MESP factors in development.

The query about the level of upregulation of Mesp2 in different mutant settings is related to the dosage-dependent effect of Mesp1/2 activity. This may be relevant to the hypothesis that’▫ both MESP1 and MESP2 are important for the early mesoderm formation, and that a sufficient amount of both proteins is required for proper cardiac formation’. That ‘It is difficult to demonstrate whether the up-regulation of Mesp2 can directly compensate for MESP1 function’▫ is a point taken. It is also understood that the ‘investigation of how these two genes ... cooperatively function in early mesoderm formation, specification and somitogenesis’▫ is outside the scope of the present study.

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**Second revision**

Author response to reviewers' comments

Editor's Note: The choice of enhancer for this study was founded on the findings that the conserved HCR1 region contains the P1-PSME with TCF/LEF sites that is required for maintaining (but not the onset of) Mesp1 expression and that another enhancer region (HCR2) may regulate Mesp2 expression during early mesoderm formation. Deletion of the EME and EME + upstream HCR1 both affect Mesp1 expression at advanced stage of mesoderm development, while deletion of EME+HCR2 affects Mesp2 expression in early mesoderm but not in the lateral mesoderm. In addition to the mesoderm defect, EME+HCR2 mutants displayed heart defects. The complexity of the role of enhancers in initiating/maintaining Mesp1/2 expression begs the question of how these enhancers are regulated and act on the target genes (Reviewer 2). For example, what are the critical target sites for upstream factors (e.g., TCF/LEF, T-box/Eomes/T) in EME, HCR1 and HCR2 that independently and cooperatively drive the "dosage-dependent" expression of Mesp1 and Mesp2? It was inferred that the TCF/LEF1 enhancer in EME is essential for Mesp1 activation, and the T-box enhancers in HCR1 and HCR2 are "strongest candidates" for activation of both Mesp1 and Mesp2. Is the E-box in EME has any role in transcriptional regulation by the two bHLH factors? Given the dual requirement of Mesp1 and Mesp2 activity in a relatively strict spatio-temporal context in vivo, It would be useful to find out if the activation of Mesp1 and Mesp2 requires the cooperativity of all enhancer components or only subsets of enhancers contained within the EME/HCR1/HCR2. This information may be gleaned from studies of luciferase reporter in cell lines, as previously performed for EME and HCR1. Whether they are distinctly from the putative "another enhancer" for somitogenesis is not a critical issue.

Response: We do not think that your suggestion to examine the enhancer activity using a luciferase reporter in cell lines is a suitable method to examine the cooperation among enhancers for Mesp1 and Mesp2 genes. Due to the nature of in vitro experiments, even if enhancer activity is observed, we will not be able to distinguish whether the activity is for Mesp1 or Mesp2. We may be able to clarify which transcriptional factors and binding sites are important for the enhancer activity, but we will not be able to determine which gene's expression is being controlled. The most direct way to examine the enhancer activity is to introduce mutations into the putative transcriptional factor binding sites in vivo, and examine Mesp1 and Mesp2 expression. However, we believe that this is beyond what we were asked to address for the initial revision of this paper. We would like to conduct such experiments in a future study.

Editor's suggestion: As previously discussed, the demonstration of a role of WNT signalling (via the TCF/LEF1 site in EME) in the maintenance of Mesp1 transcription were not of sufficient interest in the context of requirement of both Mesp genes in mesoderm differentiation, and it did not address the question of how the transcription of Mesp1 and Mesp1 is jointly regulated or that of Mesp2 is separately regulated in the absence of Mesp1 function. The hypothesis raised in the paper is that "tissue-specific expression of Mesp1 and Mesp2 continuous activation of canonical WNT signalling" (Introduction) and that there may be "common enhancers between Mesp1 and Mesp2 that compete for (upstream) transcription factors" (line 302-303) and that Mesp2 expression may not be directly regulated by Wnt signalling" (line 305-351). Bioinformatic analysis of the promoter and enhancer regions of Mesp2, as well as the conserved regulatory regions of Mesp1 and Meps2 may reveal the candidate enhancer/promoter elements whose activity may be disrupted by the neo insertion in the Mesp1-neo/neo mutant. At the least, the in-silico findings would add knowledge of how Mesp2 transcription may be initiated/maintained to compensate for the LOF of Mesp1. If the "strong" or "competing" candidate/s (e.g., T-box, E-box) could be validated functionally, it would further enhance the merit of this study.

Response: As the editor suggested, we conducted bioinformatic analyses, such as searching for E-box and T-box transcriptional binding sites between Mesp1 and Mesp2, and looking into the previously reported RNA-seq and ChIP assay data. Based on these bioinformatic analyses, we discussed extensively about the possible mechanisms for transcriptional regulation and rescued expression of Mesp1 and Mesp2 in the Discussion (line 384-439). In the Tosic et al. 2019 paper, EOMES and BRACHYURY were demonstrated to act as upstream transcriptional factors, as knock-out of both Eomes and Brachyury genes caused the loss of Mesp1 and Mesp2 expression in differentiating ES cells. Theirs and several other studies revealed the interaction of these T-box transcriptional factors with the T-box binding sites between Mesp1 and Mesp2 genes, and consistent with our study, some of the binding sites were located in the enhancers we deleted in vivo and affected Mesp1 and/or Mesp2 expression.

We removed the description “Mesp2 expression may not be directly regulated by Wnt signalling” (line 305-351) as it is difficult to conclude whether Wnt signaling regulates only Mesp1 expression or that of both Mesp1 and Mesp2 via TCF/LEF binding sites in the EME because the enhancers are located between Mesp1 and Mesp2 genes.

Editor’s Note: As the first round Reviewer 3 and second round reviewer 2 indicated,” the results shown by the newly generated mutants themselves seems not match with broad interest to the developmental biology". The more interesting finding is that the upregulation of Mesp2 compensates for the loss of Mesp1 in mesoderm development. The phenotypic outcome of the single and compound mutants points positively to the phenomenon of genetic rescue but provides no information of the underpinning molecular activity (re: your response - “Mesp1ΔNeo/ΔNeo mice developed normally, we considered that “the mutant replicates the “normal” pattern of expression in the wild type”). The expression pattern of Mesp2 in the Mesp1-delneo/delneo deviates somewhat from the wild-type pattern but appeared to have rescued much of the Mesp1/2-null phenotype though not the delay in mesoderm differentiation. It is reasonable to take the view that it is neither informative nor productive to examine why/how the NeoR cassette has disrupted the heart development in the Mesp1-neo mutants. However, it would be imperative to understand the impact of Mesp2 activity (up-regulated or otherwise) on restoring/compensating the molecular/gene activity that are disrupted by the Mesp1-delneo alleles. In essence, what gene/molecular activity is carried by Mesp2 up-regulation? This molecular activity that are restored by Mesp2 up-regulation in the Mesp1-del neo mutant versus those not restored in the Mesp1:Mesp2-dko mutant, would provide the evidence of the separate contribution of Mesp1 and Mesp2 to the molecular mechanism regulating early mesoderm differentiation and the persisting delayed differentiation. This information would add to the claim of “formal proof” of the dual requirement of these two MESP factors in development.

Response: We agree that comparing the abnormal gene expression in Mesp1/2 dKO with that in Mesp1-delneo/delneo and determining which gene activities are restored would provide evidence of a role of MESP2 in early mesoderm formation. We found that several genes that are important for early mesoderm formation are abnormally expressed in Mesp1/2 dKO, as shown in Figure 3. We are considering examining the expression of the following genes in Mesp1-delneo/delneo embryos: Wnt3 as an essential factor for primitive streak formation; Eomes and Wnt3a as anterior and paraxial mesoderm regulators, respectively; and Snail as an MESP1 target and important factor for the EMT in mesodermal cells.”

Editor’s suggestion:
Findings of this study pointed to that Mesp2 is up-regulated in Mesp1-LOF embryo, and reciprocally, Mesp1 is up-regulated in Mesp2-LOF embryo, and Mesp1/2 DKO displayed mesoderm defects. Instead of examining the expression pattern of a couple of candidate genes (e.g., Wnt3, Eomes and Snail), it would be informative to analysis the transcriptome by sequencing analysis of embryos of Mesp1-frameshift (or Mesp1-delneo/delneo), Mesp2-frameshift (or Mesp2-KO) and Mesp1/2 dKO. The results would inform of the gene activity that is compensated by Mesp2 in Mesp1-LOF (leading to no mesoderm phenotype), by Mesp1 in Mesp2-LOF (that still manifests caudal somite defects) and full spectrum of loss/downregulated gene activity (that is associated with the DKO phenotype). This would provide a more comprehensive insights into the downstream activities of Mesp1 and Mesp1 respectively, some of which may be relevant to the functional co-requirement in mesoderm development.

Response: As the editor suggested, we performed transcriptome analysis using control and Mesp1Δneo/Δneo embryos, and noted no difference in the pattern of expression other than the up-regulation of Mesp2. This suggested that Mesp2 up-regulation can fully restore/compensate for the molecular/gene activity that is disrupted by the Mesp1Δneo/Δneo alleles, and it is unlikely that there is “separate contribution of MESP1 and MESP2 to the molecular mechanism regulating early mesoderm differentiation”. If this were the case, the purpose of the transcriptome analysis using Mesp1/2 dKO would be to simply look for MESP1 and MESP2 targets, which is outside the scope of this paper. We agree that transcriptome analysis using Mesp1/2 dKO would give more comprehensive insight into the MESP1 and MESP2 functions in early mesoderm formation, but we would like to conduct this in our next study.

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Third decision letter

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ARTICLE TYPE: Research Article

I am satisfied with the response, the provision of additional data and revision of the discussion, This manuscript is accepted for publication in Development, pending our standard ethics checks.