Cell lineage specification and signalling pathway use during development of the lateral plate mesoderm and forelimb mesenchyme

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First decision letter

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MS TITLE: Cell lineage specification during development of the anterior lateral plate mesoderm and forelimb mesenchyme

AUTHORS: Axel H Newton, Sarah M Williams, Andrew T Major, and Craig A Smith

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, 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

The authors established transcriptional atlas in the lateral plate mesoderm development in the chick using single cell RNA-Seq. The results revealed that the lateral plate mesoderm can be subdivided into several types of cell cluster and how gene expression pattern is altered from the primitive mesoderm to the limb bud mesoderm.
This atlas enables to predict utilization of signaling pathways and cellular lineage in the lateral plate mesoderm.
Furthermore, the authors indicated that the transcriptional atlas was well matched with actual gene expression in the chick such as TWIST1 and that BMP signaling was required for adequate forelimb bud formation using BMP inhibitor NOGGIN.
Although this report is of interest and will contribute to future developmental research and single cell analyses, several important points should be improved.
Especially the authors should clearly describe what was an important problem in the research field and how the report could solve the problem.

Comments for the author

Major points
1. As the authors mentioned in the text, the transcriptional atlas was well matched with the previous reports in the lateral plate mesoderm. However, the authors should clarify novel finding and advantage from the transcriptional atlas.
For example a. Proposal for novel cell population such as somatic/splanchnic-bipotent cells or already committed cells at an early stage.
b. Finding novel tissue-specific markers for primitive, somatic or splanchnic mesoderm (the authors indicated TWIST1 but I think that TWIST1 is not “a new marker” because it was already well known as the authors mentioned in the text).
c. Identification and experimental demonstration of novel factor(s) to regulate subdivision or EMT in the lateral plate mesoderm.
a-c are just suggestions and not mandatory. No matter what biological events the authors focused on, however, the authors should verify that the transcriptional atlas is useful to uncover new insight that would deepen our understanding of developmental biology.
2. In Figure 5, electroporation experiments should be properly controlled.
A part of GFP-control embryos and all NOGGIN-GFP embryos look like abnormal or developmental delay.
The authors need to optimize electroporation condition to minimize damage and to avoid undesigned gene induction out of forelimb region.
The authors did not indicate sample numbers used for each electroporation experiments in the manuscript but reliable number technical replicates were required.
3. In Figure 4 and 5, cellular level images are required to apply the single cell transcriptome data to wet results.
Tissue level images that the authors showed, were not suitable to compare single cell transcriptome data. For example, correlation between TWIST1 and TBX5 in single cell resolution strengthen reliability of single cell transcriptome.

Minor points:
1. Line 73-74: Thus, while FOXF1 and HAND1 play important roles in splanchnic LPM development, those that promote somatic LPM differentiation remain unclear.
Line 96-97: However, the events immediately preceding the TBX5-dependant limb regulatory pathway in the somatic LPM are less well understood.
Line 101-103: Importantly however, details regarding the cellular decisions that underlie LPM differentiation, subdivision, and commitment to a limb fate remain undetermined.
If authors discuss unanswered problems, the report should answer the problems such as FOXF1 and HAND1 promotion for somatic LPM differentiation, TBX5-dependant limb regulatory pathway in the somatic LPM and cellular decisions that underlie LPM differentiation, subdivision, and commitment to a limb fate.
Otherwise, the text should be corrected to clarify what was the problem and how the report answered.

2. Line 109: but also reveal novel tissue-specific markers…
Line 111-112: Notably, we identify TWIST1 as an early marker of somatic LPM development with a likely role underlying EMT of the limb bud mesenchyme.
Line 430-431: TWIST1 has known expression in the somatic LPM (Gitelman, 1997; Tavares et al., 2001).
These sentences contradict. Line 109 and line 111-112 should be corrected.
3. Line 214: Figure 2b It should be Figure 2c.
4. Line 330-331: We therefore investigated whether these transcription factors may play a role during EMT and initiation of the chicken forelimb.
Line 337-338: The molecular role of TWIST1 was further examined in the somatic LPM through immunofluorescence.
Line 490-492: Our data highlight a previously unidentified role for TWIST1 as an early mediator of somatic LPM development, though additional work is required to define its precise role.
Molecular roles cannot be revealed by expression analyses. The authors should experimentally demonstrate the molecular roles, otherwise they should be corrected.
5. Line 372: Figure5a It should be Figure5b.
6. In all figures, legends and text, labeling for each panel should be same. For example, (A) in the figure legend should be changed into a) in accordance with the figure and text.
7. Line 499: (C) It should be bold letter.
8. Line 510: ectoderm When the authors use the word “ectoderm”, the authors should clarify which ectoderm (c2 ectoderm or c11 AER/ectoderm) they indicate.
9. Line 510: the early mesoderm The authors should define difference between the early mesoderm and primitive mesoderm, otherwise they should be corrected through the manuscript.
10. Line 555-559: Note, TWIST1 does not appear to be as significantly affected by NOGGIN-GFP, as though expression appears reduced in treated limb buds, levels of mRNA expression in limb sections appear unchanged compared to the control side similar to observations of protein localization (A).
This sentence includes repetition and is somehow difficult to understand. It should be simplified to indicate only two facts: 1. reduction of limb bud thickness, 2. no change in expression level.
11. Line 563: while emu eggs were collected at E3.5 (HH10) e4.5 (HH14) and e5.5 (HH18)
There is no data for emu. It should be corrected.
12. Line 641-642: Tissues were permeabilized in 10mg/mL proteinase K for up to 1 hour, depending upon size then re-fixed in glutaraldehyde/ 4% PFA.
I think that proteinase K concentration is too high for this treatment (in my knowledge, proteinase K concentration is usually 1-10 µg/ml for in situ hybridization). The authors should confirm it.
13. In the section: “gene expression analysis by in situ hybridization and immunofluorescence” in methods, information of antibodies such as supplier company, catalogue number and concentration is required.
14. Line 660: Antigen retrieval The authors should describe the protocol.
15. Black arrows in Fig. 4a, white arrows in Fig. 5b and asterisks in Fig. 5a, b should be explained.
16. In all supplementary figures, the authors should include figure title and legends.
17. In figure S4, Figure X should be Figure S4.

Reviewer 2

Advance summary and potential significance to field

In the submitted manuscript, Nelson and colleagues tackle a major gap in the field of limb formation: the initial stages of limb bud initiation and outgrowth from the somatic LPM. In addition, the structure and subdivision of the LPM into its splanchnic and somatic parts remains understudied, both by a lack of conceptual and of mechanistic tools.
In their work, the authors use the chicken embryo and scRNA-seq to reveal new details about the cellular heterogeneity and active processes in the somatic LPM at these critical time points. The authors document possible signaling interactions within the early partitioning LPM and show that the EMT regulator Twist1 has select expression already earlier than anticipated in the emerging forelimb field. Through their data presentation and discussion, the authors provide a conceivable framework for earliest (fore)limb initiation following LPM splitting, which is highly timely and complementary to existing work in the field.

Despite these strengths and at times beautiful execution of the involved bioinformatics work, the functional testing of the conceived models is limited to BMP inhibition by Noggin electroporation. These experiments are performed at an interesting stage of development, yet limited in scope, documentation, and their readouts, leaving the reader (or at least the reviewer) wanting for more info. Functional testing of RA perturbations with/without BMP inhibition testing of an impact on additional genes found differentially expressed in the scRNA-seq, but already inclusion of further controls would greatly augment the manuscript. The impact at different time points and read out by various markers would provide more depth, too.
Overall, while the authors spend significant effort in describing the underlying biology with their scRNA-seq, their final in vivo conclusions are overly reductionistic again only address one or two players in the entire process.

The reviewer does not have the computational or biostatistical expertise to comment on the details and rigor of the performed scRNA-seq and follow-up analyses.

Comments for the author

Major comments
1) Twist1 is one of the major findings from all the transcriptomics analysis yet the in vivo experiments connecting the individual parts of the authors'
model remain limited. Suggestions for additional experiments:
a) limb bud region-specific Twist1 knockdown to determine role in limb bud initiation by checking then downstream genes, i.e. is there an interaction between Twist1 and Tbx5?
b) Effect of RA signaling on Twist1, towards answering the question if BMP knockdown doesn't affect Twist1, what does?
c) Ectopically express Twist1 in the future limb bud region d) How are FGF genes de-regulated by the treatment, i.e. is there a later impact of the electroporation on the AER or other structures?
2) Related, in Figure 5 the authors state that Noggin expression results in reduced Prrx1. From the presented pictures, another possible conclusion could be that the Noggin-transduced side could simply have less cells, resulting in a weaker in situ staining when seen from dorsal. In the section, the area is as described hyoptrophic/hypoplastic, but Prrx1 mRNA seems still solidly expressed. The same holds true for the Twist1 ISH and associated claims.
The authors should provide close-up images of the area indicated in the section
(i.e. as additional row of images in this overall nicely presented figure).
Ideally, some form of quantification to solidify the claims would strengthen this entire figure. Further, the authors are encouraged to add quantifications to their figure panels and throughout. As last touch, labeling of splanchnic vs somatic LPM in 5a as well as labeling of observed stages in the figure itself would greatly guide the reader.
3) The authors add a superb model schematic in figure 2 and should have a summarizing model of this kind at the end of their manuscript to guide the reader.

Minor
1) References are light, often one of two references for major topics with an over-reliance on individual reviews at times. The authors are encouraged to flesh out their references to provide the reader with context of this (at times complex) research field.
2) Lines 57-59: Dampen the statement that these TF genes "initiate" these fates; their expression coincides with the onset of individual cell fate development and their activities might be required, but they are not master-regulatory.
3) Lines 96-97: "However, the events immediately preceding the TBX5-dependant limb regulatory pathway in the somatic LPM are less well understood." This is a central conundrum driving the work and a major open question in the field. The authors are encouraged to emphasize this point throughout their manuscript.
4) Line 156: The authors find Cdx4 among the enriched transgenes; yet, in common understanding this is a caudal/posterior gene. Can the authors reference work describing Cdx4 expression outside the tail region to provide context?
5) Line 164: The authors are encouraged to add a brief summarizing statement at the end of this important section.
6) Lines 188-190: That the LPM features complex interactions seems obvious, the authors should revisit the wording here for a more instrumental statement supporting their work in context of what's already known/assumed.

7) Lines 235-236: Quite a number of downstream targets for these signaling pathways are known, but not necessarily in the tissues the authors describe; rephrase accordingly.
8) Line 347ff: the authors only sampled a small section of the LPM, and statements describing expression specificity and selectivity should be made in that context; can the authors refer to wholemount stainings for Twist1 (and other genes) to emphasize/contextualize specificity (i.e. controls in Figure 5)?
9) Line 400ff: great statement!
10) Lines 448-449: References for this statement?
11) Line 456 ff: Also related to the experiments (see above), can the authors check more candidate genes and how they chance? For instance, what happens to FGF ligands, and does the perturbation cause a failure in AER induction, etc.
12) Figures overall: the labeling of the scRNA-seq data could be made clearer by better emphasizing different stages in different outputs, as well as homogenization of gene nomenclature throughout the text and figures.
13) Supplemental figure legends seem missing from the reviewer's documents.

Reviewer 3

Advance summary and potential significance to field

In this paper, Newton et al., use single cell transcriptomics to investigate how the anterior-most portion of the posterior lateral plate mesoderm (LPM) is specified into splanchnopleural and somatopleural compartments and how forelimb progenitors emerge from it to trigger limb initiation. The authors establish a transcriptional atlas revealing the involvement of signaling pathways and transcription factors, from an unbiased standpoint, confirming already demonstrated functions and also uncovering new potential regulators. Particular emphasis is put on Twist1, which is found to be expressed together with Prrx1 and prior to Tbx5. Its function during limb initiation is discussed, although not directly tested. Finally, the authors perturb BMP signaling by electroporating Noggin in the ectoderm and observe the effect on LPM subdivision and limb bud formation.
Overall the data is convincing and nicely presented. It provides important -unbiased- insights into processes (LPM and limb formation), which have been historically investigated from a -biased- candidate gene approach.

Comments for the author

My major concern is with the functional perturbation using the electroporation technique. It is unclear whether the effect the authors observe are specific to the construct electroporated or due to non-specific malformations, which are known to occur with the electroporation. The embryos appear to be severely malformed with defects which are difficult to relate to the loss of BMP function in the lateral ectoderm (e.g. neural tube defects). As a result these experiments are not convincing.
Furthermore, it would be worth checking that ectodermal cells and not periderm cells are indeed targeted by the electroporation.

Other comments:
- There seems to be a confusion between the anterior LPM (aLPM) from which the cardiac mesoderm form and the posterior LPM (pLPM) which is subdivided into somatopleure and splanchnopleure and from which limbs form (see Tanaka et al 2016). The authors refer to the anterior-most pLMP and should clarify the terminology.
- P16 l.442 : I do not understand this sentence.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

The authors established transcriptional atlas in the lateral plate mesoderm development in the chick using single cell RNA-Seq. The results revealed that the lateral plate mesoderm can be subdivided into several types of cell cluster and how gene expression pattern is altered from the primitive mesoderm to the limb bud mesoderm.

This atlas enables to predict utilization of signalling pathways and cellular lineage in the lateral plate mesoderm. Furthermore, the authors indicated that the transcriptional atlas was well matched with actual gene expression in the chick such as TWIST1 and that BMP signalling was required for adequate forelimb bud formation using BMP inhibitor NOGGIN.

Although this report is of interest and will contribute to future developmental research and single cell analyses, several important points should be improved. Especially the authors should clearly describe what was an important problem in the research field and how the report could solve the problem.

Major points
1). As the authors mentioned in the text, the transcriptional atlas was well matched with the previous reports in the lateral plate mesoderm. However, the authors should clarify novel finding and advantage from the transcriptional atlas.

For example,
1) Proposal for novel cell population such as somatic/splanchnic-bipotent cells or already committed cells at an early stage.
2) Finding novel tissue-specific markers for primitive, somatic or splanchnic mesoderm (the authors indicated TWIST1 but I think that TWIST1 is not “a new marker” because it was already well known as the authors mentioned in the text).
3) Identification and experimental demonstration of novel factor(s) to regulate subdivision or EMT in the lateral plate mesoderm.
1-3 are just suggestions and not mandatory. No matter what biological events the authors focused on, however, the authors should verify that the transcriptional atlas is useful to uncover new insight that would deepen our understanding of developmental biology.

Response: In this study we aimed to utilize a combination of in silico and in vivo approaches to provide a multifaceted overview of the mechanisms underlying development of the LPM. We have performed substantial revision of the introduction and discussion, streamlined the text and emphasized the problem, highlighted what was known, where knowledge gaps lie and the questions that our study specifically address. This includes rewriting of the final paragraph of the introduction to specifically list each of the novel findings that we present, which in summary are:
- Construction of a gene expression atlas of the developing LPM, including defining previously uncharacterized tissue-specific marker genes.
- Annotation of global signalling pathways, tissue interactions and ligand-receptor pairs during LPM differentiation and early limb initiation. This Includes the importance of BMP signalling during LPM development and early limb initiation.
- Defining the gene expression cascades underlying subdivision and specification of the somatic and splanchnic LPM. Importantly, this includes the temporal activation of genes prior to TBX5 expression, during early limb initiation – a key focus area.
- Identification of a putative and uncharacterised role for TWIST1 during EMT of the somatic LPM – although this requires further validation.
- Demonstrated the previously unknown, yet necessary role of ectodermal BMP signalling in somatic LPM specification and limb outgrowth through activation of TBX5 and FGF10.

2. In Figure 5, electroporation experiments should be properly controlled.

A part of GFP-control embryos and all NOGGIN-GFP embryos look like abnormal or developmental delay. The authors need to optimize electroporation condition to minimize damage and to avoid undesigned gene induction out of forelimb region.

The authors did not indicate sample numbers used for each electroporation experiments in the manuscript but reliable number technical replicates were required.

Response:
We have repeated this experiment, producing improved data for both GFP controls and also provide Noggin (BMP inhibitor)-treated embryos. New data are now shown in revised Figure 5, and extended in supplementary figure 4. We make note the number of embryos used for control and Noggin-treatment which gave reproducible phenotypes. This is clearly seen where limb buds form in the presence of ectodermal GFP, but fail to form in GFP-T2A-NOG electroporations. As we report in the original m/s. we find that BMP inhibition reduces commitment of the LPM into somatic LPM, and inhibits limb initiation through descreased activation of the limb bud markers TBX5 (and subsequent FGF10 / FGF8).

3. In Figure 4 and 5, cellular level images are required to apply the single cell transcriptome data to wet results.
Tissue level images that the authors showed, were not suitable to compare single cell transcriptome data. For example, correlation between TWIST1 and TBX5 in single cell resolution strengthen reliability of single cell transcriptome.

Response:
The single cell data was validated by in situ hybridisation and immunofluorescence of select markers, which is a typical approach. Figure 4 shows the cellular distribution of TWIST1 that derived from the scRNA-seq dataset, very clearly validating that this gene is expressed specifically throughout all cells that comprise the somatic LPM cell layer, pre and post-EMT. In the context of the specific aims of this paper, no deeper cellular analysis was warranted. We did not examine PRRX1 and TBX5 protein expression due to the lack of a suitable chicken-specific antibodies. However, we do show mount in situ hybridization for PRRX1 and TBX5 during equivalent stages of development. Additionally, in figure 5 we are showing tissue level changes that occur as a result of inhibiting ectodermal BMP signalling.

Minor points:
1). Line 73-74: Thus, while FOXF1 and HAND1 play important roles in splanchnic LPM development, those that promote somatic LPM differentiation remain unclear.

Line 96-97: However, the events immediately preceding the TBX5-dependant limb regulatory pathway in the somatic LPM are less well understood.

Line 101-103: Importantly however, details regarding the cellular decisions that underlie LPM differentiation, subdivision, and commitment to a limb fate remain undetermined.

Response: This section has been rewritten in line with above comments, updated paragraph line: 53

If authors discuss unanswered problems, the report should answer the problems such as FOXF1 and HAND1 promotion for somatic LPM differentiation, TBX5-dependant limb regulatory pathway in the somatic LPM and cellular decisions that underlie LPM differentiation, subdivision, and commitment to a limb fate. Otherwise, the text should be corrected to clarify what was the problem and how the report answered.

Response: These three statements point to the fact that little is known about the events underlying early somatic LPM specification, and subsequent activation of the limb developmental pathway. We have revised these sentences in the introduction to make this point clear. That is, while FOXF1 has a characterized role in splanchnic LPM development, the genes that drive somatic LPM development are not. This major question is addressed in the results section: Gene expression dynamics underlying LPM specification and limb development.

2). Line 109: but also reveal novel tissue-specific markers…

Response: Rewritten to "… corroborating known, and identifying previously undetermined, tissue-specific marker genes". Updated line: 97

Line 111-112: Notably, we identify TWIST1 as an early marker of somatic LPM development with a likely role underlying EMT of the limb bud mesenchyme.

Response: Rewritten to state: "Further to this, we identify the sequential activation of transcription factors during limb initiation, including early activation of TWIST1, prior to TBX5 and the FGF10-FGF8 feedback loop" Lines 101-103

Line 430-431: TWIST1 has known expression in the somatic LPM (Gitelman, 1997; Tavares et al., 2001).

Response: This has been left unchanged.

These sentences contradict. Line 109 and line 111-112 should be corrected.

Response: This paragraph has been re-written to refocus the aims of the study. Particularly, in line with the reviewers comments we downplay the novelty of TWIST1 expression in the somatic LPM, but emphasize its putative role in somatic LPM EMT. This sentence has been rewritten to state: "Interrogation of TWIST1 expression and localization in the early somatic LPM revealed a putative role during EMT of the somatic LPM" Lines 103-104

3). Line 214: Figure 2b. It should be Figure 2c.

Response: Corrected in the text.

4). Line 330-331: We therefore investigated whether these transcription factors may play a role during EMT and initiation of the chicken forelimb.
Line 337-338: The molecular role of TWIST1 was further examined in the somatic LPM through immunofluorescence.

Response: Rewritten to state: "Immunofluorescent labelling in HH12 chicken forelimb fields revealed strong, specific expression of TWIST1… in somatic LPM cells prior to limb bud EMT". Line 320-321 of revised text.

Line 490-492: Our data highlight a previously unidentified role for TWIST1 as an early mediator of somatic LPM development, though additional work is required to define its precise role.

Response: Rewritten to state: "...these data implicate TWIST1 as an early mediator of somatic LPM specification and EMT, though this requires further validation in vivo.". Updated lines: 449-450.

Molecular roles cannot be revealed by expression analyses. The authors should experimentally demonstrate the molecular roles, otherwise they should be corrected.

Response: Here we show that the known EMT transcription factors PRRX1 and TWIST1 are strongly expressed in the somatic LPM prior to TBX5. While from our data we cannot conclude that these genes are responsible for limb EMT, their spatiotemporal expression profiles, known roles in zebrafish LPM migration, EMT and cancer, and lack of other EMT factors in the somatic LPM, are strongly suggestive that they work either independently or cooperatively. See updated discussion. However, as this is still speculative, we have rewritten the text to reflect this point in the absence of functional data.

5). Line 372: Figure5a. It should be Figure5b.

Response: Corrected in the text.

6). In all figures, legends and text, labelling for each panel should be same. For example, (A) in the figure legend should be changed into a) in accordance with the figure and text.

Response: These have been updated as per journal figure formatting guidelines

7.) Line 499: (C) It should be bold letter.

Response; Corrected in the text.

8). Line 510: ectoderm. When the authors use the word “ectoderm”, the authors should clarify which ectoderm (c2 ectoderm or c11 AER/ectoderm) they indicate.

Response: Corrected this to “dorsal ectoderm (c2), and line 892 to “between the limb and AER ectoderm (c11)”.

9). Line 510: the early mesoderm. The authors should define difference between the early mesoderm and primitive mesoderm, otherwise they should be corrected through the manuscript.

Response: These terms were used somewhat interchangeably, but for consistency we have changed this to either primitive mesoderm or early LPM and updated these throughout the text.

10. Line 555-559: Note, TWIST1 does not appear to be as significantly affected by NOGGIN-GFP, as though expression appears reduced in treated limb buds, levels of mRNA expression in limb sections appear unchanged compared to the control side, similar to observations of protein localization (A). This sentence includes repetition and is somehow difficult to understand. It should be simplified to indicate only two facts: 1. reduction of limb bud thickness, 2. no change in expression level.

Response: This sentence now states: “While TWIST1 expression appears reduced in Noggin antagonised limb buds, tissue sections did not show obvious reductions in either in situ mRNA levels (B) or protein localization (A) compared to GFP controls”.

11.) Line 563: while emu eggs were collected at E3.5 (HH10) e4.5 (HH14) and e5.5 (HH18). There is no data for emu. It should be corrected.

Response: This has been removed from the text.

12). Line 641-642: Tissues were permeabilized in 10mg/mL proteinase K for up to 1 hour, depending upon size then re-fixed in glutaraldehyde/ 4% PFA.

Response: This is a typo. The proteinase K was used at 10µg/mL.

13). In the section: “gene expression analysis by in situ hybridization and immunofluorescence” in methods, information of antibodies such as supplier company, catalogue number and concentration is required.

Response: Supplier information was in a supplementary table which was omitted from the initial submission. Antibody and plasmid details are now listed in Supplementary table 5

14). Line 660: Antigen retrieval. The authors should describe the protocol.

Response: This has been detailed in the revised text (lines 602-604).

15). Black arrows in Fig. 4a, white arrows in Fig. 5b and asterisks in Fig. 5a, b should be explained.

Response: Figure legends have been rewritten to include descriptions of arrows and asterisks.

16). In all supplementary figures, the authors should include figure title and legends.

Response: Supplementary figure titles and legends have been included with this manuscript revision.

17). In figure S4, Figure X should be Figure S4.

Response: corrected.

Reviewer 2 Advance Summary and Potential Significance to Field...
In the submitted manuscript, Nelson and colleagues tackle a major gap in the field of limb formation: the initial stages of limb bud initiation and outgrowth from the somatic LPM. In addition, the structure and subdivision of the LPM into its splanchnic and somatic parts remains understudied, both by a lack of conceptual and of mechanistic tools.

In their work, the authors use the chicken embryo and scRNA-seq to reveal new details about the cellular heterogeneity and active processes in the somatic LPM at these critical time points. The authors document possible signaling interactions within the early partitioning LPM and show that the EMT regulator Twist1 has select expression already earlier than anticipated in the emerging forelimb field. Through their data presentation and discussion, the authors provide a conceivable framework for earliest (fore)limb initiation following LPM splitting, which is highly timely and complementary to existing work in the field.
Despite these strengths and at times beautiful execution of the involved bioinformatics work, the functional testing of the conceived models is limited to BMP inhibition by Noggin electroporation. These experiments are performed at an interesting stage of development, yet limited in scope, documentation, and their readouts, leaving the reader (or at least the reviewer) wanting for more info. Functional testing of RA perturbations with/without BMP inhibition, testing of an impact on additional genes found differentially expressed in the scRNA-seq, but already inclusion of further controls would greatly augment the manuscript. The impact at different time points and read out by various markers would provide more depth, too.
Overall, while the authors spend significant effort in describing the underlying biology with their scRNA-seq, their final in vivo conclusions are overly reductionistic again only address one or two players in the entire process.
The reviewer does not have the computational or biostatistical expertise to comment on the details and rigor of the performed scRNA-seq and follow-up analyses.

Reviewer 2 Comments for the Author...

Major comments
1) Twist1 is one of the major findings from all the transcriptomics analysis, yet the in vivo experiments connecting the individual parts of the authors' model remain limited. Suggestions for additional experiments:
a) limb bud region-specific Twist1 knockdown to determine role in limb bud initiation by checking then downstream genes, i.e. is there an interaction between Twist1 and Tbx5?
Response: We actually attempted these experiments prior to manuscript preparation. However, using, TOL2 vectors expressing validated shRNAs, we were unable to achieve significant reduction of TWIST1 mRNA or protein. Studies have previously shed light on the phenotypic effects of TWIST1 knockdown (Chen & Behringer 1995) and some genes that are differentially regulated as a result (Leobel et al. 2002), though TBX5 was not noted as of these genes. However, we agree that this experiment is necessary to further define the role of TWIST1 during LPM development and TBX5 initiation, but have been unable to do in this manuscript. While early expression of TWIST1 was indeed a major finding, this was not on the only major finding of the study. We also noted the enrichment of BMP signaling during LPM specification and limb bud outgrowth, and chose instead to conduct in vivo studies on that aspect of LPM differentiation.

b) Effect of RA signaling on Twist1, towards answering the question if BMP knockdown doesn’t affect Twist1, what does?

Response: As per above comment.

c) Ectopically express Twist1 in the future limb bud region
Response: We completely agree this is an important experiment. In line with the above comment, we also attempted TWIST over-expression but did not observe any significant effects on limb bud morphology, at least within 48 hours after EP. We believe that this is due to the fact that targeted electroporation of the somatic LPM can only be performed at a relatively late stage of LPM

development. That is, electroporation can only be performed once a coelom has formed, and thus the somatic LPM is already specified and has strong expression of TWIST1 protein (Figure 4b). Assuming that TWIST influences EMT of the somatic LPM or early activation of other limb genes, many of these events will have already occurred by the time the coelom has formed such that TWIST1 (or siRNA) can be injected and delivered by electroporation. It is due to this reason that we instead focused on inhibiting the signals that initiate LPM subdivision and somatic LPM specification, prior to subdivision and formation of the coloem. However, ectopic TWIST will likely influence early limb development (as well as the role of different bHLH hetero- / homodimers in the somatic LPM) but believe that, again, would be better suited to a focused follow up study.

d) How are FGF genes de-regulated by the treatment, i.e. is there a later impact of the electroporation on the AER or other structures?

Response: We have repeated the experiment in line with this comment, looking at expression of FGF genes 72h after electroporation. Noggin caused a complete failure of limb bud formation, and abolished TBX5, FGF10 and FGF8 expression, see updated figure 5 and Figure S4. These data add to the recent study by Tomizawa et al. 2021 who electroporated NOGGIN into the ectoderm and noted a severe reduction in AER formation and absent FGF8 expression (>HH20). This is an important observation which complements our study, though our data highlight that is through attenuation of TBX5 prior to establishment of the feedback loop. This is a critical component of this study and has been largely expanded in the discussion, namely paragraph beginning line 453.

2) Related, in Figure 5 the authors state that Noggin expression results in reduced Prrx1. From the presented pictures, another possible conclusion could be that the Noggin-transduced side could simply have less cells, resulting in a weaker in situ staining when seen from dorsal. In the section, the area is as described hyoptrophic/hypoplastic, but Prrx1 mRNA seems still solidly expressed.

Response: There are certainly fewer cells in the side electroporated with the BMP inhibitor, Noggin. However, PRRX1 mRNA staining intensity is lower than in on the contralateral side (see revised figure 5B.). We consider that BMP antagonism leads to Prrx1 downregulation because the data agree with two key previous studies.
(1) Funayama et al. 1999 showed that in the absence of ectoderm or co-culture with NOGGIN expressing cells, PRRX1 is not activated within the LPM. (2) Ocana et al. 2012 showed that BMP2 beads induce PRRX1 expression in the LPM. Together, while it is possible that the observed decreases in mRNA are due to purely to reduced numbers of cells, we believe we are also observing decreased activation of PRRX1. One possibility is that as PRRX1 is activated as early as ~HH8 (Ocana et al. 2017), PRRX1 was already actively undergoing transcription in the LPM prior to our ectopic inhibition via NOGGIN. As such, some residual or low levels of PRRX1 may be present in the somatic LPM.

The same holds true for the Twist1 ISH and associated claims.
The authors should provide close-up images of the area indicated in the section (i.e. as additional row of images in this overall nicely presented figure). Ideally, some form of quantification to solidify the claims would strengthen this entire figure. Further, the authors are encouraged to add quantifications to their figure panels and throughout.

Response: Figure 5 has been updated with close up images of the forelimb buds as per this request. From these updated panels it is still difficult to say whether TWIST1 expression is decreased as a result of inhibited BMP signalling. However, we believe that it is not BMP responsive due to the presence of TWIST1 protein in Figure 5a, albeit in reduced numbers of cells. We have made every effort to highlight the role of BMP antagonism in Figure 5, and have updated the discussion as to the signal inputs of TWIST1 (including retinoic acid) activation, see discussion paragraph beginning line 476.

As last touch, labelling of splanchnic vs somatic LPM in 5a as well as labelling of observed stages in the figure itself would greatly guide the reader.
Response: Somatic and splanchnic labels have been added to figure 5 (and figure 4b).

3) The authors add a superb model schematic in figure 2 and should have a summarizing model of this kind at the end of their manuscript to guide the reader.

Response: we have created a new summary figure 6 in response.

Minor
1) References are light, often one of two references for major topics with an over-reliance on individual reviews at times. The authors are encouraged to flesh out their references to provide the reader with context of this (at times complex) research field.

Response: We have made a large effort to expand the use of references throughout the manuscript and remove dependence on reviews.

2) Lines 57-59: Dampen the statement that these TF genes "initiate" these fates; their expression coincides with the onset of individual cell fate development and their activities might be required, but they are not master-regulatory.

Response: This section has been reworked in accordance with the comments of reviewer one. This statement was streamlined and now includes original references. See paragraph beginning line 53 of revised text.

3) Lines 96-97: "However, the events immediately preceding the TBX5-dependent limb regulatory pathway in the somatic LPM are less well understood." This is a central conundrum driving the work and a major open question in the field. The authors are encouraged to emphasize this point throughout their manuscript.

Response: We agree, and this point has now been emphasized throughout the manuscript. Particularly, we have updated our discussion to now emphasize our finding that BMP regulates activates the TBX5-FGF10-FGF8 pathway, which was previously unknown from the literature. See updated discussion paragraph, beginning line 453.

4) Line 156: The authors find Cdx4 among the enriched transgenes; yet, in common understanding this is a caudal/posterior gene. Can the authors reference work describing Cdx4 expression outside the tail region to provide context?

Response: CDX4 is expressed in the primitive streak, neural tube, paraxial mesoderm and lateral plate in early embryos, which becomes regionalized caudally as development progresses. This is evident in the GEISHA atlas of chicken embryo gene expression (http://geisha.arizona.edu/geisha/search.jsp?entrez_gene=448850). The expression of CDX4, together with MSGN1, FGF8, EVX1 etc all suggests that our sampling included some of the very early embryonic / primitive mesoderm when we dissected our tissues. We have added a specific reference to the results to contextualize the early observed expression of these genes (Alev et al. 2010: Transcriptomic landscape of the primitive streak). “the primitive mesoderm / LPM (c4) was solely comprised of E1.5 cells and displayed strong expression of early primitive streak and mesodermal markers such as MSGN1, EVX1 and CDX4 (Alev et al., 2010), suggesting the isolation of cells earlier than HH10.” Line 146-149 of the revised text.

5) Line 164: The authors are encouraged to add a brief summarizing statement at the end of this important section.
‘Response: We have included the summarizing sentence, “Together, these clusters represent all of the known, major tissues types within the developing forelimb field, which for the first time, now possess detailed transcriptional profiles.” See lines 155-158 of revised text.

6) Lines 188-190: That the LPM features complex interactions seems obvious, the authors should revisit the wording here for a more instrumental statement supporting their work in context of what's already known/assumed.

Response: This has been updated to consistent with known data, and introduce the next section. This now reads: “While these observations indicate that differentiation of multipotent LPM progenitors involves diverse signalling crosstalk (Figure 2a), we focused on TGF-β, WNT, BMP, FGF and HH, given their known roles influencing LPM specification, subdivision and limb development (Loh et al., 2016). Line 183-186 of revised text.
We have also eluded to a potential role of SHH-FOXF1-BMP4 signalling throughout the manuscript.

7) Lines 235-236: Quite a number of downstream targets for these signaling pathways are known, but not necessarily in the tissues the authors describe; rephrase accordingly.

Response: This has been refocused throughout the text.

8) Line 347ff: the authors only sampled a small section of the LPM, and statements describing expression specificity and selectivity should be made in that context; can the authors refer to wholemount stainings for Twist1 (and other genes) to emphasize/contextualize specificity (i.e. controls in Figure 5)?

Response: We have added in text to distinguish between wholemount limb field / bud expression or somatic LPM tissue sections.

11) Line 456 ff: Also related to the experiments (see above), can the authors check more candidate genes and how they change? For instance, what happens to FGF ligands, and does the perturbation cause a failure in AER induction, etc.

Response: In line with previous comments, we have repeated this experiment to look at a later developmental stage, and include expression of FGF ligands – which both are reduced through ectopic ectodermal Noggin. This is in line with its ability to reduce FGF8 in the ectoderm (Tomizawa et al. 2021). We have expanded our discussion to accommodate this finding.

12) Figures overall: the labeling of the scRNA-seq data could be made clearer by better emphasizing different stages in different outputs, as well as homogenization of gene nomenclature throughout the text and figures.

Response: We have standardized this throughout the text and figures.

13) Supplemental figure legends seem missing from the reviewer's documents.

Response: Supplementary figure legends have been added in an accompanying document.

Reviewer 3 Advance Summary and Potential Significance to Field...

In this paper, Newton et al., use single cell transcriptomics to investigate how the anterior-most portion of the posterior lateral plate mesoderm (LPM) is specified into splanchnopleural and somatopleural compartments and how forelimb progenitors emerge from it to trigger limb initiation. The authors establish a transcriptional atlas revealing the involvement of signaling pathways and transcription factors, from an unbiased standpoint, confirming already demonstrated functions and also uncovering new potential regulators. Particular emphasis is put on Twist1, which is found to be expressed together with Prrx1 and prior to Tbx5. Its function during limb initiation is discussed, although not directly tested. Finally, the authors perturb BMP signaling by electroporating Noggin in the ectoderm and observe the effect on LPM subdivision and limb bud formation.
Overall the data is convincing and nicely presented. It provides important -unbiased- insights into processes (LPM and limb formation), which have been historically investigated from a -biased- candidate gene approach.

Reviewer 3 Comments for the Author...

My major concern is with the functional perturbation using the electroporation technique. It is unclear whether the effect the authors observe are specific to the construct electroporated or due to non-specific malformations, which are known to occur with the electroporation. The embryos appear to be severely malformed with defects which are difficult to relate to the loss of BMP function in the lateral ectoderm (e.g. neural tube defects). As a result these experiments are not convincing.

Response: In alignment with comments by the other reviewers we have repeated this experiment to ensure that our results were reproducible and genuine. We have created a new panel in figure 5 showing the influence of ectodermal Noggin on limb formation. We have included replicate numbers for GFP and GFP-T2A-NOG electroporations and added additional genes to support our data. We also observed much less influence on off-target effects, which can also been seen in Figure S4. As electroporations were performed at HH9-10, prior to closure of the neural tube, in some instances, Noggin was electroporated into the ectoderm overlaying the neural tube which caused some neural tube defects. BMP4 is important during neural tube closure and polarity and given the potency of Noggin as an BMP antagonist, these effects are somewhat expected. However, its important to note that though these effects were observed in some embryos, we only focus our analysis on effects on the somatic LPM and limb, drawing comparisons to GFP controls which did not cause neural tube (and limb) defects. This was perhaps unclear from our data, so we have repeated our GFP control electroporations to show that the EPs were not responsible for any abnormal or off target effects.

Furthermore, it would be worth checking that ectodermal cells and not periderm cells are indeed targeted by the electroporation.

Response: At stage HH9-10, the ectoderm consists of a single layer of cells, which subsequently gives rise to both the surface ectoderm and periderm. Therefore, HH9 electroporation results in transfection of both cell types. Discussed in Tomizawa et al. 2021

Other comments:
There seems to be a confusion between the anterior LPM (aLPM) from which the cardiac mesoderm form and the posterior LPM (pLPM) which is subdivided into somatopleure and splanchnopleure and from which limbs form (see Tanaka et al, 2016). The authors refer to the anterior-most pLMP and should clarify the terminology.

Response: We thank the reviewer for pointing out this error, where the forelimb field does form from the anterior aspect of the posterior LPM. We have corrected this initially at its first instance (line 84) and amended aLPM to LPM throughout the rest of the text.

-P16 l.442 : I do not understand this sentence.
Response: This sentence has been rewritten to avoid confusion.

Second decision letter

MS ID#: DEVELOP/2022/200702

MS TITLE: Cell lineage specification and signalling pathway usage during development of the lateral plate mesoderm and forelimb mesenchyme

AUTHORS: Axel H Newton, Sarah M Williams, Andrew T Major, and Craig A Smith

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the comments of Reviewer 2 can be satisfactorily addressed (please see Editor's note). Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Although there are technical limitations, the authors improved or well discussed the manuscript about the reviewers' comments, especially in vivo experiments. Now the reviewer think that the manuscript is acceptable for Development. However, there are still some minor points to be clarify before accept.

Comments for the author

The authors response: The single cell data was validated by in situ hybridisation and immunofluorescence of select markers, which is a typical approach. Figure 4 shows the cellular distribution of TWIST1 that derived from the scRNA-seq dataset, very clearly validating that this gene is expressed specifically throughout all cells that comprise the somatic LPM cell layer, pre and post-EMT. In the context of the specific aims of this paper, no deeper cellular analysis was warranted. We did not examine PRRX1 and TBX5 protein expression due to the lack of a suitable chicken-specific antibodies. However, we do show mount in situ hybridization for PRRX1 and TBX5 during equivalent stages of development. Additionally, in figure 5 we are showing tissue level changes that occur as a result of inhibiting ectodermal BMP signalling.
Line325 in the revised manuscript, "…specific expression of TWIST1 protein, and co-expression with PRRX1 (data not shown), …". This sentence should be corrected if there are no available antibodies.

The authors response: This sentence now states: "While TWIST1 expression appears reduced in Noggin antagonised limb buds, tissue sections did not show obvious reductions in either in situ mRNA levels (B) or protein localization (A) compared to GFP controls".
This sentence is still somehow confusing and should be clarify. For example, "While TWIST1 expression appears reduced in Noggin antagonised limb buds in whole mount in situ hybridization…".

Reviewer 2

Advance summary and potential significance to field

In their revised manuscript, Newton and colleagues addressed several raised comments and refined the presentation and interpretation of their submitted work.
As the data stands now, the remaining gaps are the absence of functional data for Twist1 action/function (despite the additional BMP/FGF info) and the at times challenging quantification and thus interpretation of individual mRNA in situ patterns. We believe the editor will be able to make a call what the authors should still address.

Comments for the author

Outstanding points:
1) While the reviewers are appreciative of the authors additional data regarding the effects of fgf expression from Bmp inhibition, the absence of any functional data for Twist1 remains a major gap that is unaddressed in this study. We defer to the editor's decision regarding this aspect.
2) The authors added powerful additional panels with the limb bud closeups and labeling. However, some of the ISH are still inconclusive as presented right now particularly TWIST1. By the authors own admission these are difficult to interpret. No quantifications have been included to support the claims of decreased expression for PRRX1 or TWIST1 and no supporting data is included to address concerns of cell # vs. decreased expressing. We again defer to the editor's decision regarding this aspect. The authors are encouraged to dampen their interpretations and statements, ideally discussing the experimental caveats of these findings in the text.
3) Wonderful schematic that will be instrumental to the readers!
4) All minor points have been addressed and are satisfactory.

Reviewer 3

Advance summary and potential significance to field

The Authors have addressed all my concerns.

Comments for the author

The paper is, in my vie, ready for publication.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
Although there are technical limitations, the authors improved or well discussed the manuscript about the reviewers' comments, especially in vivo experiments. Now the reviewer think that the manuscript is acceptable for Development. However, there are still some minor points to be clarify before accept.

Reviewer 1 Comments for the Author:
The authors response: The single cell data was validated by in situ hybridisation and immunofluorescence of select markers, which is a typical approach. Figure 4 shows the cellular distribution of TWIST1 that derived from the scRNA-seq dataset, very clearly validating that this gene is expressed specifically throughout all cells that comprise the somatic LPM cell layer, pre and post-EMT. In the context of the specific aims of this paper, no deeper cellular analysis was warranted. We did not examine PRRX1 and TBX5 protein expression due to the lack of a suitable chicken-specific antibodies. However, we do show mount in situ hybridization for PRRX1 and TBX5 during equivalent stages of development. Additionally, in figure 5 we are showing tissue level changes that occur as a result of inhibiting ectodermal BMP signalling.

Line325 in the revised manuscript, "…specific expression of TWIST1 protein, and co-expression with PRRX1 (data not shown), …". This sentence should be corrected if there are no available antibodies.
Response: As the PRRX1 antibody was not used further due to non-specific background, we have removed the statement " .. and co-expression with PRRX1 (data not shown) .." from the text. This has also been removed from the methods.

The authors response: This sentence now states: "While TWIST1 expression appears reduced in Noggin antagonised limb buds, tissue sections did not show obvious reductions in either in situ mRNA levels (B) or protein localization (A) compared to GFP controls".

This sentence is still somehow confusing and should be clarify. For example, "While TWIST1 expression appears reduced in Noggin antagonised limb buds in whole mount in situ hybridization…".
Response: In response to reviewer 2s concerns, more below, we have updated figure 5 with tissue section panels that which show that TWIST1 is not affected by BMP signalling, where there are no changes in either mRNA or protein expression in Noggin treated embryos. In response, we have updated the text, which now simply reads: "TWIST1 was not affected by BMP inhibition, showing no obvious reductions in mRNA expression (C) or protein localization (A) compared to GFP controls."

Reviewer 2 Advance Summary and Potential Significance to Field:
In their revised manuscript, Newton and colleagues addressed several raised comments and refined the presentation and interpretation of their submitted work. As the data stands now, the remaining gaps are the absence of functional data for Twist1 action/function (despite the additional BMP/FGF info) and the at times challenging quantification and thus interpretation of individual mRNA in situ patterns. We believe the editor will be able to make a call what the authors should still address.

Reviewer 2 Comments for the Author:
Outstanding points:

1) While the reviewers are appreciative of the authors additional data regarding the effects of fgf expression from Bmp inhibition, the absence of any functional data for Twist1 remains a major gap that is unaddressed in this study. We defer to the editor's decision regarding this aspect.
Response: We regret that we were unable to ascertain the role of TWIST1 in this study, and agree that this is an important component of the story that still needs to be resolved. However, as we found that *TWIST1* was not BMP responsive (see below comment) which was the main focus of our experimentation, any additional TWIST1 functional data falls outside the scope of this study. We take your concerns on board and have reduced any conclusions about the role of TWIST in the text, and have updated the second last paragraph of the discussion to point out that further investigation is required to ascertain the role of TWIST1 during somatic LPM specification.

2) The authors added powerful additional panels with the limb bud closeups and labelling. However, some of the ISH are still inconclusive as presented right now, particularly TWIST1. By the authors own admission these are difficult to interpret. No quantifications have been included to support the claims of decreased expression for PRRX1 or TWIST1 and no supporting data is included to address concerns of cell # vs. decreased expressing. We again defer to the editor's decision regarding this aspect. The authors are encouraged to dampen their interpretations and statements, ideally discussing the experimental caveats, of these findings in the text.
Response: We appreciate the reviewers concern on making sure that this figure and the data was as clear as possible. In response, we have sectioned through the wholemount *in situ* stained limb buds to show the mRNA localization patterns at the tissue-level. From these, and in association with the whole mount embryos, it is evident that the limb bud mesenchyme fails to form from the somatic LPM, *PRRX1* is downregulated, *TBX5* activation is reduced and as a result *FGF10* and *FGF8* are not activated, failing to form the feedback loop and drive proliferation of the mesenchyme. From this additional data, it is now clear that TWIST1 (RNA and protein) is not affected by BMP antagonism. Additionally, by utilizing a later timepoint for collection, these differences in somatic LPM / limb bud thickness are much more obvious. We believe that these are now address the issue of "cell # vs. decreased expression".

3) Wonderful schematic that will be instrumental to the readers!
Response: We thank the reviewer for this comment.

4) All minor points have been addressed and are satisfactory.

Reviewer 3 Advance Summary and Potential Significance to Field:
The Authors have addressed all my concerns.

Reviewer 3 Comments for the Author:
The paper is, in my vie, ready for publication.

Third decision letter

MS ID#: DEVELOP/2022/200702

MS TITLE: Cell lineage specification and signalling pathway usage during development of the lateral plate mesoderm and forelimb mesenchyme

AUTHORS: Axel H Newton, Sarah M Williams, Andrew T Major, and Craig A Smith
ARTICLE TYPE: Research Article

I am satisfied with the response and revision of the manuscript. Your manuscript has been accepted for publication in Development, pending our standard ethics checks.