Defining the signalling determinants of a posterior ventral spinal cord identity in human neuromesodermal progenitor derivatives

Matthew Wind, Antigoni Gogolou, Ichcha Manipur, Ilaria Granata, Larissa Butler, Peter W. Andrews, Ivana Barbaric, Ke Ning, Mario R. Guarracino, Marysia Placzek and Anestis Tsakiridis
DOI: 10.1242/dev.194415

Editor: Francois Guillemot

Review timeline

Original submission:	24 June 2020
Editorial decision:	10 August 2020
First revision received:	22 January 2021
Editorial decision:	16 February 2021
Second revision received:	22 February 2021
Accepted:	23 February 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/194415

MS TITLE: Defining the signalling determinants of a posterior ventral spinal cord identity in human neuromesodermal progenitor derivatives

AUTHORS: Matthew Wind, Antigoni Gogolou, Ichcha Manipur, Ilaria Granata, Larissa C Butler, Peter W Andrews, Ivana Barbaric, Ke Ning, Mario R Guarracino, Marysia A. Placzek, and Anestis Tsakiridis

I have now received the reports of three referees on your manuscript and I 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 great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request a more direct comparison with previously published protocols and a more thorough analysis of the cell types generated using your protocol. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their 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 the manuscript by Wind et al., the authors aim to develop a protocol that improves the in vitro generation of human motor neurons with a posterior identity. The ability to generate defined cell types in vitro from human pluripotent cells is of major relevance to the development and regenerative medicine fields. However, the data presented are too preliminary to firmly conclude how the approaches have improved upon previously published methods, and how useful the system will be for understanding differences in motor neurons with distinct axial identities.

Comments for the author

The main objective of the manuscript by Wind et al. is to develop an improved protocol for the generation of human motor neuron progenitors in vitro, that display posterior (spinal cord) character. What is not clear from the data presented is how the protocol advances previously established approaches, which are already quite similar (eg. Verrier et al 2018 Development and Lippmann et al 2015). Without a more direct comparison and extensive characterisation of what cell types are generated in this protocol, it is unclear how the efficiency in generating spinal cord motor neuron progenitors has been improved over existing methods, eg, whether by improving the proportion of neural progenitorsgenerated and/or the proportion of neural cells that demonstrate the desired (ventral and posterior) motor neuron identity, as a result of the culture conditions described in the manuscript. Further comments are provided below.

In Fig1a, the replating of cells after day 3 seems to decrease TBXT in all 3 conditions presented (low levels in all day 4 conditions). The levels of TBXT then seems to rise in all three conditions over time as the cells expand, which is more obvious in the high CHIR/FGF condition. This seems at odds with the authors conclusions that high CHIR/FGF treatment sustains TBXT levels, shown by qPCR in S1A.

The authors conclude early on (line 155) that changing the regime of exposure to CHIR/FGF limits mesodermal differentiation. It was not clear at this point how the authors conclude this from the data presented in figure 1 (as loss of TBXT may suggest that the cells have differentiated further, and now express more mature mesoderm markers).

As NMPs do not express neural markers, and the transcriptome data presented are from bulk RNA-seq, it is difficult to interpret the relevance of the fold changes presented in Figure 2D and how they may explain the cell types present in the dish. The Sox2 staining in Figure 2A is clear, but its relevance to interpreting what cells are present in the dish is difficult to conclude as Sox2 is expressed in many cell types, including epiblast, preneural, neural progenitors and neural crest cells, which the authors later note and have previously shown are derivatives of NMPs.

The Cdx2 IF images raise further questions about cell type and more generally how to interpret the RNA-seq data. It appears as though the Cdx2 protein is decreasing substantially by day 7 in the image shown in Fig 2A, although perhaps not entirely throughout the dish. This contrasts with the RNA-seq analysis which as currently presented, shows a more stable maintenance of Cdx2 transcript at the population level, when comparing day 3 and 7. Additional single cell approaches to conclude what cell types are present in the dish would help to interpret the data.

The interpretation that the simultaneous blocking of BMP/TGF beta signalling improves the proportion of ventral neural cells generated in response to Shh signalling, is unclear. Dual smad inhibition is a well-established method to block the differentiation of non-neural cells (c.f. Chambers et al., 2009). It is therefore not possible to exclude the possibility that the combined

activation of Shh signalling together with TBFb/BMP inhibition is increasing the proportion of ventral cell types due to a decrease in non-neural cells and not due to an explicit requirement for inhibition in neural cells to obtain a ventral fate. The decrease in expression of "dorsal" genes in Fig3 may be the result of decreased mesodermal cell types, as Pax3 and Msx1 are not exclusively expressed in the dorsal neural tube.

Reviewer 2

Advance summary and potential significance to field

The success of pluripotent stem cells for clinical applications rests on our ability to generate the appropriate cell types. Specifically, generating caudal CNS was a historic challenge in the field. Neuromesodermal progenitors (NMPs) contribute to the caudal spinal cord, and thus the aim of guiding human pluripotent stem cells into MNPs is a worthwhile goal.

Different from the “classic” MN differentiation protocol that produces rostral MN fates, the authors test different signaling regimes to induce NMPs from human pluripotent stem cells and generate thoracic motor neurons. Supported by their demonstrated in vivo and in vitro patterning properties, WNT-FGF caudalizing signals plus BMP inhibition and Shh activation to ventralize are the appropriate signaling cues. In agreement with previous publications, the authors find WNT-FGF induces caudal Hox gene expression. Moreover, the WNT-FGF activation timing expression favoring neuronal fates. The dual ventralization regimen is clever and effective.

Additionally, the authors test the protocol in more than one cell line adding value to their work. Performing grafts is demanding and always a welcome addition to these studies. Some human neurites exit the spinal cord and navigate the different paths mouse-derived MNs do.

Although there is a clear effort refining the protocol to improve efficiency, I am less clear about the conceptual or mechanistic novelty. The signaling molecules, and even some concentrations and times, have been already reported to generate NMPs and MNs from human pluripotent stem cells. Moreover, the MN production efficiency is on par with previous protocols, although of different positional identity. Additionally, there is no in-depth MN fate characterization to support an efficient thoracic MN fate differentiation protocol.

In short, this work condenses a lot of know-how into a valuable resource.
However, I believe it will benefit from some improvement in the conceptual and some technical characterization.

Comments for the author

The success of pluripotent stem cells for clinical applications rests on our ability to generate the appropriate cell types. Specifically, generating caudal CNS was a historic challenge in the field. Neuromesodermal progenitors (NMPs) contribute to the caudal spinal cord, and thus the aim of guiding human pluripotent stem cells into MNPs is a worthwhile goal.

Different from the “classic” MN differentiation protocol that produces rostral MN fates, the authors test different signaling regimes to induce NMPs from human pluripotent stem cells and generate thoracic motor neurons. Supported by their demonstrated in vivo and in vitro patterning properties, WNT-FGF caudalizing signals plus BMP inhibition and Shh activation to ventralize are the appropriate signaling cues. In agreement with previous publications, the authors find WNT-FGF induces caudal Hox gene expression. Moreover, the WNT-FGF activation timing expression favoring neuronal fates. The dual ventralization regimen is clever and effective.

Additionally, the authors test the protocol in more than one cell line adding value to their work. Performing grafts is demanding and always a welcome addition to these studies. Some human neurites exit the spinal cord and navigate the different paths mouse-derived MNs do.

Although there is a clear effort refining the protocol to improve efficiency, I am less clear about the conceptual or mechanistic novelty. The signaling molecules, and even some concentrations and times, have been already reported to generate NMPs and MNs from human pluripotent stem cells. Moreover, the MN production efficiency is on par with previous protocols, although of different positional identity. Additionally, due to the lack on specific MN type marks analysis, there is no in-depth MN fate characterization to support an efficient thoracic MN fate differentiation protocol. In short, this work condenses a lot of know-how into a valuable resource.

However, I believe it will benefit from some improvement in the conceptual and some technical characterization.

Given the interneuron generation, at a minimum an Isl1/2 and Hb9 containing and quantification will solidify MN (double positive) versus interneuron (some are Isl1/2) identities. On that vein, the generation of PGC MN fate from pluripotent stem cells has been the notorious difficult fate to obtain.

Perhaps this protocol produces it. A careful examination with MN markers could reveal it.
The authors are correct on stating that Hoxc9+ MNs are of thoracic axial identity. However, it is expected they downregulate rostral Hox gene expression, which is maintained at high levels based on the presented data.

Moreover, there is no MN fate markers characterization to gain some MN thoracic fate confidence by excluding alternative axial MN fates. Many of these markers can be extracted already from their RNA-seq data, and perhaps a simple comparison with published data or their own generated MNs would help.

I was confused by this statement: “containing only low numbers of OLIG2+/ISL1/2+/MNX1+ MN progenitors/MNs”. It is not clear to me if this is a triple positive population is the one reported because it seems single positive cells from the figure. Moreover, Mnx1 and Olig2 are co-expressed in a narrow window of time. Perhaps just using MNp (Nkx6.1/Olig2) and MNs (Isl1/2 / Mnx1 or NF) would make this quantification easer to the reader if that is the goal.

Of note, Olig2/Nkx2.2 cells are expected to remain in culture but with an oligodendrocyte progenitor fate. Thus, Olig2-only staining requires careful interpretation.

Minor:
Error bars on biological replicates on Fig 1B will help the reader know the expected protocol variability.

Adding CDX2 to Fig 2B quantification would also give a sense of variability to the reader.
There are a few human NMP protocols, some of them even with Day 3 transcriptomics. Since this work aims to synthesize and refine human NMP generation, it would be good to compare the overall transcriptomic analysis with others. Although more demanding, extracting signatures from a more detailed mouse study and benchmark the human NMPs would be a great addition.

On that vein, adding more genes to Fig 2C will make fate choice more evident without the need to go to the table or reanalyze the original data. Perhaps using Day 3 as a reference will make the upregulation and downregulation clearer.

I find the representation format change from Fig 2C to 2D confusing.

Although it is now normalized to D3, it changes to format and removes pluripotent cells.

I find it reassuring that Hoxb cluster genes are induced earlier as it does during early development. Grafting experiments are demanding, and a great complement, and thus I am not proposing any repetition. However, it should be noted that Fig 5B ii might contain neurites projecting into limbs. Where these Hoxc9 positive, as shown in Fig S4?

Reviewer 3

Advance summary and potential significance to field

Wind et al present a refined protocol to produce human thoracic motor neurons from human pluripotent stem cells (hPSCs). hPSC-derived motor neurons are usually of a cervical identity and are differentiated via a neural rosette stage. This team take advantage of their expertise in so-called neuromesodermal progenitors (NMPs) and manipulation of developmentally relevant signalling pathways to produce motor neurons with a more caudal identity. This makes the work highly novel and interesting from a developmental biology perspective, and it also could be relevant to regenerative medicine in the future.

Comments for the author

In general the differentiation protocols and marker analysis is well performed, and the chick grafting experiments are very informative as to the potential of the hPSC-derived motor neurons in vivo. The electrophysiological experiments are quite basic and preliminary making it difficult to draw any conclusions from them.

Specific issue to address include:

1. Figures 1 and 2: It is difficult to discern how representative the data in these two figures are. There is no indication about biological and/or technical repeats, nor are any statistics performed. For, example in line 150, high FGF2 (100 ng/ml) is claimed to further enhance reduction of T levels, but the data to support this claim is absent. Furthermore, the data in Figure 1B that directly compares 20 ng/ml and 100 ng/ml FGF2 does not show any significant difference.
2. Figure 1B: Please include the quantification of T+ve cells in CHIR (8 uM) + FGF2 (40 ng/ml) up to Day 5.
3. Figure 3, and lines 188-244. The discussion/narrative of how dual-SMAD inhibition was arrived at is very confusing. If this could be simplified with less jumping around between the different panels in Figure 3, that would significantly improve the readability. Dual-SMAD inhibition for neural induction has been reported since 2009, and the TGFb-inhibitor likely promotes exit from pluripotency. Why is this inhibitor added at Day 3, while the BMP inhibitor is added at Day 0?
4. Figure 5C. This experiment compares cervical to thoracic motor neurons by looking at current-induced, not spontaneous, action potentials. This is the most basic electrophysiology experiment that could be performed and does not show they are “equivalent in terms of electrophysiology” as stated in line 342, or they are “behaving similarly” as stated in line 359. Unless more electrophysiology experiments can be provided, the most that can be stated is that both preparations exhibit properties of immature neurons.
5. Discussion: Motor neurons of lumbosacral identity still appear to be elusive. How could this protocol be further modified to promote the differentiation of motor neurons with the most posterior of identities?

First revision

Author response to reviewers' comments

Below is a detailed, point-to-point response to each of the issues raised by the reviewers-a PDF version can also be found as part of the supplementary information.

Reviewer #1
1.1. However, the data presented are too preliminary to firmly conclude how the approaches have improved upon previously published methods, and how useful the system will be for understanding differences in motor neurons with distinct axial identities.

We appreciate the reviewer's concern and have now included new data as discussed under the reviewer's specific points below.

1.2. What is not clear from the data presented is how the protocol advances previously established approaches, which are already quite similar (eg. Verrier et al 2018 Development and Lippmann et al 2015). Without a more direct comparison and extensive characterisation of what cell types are generated in this protocol, it is unclear how the efficiency in generating spinal cord motor neuron progenitors has been improved over existing methods...

As mentioned above we have now added new data (Figures 4E, 4F, S5) showing that our approach (prolonged culture of NMP-like cells in WNT-FGF agonists combined with BMP-TGFβ inhibitors and pro-neural/ventralising signals) yields thoracic MNs of a PGC identity while a conventional differentiation strategy that is based on the immediate culture of NMP-like cells in pro-neural/ventralising conditions (Gouti et al., 2014; Rayon et al., 2020; Verrier et al., 2018) does not. As noted by Reviewer #2, PGC, it has been challenging to generate MNs from hPSCs using current protocols and thus their efficient production in vitro is likely to provide important insights into their biology and open new avenues for disease modelling/cell replacement applications in future studies.

1.3. In Fig1a, the replating of cells after day 3 seems to decrease TBXT in all 3 conditions presented (low levels in all day 4 conditions). The levels of TBXT then seems to rise in all three conditions over time as the cells expand, which is more obvious in the high CHIR/FGF condition. This seems at odds with the authors conclusions that high CHIR/FGF treatment sustains TBXT levels, shown by qPCR in S1A."

We have now updated the graph (New Figure 1D) showing the quantification of TBXT+ cells, to include more biological replicates and all the three culture regimes depicted in Fig. 1. Our new data/analysis reveals that the reduction in TBXT in cells exposed to high CHIR/FGF is not statistically significant, and is transient (between days 3-4), subsequently recovering (between days 4-5). The other two WNT-FGF agonist concentrations gave rise to a steadily decreasing population of TBXT+ cells (see new Figures 1D and S1C). The lack of precise correlation between TBXT protein and transcript levels over days 3-4 may reflect post-transcriptional/-translational mechanisms influencing mRNA and protein stability. This appears to be a common phenomenon and it is well documented that the relationship between cellular protein levels and mRNA abundance is often complex and affected by a wide array of biological and experimental parameters (Liu et al., 2016).

1.4. The authors conclude early on (line 155) that changing the regime of exposure to CHIR/FGF limits mesodermal differentiation. It was not clear at this point how the authors conclude this from the data presented in figure 1 (as loss of TBXT may suggest that the cells have differentiated further, and now express more mature mesoderm markers).

We agree with the reviewer and we have now removed this statement rephrasing our conclusion to: "…lower WNT and high FGF levels drive the gradual extinction of TBXT+ progenitors" (lines 158-159 in the revised manuscript version) instead of "…lower WNT and high FGF levels drive the extinction of TBXT+SOX2+ progenitors and limit mesodermal differentiation".

1.5. As NMPs do not express neural markers, and the transcriptome data presented are from bulk RNA-seq, it is difficult to interpret the relevance of the fold changes presented in Figure 2D and how they may explain the cell types present in the dish. The Sox2 staining in Figure 2A is clear, but its relevance to interpreting what cells are present in the dish is difficult to conclude as Sox2 is expressed in many cell types, including epiblast, preneural, neural progenitors and neural crest cells, which the authors later note and have previously shown are derivatives of NMPs.

We did not intend to imply that the cultures emerging following prolonged culture of NMP-like cells in WNT and FGF agonists are composed exclusively of early neural cells but rather that these signals "steer NM-potent spinal cord progenitors toward a pre-neural/early neural state exhibiting dorsal neural tube features…" (Lines 183-186 in originally submitted manuscript version). We summarise below our arguments for this conclusion, based on three lines of evidence. Nonetheless, we have now also added new supporting data:

(i) Significant downregulation of paraxial mesoderm/somite/NMP markers indicated by bulk RNA-seq data and immunostaining (New Figures 1, 2D). We have now also included an additional graph depicting the expression changes of a larger number of paraxial mesoderm/somite markers (selection was based on single cell transcriptome data from (Diaz-Cuadros et al., 2020)) between days 3 (NMP stage) and day 7 (early pre-neural/neural state) obtained from our bulk RNA-seq analysis (New Figure S2A). This shows almost all of them are significantly downregulated with the exception of three (MEOX1, CADM1 and FBN2), which have been reported to also mark neural crest and its derivatives (Ansari et al., 2014; Candia et al., 1992; Lumb et al., 2017; Shimada et al., 2012). This suggests that the culture conditions are largely incompatible with differentiation toward paraxial mesoderm/somite but may promote neural crest specification.

(ii) The significant upregulation of pre-neural/early neural/dorsal markers (New Figures 2E and S2B) and the single cell protein analysis by immunofluorescence showing that the cultures are predominantly SOX2+ (~90% of total cells) and CDX2+ positive ~85% of total cells) (new Figure 2A-C) both indicate the acquisition of a pre-neural/early dorsal spinal cord character by most cells in the culture.

(iii) We previously demonstrated that pluripotent epiblast markers (NANOG, OTX2) are already extinguished at day 3 (Frith et al., 2018), which suggests that the presence of SOX2+ epiblast cells is unlikely.

To capture more accurately these conclusions as well as the possible existence of minor unidentified cell subpopulations we have introduced the following text changes:

- We show that combined WNT-FGF activities drive a posterior dorsal pre-/early neural state (lines 34-35 in the revised manuscript version).
- WNT-FGF-induced pre-neural/early neural progenitors (lines 123-124).
- “WNT and FGF induce a posterior pre-/early neural state from human NMPs” (line 135).
- “We found that high levels of CHIR (8 μM) and FGF2 (40 ng/ml) promoted predominantly the generation of presomitic/paraxial mesoderm cells” (Lines 148-149).
- “…a combination of WNT and high FGF signalling activities steer human NMP-like cells predominantly toward a pre-neural/early neural state exhibiting dorsal neural tube features …” (lines 189-190).
- “We next tested culture conditions for the efficient ventralisation of pre-neural/early spinal cord progenitors ….” (lines 196-197).
- “…we tested the presence of dorsal neural cell types/prospective neural crest…” (line 228).
- “…day 7 WNT-FGF-induced cultures are composed mainly by a mixture of such pre-neural and early neural progenitors, which are likely to exhibit neural crest-forming potential. …” (lines 434-436).

Finally we would like to note that very similar findings i.e. continued WNT-FGF treatment promotes the transition of hPSC-derived NMP-like cells toward SOX2+CDX2+ pre-neural/early neural cells, have been reported recently by another study (Cooper, 2020) thus independently confirming our conclusions. We have now modified our discussion to reflect this new report (see lines 441-444).

1.6. The Cdx2 IF images raise further questions about cell type and more generally how to interpret the RNA-seq data. It appears as though the Cdx2 protein is decreasing substantially by day 7 in the image shown in Fig 2A, although perhaps not entirely throughout the dish. This contrasts with the RNA-seq analysis, which as currently presented, shows a more stable maintenance of Cdx2 transcript at the population level, when comparing day 3 and 7. Additional single cell approaches to conclude what cell types are present in the dish would help to interpret the data.

As mentioned above a lack of correlation between protein and transcript levels is not uncommon and CDX2 levels in particular have been previously shown to be regulated by various post-transcriptional and post-translational mechanisms (Boulanger et al., 2005; Pereira et al., 2013; Rings et al., 2001) and are thus likely to cause the protein/mRNA discrepancy noted by the reviewer. Again we would like to stress that the induction of CDX2+SOX2+ cells from NMP-like cells following culture in WNT-FGF agonists has been confirmed independently by another recent study (Cooper, 2020) thus strengthening our conclusions.

1.7. The interpretation that the simultaneous blocking of BMP/TGF beta signalling improves the proportion of ventral neural cells generated in response to Shh signalling, is unclear. Dual smad inhibition is a well-established method to block the differentiation of non-neural cells (c.f. Chambers et al., 2009). It is therefore not possible to exclude the possibility that the combined activation of Shh signalling together with TBFb/BMP inhibition is increasing the proportion of ventral cell types due to a decrease in non-neural cells and not due to an explicit requirement for inhibition in neural cells to obtain a ventral fate. The decrease in expression of "dorsal" genes in Fig3 may be the result of decreased mesodermal cell types, as Pax3 and Msx1 are not exclusively expressed in the dorsal neural tube.

The reviewer raises a valid point. To address this we quantified the number of SOX2+ cells obtained in the absence or presence of TGFβ/BMP inhibitors at an early timepoint of differentiation (day 8). Our rationale was that if TGFβ-BMP inhibition eliminates “contaminant” SOX2-negative, PAX3+/MSX1+ mesodermal cell types in favour of a neural fate, then we should observe an early increase in the SOX2+ fraction in parallel with a reduction in dorsal markers such as PAX3/MSX1. However, following treatment with TGFβ/BMP inhibitors we observed a decrease in the number of SOX2+ cells and a simultaneous increase in the percentage of cells expressing SOX1, a later neural progenitor marker. These data, which have now been added to the manuscript (New Figure S3), suggest that dual TGFβ-BMP inhibition acts on a cell entity that is already committed to a pre-neural fate and enhances neural commitment by restricting the acquisition of a dorsal neural/neural crest character but also potentially via acceleration of transition toward a neural fate. To reflect this finding we have added the following new paragraph/sentences in the manuscript:

- “…suppression of TGFβ-BMP signalling pathways promotes a ventral identity and neural commitment” (lines 36-37 in the revised manuscript version).
- “…we demonstrate that efficient ventralisation and further neural commitment of WNT-FGF-induced pre-neural/early neural progenitors requires active suppression of the pro-dorsal TGFβ and BMP signalling pathways…” (lines 122-124).
- “This finding suggests that blocking of BMP-TGFβ activities enhances neural differentiation of SOX2+ early spinal cord progenitors potentially by restricting the emergence of neural crest cells as mentioned earlier and also accelerating their transition toward a “later” neural fate in line with previous reports showing that these signalling pathways are critical for the temporal control of neurogenesis . (Dias et al., 2014; Ma et al., 2020; Sagner, 2020)” (lines 262-267).
- “This relies on the induction of posterior neurectoderm (via WNT-FGF-RA stimulation/BMP-TGFβ inhibition)…” (lines 271-272).
- “Moreover, our data indicate that BMP-TGFβ inhibitors also improves differentiation efficiency by promoting neural differentiation of early SOX2+ progenitors, potentially at the expense of neural crest specification and via the acceleration of the rate of neural commitment, as indicated by the increase in the number of SOX1+ cells following treatment.” (lines 473-477).

Reviewer #2

2.1 "Although there is a clear effort refining the protocol to improve efficiency, I am less clear about the conceptual or mechanistic novelty. The signalling molecules, and even some concentrations and times, have been already reported to generate NMPs and MNs from human pluripotent stem cells. Moreover, the MN production efficiency is on par with previous protocols, although of different positional identity. Additionally, due to the lack on specific MN type marks analysis, there is no in-depth MN fate characterization to support an efficient thoracic MN fate differentiation protocol."

We appreciate he reviewer’s concerns and have now included new data to demonstrate that our differentiation strategy is an efficient route toward the induction of thoracic/PGC MNs in contrast to a previously published NMP-based strategy (new Figure panels 4E, 4F, new Figure S5) thus addressing the reviewer’s issues about in-depth MN fate characterization/comparison with previous approaches. We also added new data (new Figure S3), which show that BMP-TGFβ inhibitors improve MN differentiation efficiency by augmenting neural differentiation of early SOX2+ progenitors at the expense of neural crest/dorsal neural cells (as indicated by Figure 3) and via the acceleration of the rate of neural commitment (new Figure S3B, S3C; see also our response above) thus providing a novel mechanistic insight into their mode of action.

2.2. On that vein, the generation of PGC MN fate from pluripotent stem cells has been the notorious difficult fate to obtain. Perhaps this protocol produces it. A careful examination with MN markers could reveal it.

We thank the reviewer for this suggestion, and have responded as detailed above. We do indeed find evidence for thoracic/PGC MNs (See new Figures 4E, 4F, S5).

2.3. The authors are correct on stating that Hoxc9+ MNs are of thoracic axial identity. However, it is expected they downregulate rostral Hox gene expression, which is maintained at high levels based on the presented data.

We believe that the simultaneous detection of HOX transcripts belonging to paralogous groups (PG) 6-10 is likely to reflect the presence of brachial and lumbar MN subpopulations in our cultures in addition to thoracic MNs. This is in line with other recent studies showing expression of HOX PG(6-10) members within the same hPSC-derived MN cultures, both at the transcript and protein level (Faustino Martins et al., 2020; Mouilleau, 2020). It should be noted that in one of these studies the detection of HOXC6 transcripts did not correlate with HOXC6 protein positivity (Faustino Martins et al., 2020) pointing to the existence of post-transcriptional/-translational mechanisms controlling HOX expression. Unfortunately, our efforts to dissect the heterogeneity of our MN cultures at the single cell level using immunostaining have been unsuccessful as we did not manage to find reliable antibodies against HOX PG6 members.

2.4. Moreover, there is no MN fate markers characterization to gain some MN thoracic fate confidence by excluding alternative axial MN fates. Many of these markers can be extracted already from their RNA-seq data, and perhaps a simple comparison with published data or their own generated MNs would help.

We show that our MN cultures express the PGC marker nNOS (see above) indicating robust induction of a thoracic axial identity. Please note that we do not have any RNA-seq data corresponding to day 24 MN cultures that could aid further verification of their axial level/columnar identity.

2.5. I was confused by this statement: “containing only low numbers of OLIG2+/ISL1/2+/MNX1+ MN progenitors/MNs”. It is not clear to me if this is a triple positive population is the one reported because it seems single positive cells from the figure.

We apologise for the lack of clarity. The sentence in question refers to single positive populations. We have now changed it to:

- “…containing only low numbers of OLIG2+ MN progenitors and ISL1/2+ or MNX1+ MNs (≤5% of total) respectively” (lines 222-223 in the revised manuscript version).

2.6. Error bars on biological replicates on Fig 1B will help the reader know the expected protocol variability.

We have now included error bars in the new Figure 1D.

2.7. Adding CDX2 to Fig 2B quantification would also give a sense of variability to the reader.

We have now included quantification of CDX2+ cells in the new Figure 2C.

2.8. There are a few human NMP protocols, some of them even with Day 3 transcriptomics. Since this work aims to synthesize and refine human NMP generation, it would be good to compare the overall transcriptomic analysis with others. Although more demanding, extracting signatures from a more detailed mouse study and benchmark the human NMPs would be a great addition.

We thank the reviewer for this good suggestion and we have now included a list of all significantly up- and downregulated transcripts in day 3 NMP-like cells (vs day 0 undifferentiated hPSCs) in the new table S1 as a resource that can be used by other groups for future comparative analyses. However, we feel that extracting a human NMP transcriptome signature is out of the scope of this

manuscript, which focuses on the differentiation of human NMP-like cells toward ventral spinal cord.

2.9. On that vein, adding more genes to Fig 2C will make fate choice more evident without the need to go to the table or reanalyze the original data. Perhaps using Day 3 as a reference will make the upregulation and downregulation clearer.

As mentioned above we have now included a graph depicting log fold gene expression changes of a larger number of paraxial mesoderm/somite-associated markers using day 3 as a reference (New Figure S2A).

2.10. I find the representation format change from Fig 2C to 2D confusing. Although it is now normalized to D3, it changes to format and removes pluripotent cells.

We have now adopted the same format for representing expression changes for all sets of genes examined in new Figure 2. We have moved the graph showing log fold expression changes of selected neural/dorsal/early neural crest markers normalised to day 3 (originally Figure 2D) to the new supplementary Figure S2B.

2.11. However, it should be noted that Fig 5B ii might contain neurites projecting into limbs. Where these Hoxc9 positive, as shown in Fig S4?

Unfortunately we did not carry out immunostaining against HOXC9 on this specific section though adjacent sections obtained from the same embryo contained HOXC9+ donor cells.

Reviewer #3
3.1. Figures 1 and 2: It is difficult to discern how representative the data in these two figures are. There is no indication about biological and/or technical repeats, nor are any statistics performed. For, example in line 150, high FGF2 (100 ng/ml) is claimed to further enhance reduction of T levels, but the data to support this claim is absent. Furthermore, the data in Figure 1B that directly compares 20 ng/ml and 100 ng/ml FGF2 does not show any significant difference.

We appreciate these points and have now included error bars and information about biological replicates in new Figure 1D to address this issue. We have also incorporated new data (New Figure S1C) comparing mean fluorescence intensity of TBXT protein expression in cultures generated using 20 ng/ml vs 100 ng/ml FGF2; these show that the higher FGF2 concentration further enhances reduction of TBXT levels.

3.2. Figure 1B: Please include the quantification of T+ve cells in CHIR (8 uM) + FGF2 (40 ng/ml) up to Day 5.

These data have now been added into new Figure 1D.

3.3. Figure 3, and lines 188-244. The discussion/narrative of how dual-SMAD inhibition was arrived at is very confusing. If this could be simplified with less jumping around between the different panels in Figure 3, that would significantly improve the readability.

We have amended the text in this section and also changed the colour coding of the different inhibitor treatments and structure in corresponding figure 3 to improve readability.

3.4. Dual-SMAD inhibition for neural induction has been reported since 2009, and the TGFb-inhibitor likely promotes exit from pluripotency. Why is this inhibitor added at Day 3, while the BMP inhibitor is added at Day 0?

As stated earlier we have now added new data (New Fig. S3) indicating that dual BMP-TGFβ inhibition promotes neural differentiation of early SOX2+ progenitors potentially at the expense of neural crest rather than eliminating the emergence of non-neural cell types. As already mentioned in the results section of the manuscript we “included a BMP pathway inhibitor (LDN 193189) during the initial induction of NMP-like cells from hPSCs (D0-3) to suppress emergence of neural crest cells, as we previously showed that endogenous BMP activity during that stage can promote early

neural crest features at the expense of central nervous system neural cells (Frith et al 2018)" (Lines 197-201 in original submission). The TGFβ inhibitor SB431542 is added later, at day 3, after induction of TBXT+SOX2+ cells as earlier treatment of cultures has been shown to be deleterious on the generation of NMP-like cells, which appear to be dependent on Nodal signalling (Edri et al., 2019; Turner et al., 2014).

3.5. Figure 5C. This experiment compares cervical to thoracic motor neurons by looking at current-induced, not spontaneous, action potentials. This is the most basic electrophysiology experiment that could be performed and does not show they are "equivalent in terms of electrophysiology" as stated in line 342, or they are "behaving similarly" as stated in line 359. Unless more electrophysiology experiments can be provided, the most that can be stated is that both preparations exhibit properties of immature neurons.

Whole-cell current clamp recordings have been employed widely in comparative type experiments e.g. aiming to assess the firing rate/excitability of in vitro derived MNs carrying ALS-associated mutations vs their "healthy" counterparts (For some examples see (Sareen et al., 2013; Selvaraj et al., 2018; Wainger et al., 2014)) and thus we believe is a valid approach to also compare the electrophysiological properties of our anterior/posterior MNs. However, we agree with the reviewer that more extensive analysis is required in order to extract more definitive conclusions about how similar the two populations are. To reflect this we have amended the text/added the following sentences:

- "Together these data indicate that anterior and posterior hPSC-derived MNs are likely to be equivalent in terms of electrophysiology" (lines 394-395 in the revised manuscript version).

- "We found that the posterior MNs produced using our approach exhibit electrophysiological properties that are crudely similar to those shown by hPSC-derived MNs of a hindbrain/anterior spinal cord character: further comparative characterisation of their electrophysiological properties is now required to thoroughly compare the two populations." (lines 416-419).

3.6. Discussion: Motor neurons of lumbosacral identity still appear to be elusive. How could this protocol be further modified to promote the differentiation of motor neurons with the most posterior of identities?

The detection of HOXC10 expression (New Fig. 4/S5) indicates the potential presence of lumbar MNs in our cultures although we did not investigate this further e.g. using antibody staining against HOXC10 together with lateral motor column markers such as FOXP1. We suspect lumbar MNs are a minor fraction as their generation has been reported to be enhanced by addition of GDF11 (Lippmann et al., 2015; Mouilleau, 2020), a signal that is not included in our differentiation protocol and therefore GDF11 incorporation into our strategy would probably enhance the generation of MNs exhibiting this axial character. We have added a sentence in the discussion section to comment on this issue:

- "The detection of HOXC10 transcripts in our MN cultures also indicates the presence of cells corresponding to the lumbar level of the spinal cord, although these are likely to represent a minor entity given that our protocol does not involve the addition of the TGFβ family member GDF11, an established driver of lumbosacral axial identity in hPSC-derived MNs (Lippmann et al., 2015; Mouilleau, 2020)" (lines 411-415 in the revised manuscript version).

References

Ansari, M., Rainger, J. K., Murray, J. E., Hanson, I., Firth, H. V., Mehendale, F., Amiel, J., Gordon, C. T., Percesepe, A., Mazzanti, L., et al. (2014). A syndromic form of Pierre Robin sequence is caused by 5q23 deletions encompassing FBN2 and PHAX. Eur J Med Genet 57, 587-595.
Boulanger, J., Vezina, A., Mongrain, S., Boudreau, F., Perreault, N., Auclair, B. A., Laine, J., Asselin, C. and Rivard, N. (2005). Cdk2-dependent phosphorylation of homeobox transcription factor CDX2 regulates its nuclear translocation and proteasome-mediated degradation in human intestinal epithelial cells. J Biol Chem 280, 18095-18107.
Candia, A. F., Hu, J., Crosby, J., Lalley, P. A., Noden, D., Nadeau, J. H. and Wright, C. V. (1992). Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. Development 116, 1123-1136.

Cooper, F., Gentsch, G. E., Mitter, E., Bouissou, C., Healy, L., Hernandez-Rodriguez, A., Smith, J. C. and Bernardo, A. S. (2020). Rostrocaudal Patterning and Neural Crest Differentiation of Human Pre-Neural Spinal Cord Progenitors in vitro. bioRxiv.
Diaz-Cuadros, M., Wagner, D. E., Budjan, C., Hubaud, A., Tarazona, O. A., Donelly, S., Michaut, A., Al Tanoury, Z., Yoshioka-Kobayashi, K., Niino, Y., et al. (2020). In vitro characterization of the human segmentation clock. Nature.
Edri, S., Hayward, P., Baillie-Johnson, P., Steventon, B. J. and Martinez Arias, A. (2019). An epiblast stem cell-derived multipotent progenitor population for axial extension. Development 146.
Faustino Martins, J. M., Fischer, C., Urzi, A., Vidal, R., Kunz, S., Ruffault, P. L., Kabuss, L., Hube, I., Gazzerro, E., Birchmeier, C., et al. (2020). Self-Organizing 3D Human Trunk Neuromuscular Organoids. Cell Stem Cell 26, 172-186 e176.
Frith, T. J., Granata, I., Wind, M., Stout, E., Thompson, O., Neumann, K., Stavish, D., Heath, P. R., Ortmann, D., Hackland, J. O., et al. (2018). Human axial progenitors generate trunk neural crest cells in vitro. Elife 7.
Gouti, M., Tsakiridis, A., Wymeersch, F. J., Huang, Y., Kleinjung, J., Wilson, V. and Briscoe, J. (2014). In vitro generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity. PLoS Biol 12, e1001937.
Lippmann, E. S., Williams, C. E., Ruhl, D. A., Estevez-Silva, M. C., Chapman, E. R., Coon, J. J. and Ashton, R. S. (2015). Deterministic HOX patterning in human pluripotent stem cell-derived neuroectoderm. Stem Cell Reports 4, 632-644.
Liu, Y., Beyer, A. and Aebersold, R. (2016). On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165, 535-550.
Lumb, R., Buckberry, S., Secker, G., Lawrence, D. and Schwarz, Q. (2017). Transcriptome profiling reveals expression signatures of cranial neural crest cells arising from different axial levels. BMC Dev Biol 17, 5.
Mouilleau, V., Vaslin, C., Gribaudo, S., Robert, R., Nicolas, N., Jarrige, M., Terray, A., Lesueur, L., Mathis, M. W., Croft, G., Daynac, M., Rouiller-Fabre, V., Wichterle, H., Ribes, V., Martinat, C. and Nedelec, S. (2020). Dynamic extrinsic pacing of the HOX clock in human axial progenitors controls motor neuron subtype specification. bioRxiv.
Pereira, B., Sousa, S., Barros, R., Carreto, L., Oliveira, P., Oliveira, C., Chartier, N. T., Plateroti, M., Rouault, J. P., Freund, J. N., et al. (2013). CDX2 regulation by the RNA-binding protein MEX3A: impact on intestinal differentiation and stemness. Nucleic Acids Res 41, 3986-3999.
Rayon, T., Stamataki, D., Perez-Carrasco, R., Garcia-Perez, L., Barrington, C., Melchionda, M., Exelby, K., Lazaro, J., Tybulewicz, V. L. J., Fisher, E. M. C., et al. (2020). Species-specific pace of development is associated with differences in protein stability. Science 369.
Rings, E. H., Boudreau, F., Taylor, J. K., Moffett, J., Suh, E. R. and Traber, P. G. (2001). Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. Gastroenterology 121, 1437-1450.
Sareen, D., O'Rourke, J. G., Meera, P., Muhammad, A. K., Grant, S., Simpkinson, M., Bell, S., Carmona, S., Ornelas, L., Sahabian, A., et al. (2013). Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. Sci Transl Med 5, 208ra149.
Selvaraj, B. T., Livesey, M. R., Zhao, C., Gregory, J. M., James, O. T., Cleary, E. M., Chouhan, A. K., Gane, A. B., Perkins, E. M., Dando, O., et al. (2018). C9ORF72 repeat expansion causes vulnerability of motor neurons to Ca(2+)-permeable AMPA receptor-mediated excitotoxicity. Nat Commun 9, 347.
Shimada, K., Tachibana, T., Fujimoto, K., Sasaki, T. and Okabe, M. (2012). Temporal and Spatial Cellular Distribution of Neural Crest Derivatives and Alpha Cells during Islet Development. Acta Histochem Cytochem 45, 65-75.
Turner, D. A., Hayward, P. C., Baillie-Johnson, P., Rue, P., Broome, R., Faunes, F. and Martinez Arias, A. (2014). Wnt/beta-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells. Development 141, 4243-4253.
Verrier, L., Davidson, L., Gierlinski, M., Dady, A. and Storey, K. G. (2018). Neural differentiation, selection and transcriptomic profiling of human neuromesodermal progenitor-like cells in vitro. Development 145.
Wainger, B. J., Kiskinis, E., Mellin, C., Wiskow, O., Han, S. S., Sandoe, J., Perez, N. P., Williams, L. A., Lee, S., Boulting, G., et al. (2014). Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep 7, 1-11.

Second decision letter

MS ID#: DEVELOP/2020/194415

MS TITLE: Defining the signalling determinants of a posterior ventral spinal cord identity in human neuromesodermal progenitor derivatives

AUTHORS: Matt Wind, Antigoni Gogolou, Ichcha Manipur, Ilaria Granata, Larissa C Butler, Peter W Andrews, Ivana Barbaric, Ke Ning, Mario R Guarracino, Marysia A. Placzek, and Anestis Tsakiridis

I have now received the reports of the three referees who reviewed the earlier version of your manuscript and I 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 you satisfactorily address the remaining suggestions and comments of referee 1, in particular regarding the inclusion of a statistical analysis. 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

Wind et al provide three main advances

1) Sox2/T cells exposed to sustained WNT and FGF drives them to form dorsal / pre-neural / early neural (agreeing with recent findings by Cooper et al., 2020/2021 BioRxiv)

2) The combination of blocking of TGFb and activating SHH signalling improves the induction of a ventral spinal cord fate when added to pre-neural cells.

3) WNT/FGF influences axial identity in pre-neural cells and this impacts the differentiation of MN subtypes that later develop from these progenitors.

Comments for the author

Thank you to the authors for the inclusion of the new data in this version of the manuscript. I found it a valuable contribution which helped to clarify the main points of the paper, and demonstrate how this protocol can be distinguished from previous iterations.

I suggest to include a statistical analysis in Figure 1D, where the quantification of T positive cells appears variable. It would be useful to clarify whether a change in FGF levels (between the low and high FGF regime with the same CHIR amount) produces a significant difference in the proportion of T positive cells.

At line 120, I suggest to add a sentence to clarify that the addition of RA can subsequently promote the progression to a neural progenitor state (and decrease in the pre-neural state).

At line 455 - ".....independently of the lineage identity of the cells these pathways act on...." was a bit unclear. Is this a reference to the ability of WNT/FGF to mediate multiple roles during development, that can impact either axial identity or, for, instance, germ layer commitment? Perhaps this can be referred to as an "uncoupling" of axial versus lineage identity.

Reviewer 2

Advance summary and potential significance to field

In the revised version, the authors have improved result representation and clarified some concepts. PGC generation from human or any pluripotent stem cell has been challenging. The authors now stress the ability to generate thoracic-level motor neurons from human pluripotent stem cells. The wok is now straightforward in that sense.

Comments for the author

However, I am still a bit concerned about this work's novelty as a whole for the field. Wnt/Fgf patterning ability and concertation curves have been available for some years now. TGFb-BMP patterning is not a novel concept either. Additionally. TGFb is has been a well-described factor to neutralize differentiating cells. Having said that, the protocol is well described with some differences to what is published and perhaps an opportunity for others to test it.

Reviewer 3

Advance summary and potential significance to field

Wind and colleagues describe novel methods and manipulation of signaling pathways to produce posterior ventral motor neurons (MNs) from human pluripotent stem cells via a neuromesodermal progenitors (NMP). Anterior MN production is most commonly reported, and easiest to produce. This new study details mechanisms to produce lower-level MNs via an NMP intermediate.

Comments for the author

The new version of the manuscript thoroughly addresses comments from all 3 reviewers. The new data in Figures 2 and 4, and new Supplementary Figures S4, S5, S6, and S7 add clarity and increases depth to the findings. The authors have either toned down some of their claims and/or more cautiously interpreted their data, which has improved the manuscript. They have also added a more transparent description of biological and technical replicates in the figure legends. However, statistical tests are still not being performed.
The Discussion has been improved and includes/acknowledges the new and related Cooper at al 2020 study that has come out while this manuscript was under review.
Overall, this new version of the manuscript is signficantly improved and comprehensive.

Second revision

Author response to reviewers' comments

We would like to thank the reviewers for their overall positive assessment of our manuscript. We have now made the following changes in the figures and text (text changes shown in yellow) to address the few remaining issues raised:

Reviewer #1
1.1. I suggest to include a statistical analysis in Figure 1D, where the quantification of T positive cells appears variable. It would be useful to clarify whether a change in FGF levels (between the low and high FGF regime with the same CHIR amount) produces a significant difference in the proportion of T positive cells.

We have now included statistical analysis comparing the effect of the two culture conditions pointed by the reviewer. This showed that the higher amount of FGF2 (100 ng/ml) combined with

CHIR treatment promotes a significantly faster, greater decline in the number of TBXT- positive cells between days 3-5 of differentiation compared to the regimen employing a lower FGF2 amount (20 ng/ml) in combination with CHIR (see new Figure 1D). However, the resulting percentages of TBXT-positive cells at day 7 are not statistically different between the two conditions.

1.2. At line 120, I suggest to add a sentence to clarify that the addition of RA can subsequently promote the progression to a neural progenitor state (and decrease in the pre- neural state).
We have added a sentence to reflect the effect of RA in the introduction as suggested by the reviewer:
"…toward a pre-neural/early neural state in the absence of exogenous RA supplementation, which promotes further commitment of pre-neural cells to a definitive neural fate" (Lines 120- 121).

1.3. At line 455 - ".....independently of the lineage identity of the cells these pathways act on...." was a bit unclear. Is this a reference to the ability of WNT/FGF to mediate multiple roles during development, that can impact either axial identity or, for, instance, germ layer commitment? Perhaps this can be referred to as an "uncoupling" of axial versus lineage identity.
We have now rephrased this sentence to clarify:
"Collectively, these findings suggest that WNT and FGF stimulation can "program" a posterior axial identity (marked by posterior HOX gene expression) and further work is required to decipher how this is coupled to the induction of distinct NMP-derived lineages under the influence of the same signalling pathways" (Lines 455-457).

Reviewer #3
However, statistical tests are still not being performed.
We have now included statistical analysis of the qPCR data shown in figures 3B, 4F/S5B to strengthen our conclusions about the positive effect of combined BMP/TGFß signalling inhibition on the induction of ventral spinal cord/MN markers (Fig. 3B) and the efficiency of our differentiation strategy in producing thoracic spinal cord cells/PGC MNs (Fig. 4F, S5B).

Third decision letter

MS ID#: DEVELOP/2020/194415

MS TITLE: Defining the signalling determinants of a posterior ventral spinal cord identity in human neuromesodermal progenitor derivatives

AUTHORS: Matt Wind, Antigoni Gogolou, Ichcha Manipur, Ilaria Granata, Larissa C Butler, Peter W Andrews, Ivana Barbaric, Ke Ning, Mario R Guarracino, Marysia A. Placzek, and Anestis Tsakiridis
ARTICLE TYPE: Research Article

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