Dynamic extrinsic pacing of the HOX clock in human axial progenitors controls motor neuron subtype specification

Vincent Mouilleau, Célia Vaslin, Rémi Robert, Simona Gribaudo, Nour Nicolas, Margot Jarrige, Angélique Terray, Lea Lesueur, Mackenzie W. Mathis, Gist Croft, Mathieu Daynac, Virginie Rouiller-Fabre, Hynek Wichterle, Vanessa Ribes, Cécile Martinat and Stéphane Nedelec
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MS TITLE: Dynamic extrinsic pacing of the *HOX* clock in human axial progenitors controls motor neuron subtype specification

AUTHORS: Vincent Mouilleau, Celia Vaslin, Simona Gribaudo, Remi Robert, Nour Nicolas, Margot Jarrige, Angelique Terray, Lea Lesueur, Mackenzie W Mathis, Gist Croft, Mathieu Daynac, Virginie Rouiller-Fabre, Hynek Wichterle, Vanessa Ribes, Cecile Martinat, and Stephane Nedelec

I have now received the reports of three referees on your manuscript and I have reached a decision. The reports are appended below and 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, all the referees express considerable interest in your work, but they also have 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 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

This paper describes the differentiation of hPSCs into Motor Neuron (MN) fates of different rostrocaudal identities, by adjusting signalling exposure. Importantly it suggests that FGF2/GDF11 exposure acts as an extrinsic regulator of the dynamics of HOX gene progression, which provides some answer to a fundamental question of timing in developmental patterning; namely, whether HOX gene activation progresses as an intrinsic 'clock' or whether the tempo of the process is regulated extrinsically. As such, it is significant not only for providing new insights into an old question, but it also has implications for directed engineering of in vitro approaches towards MN of different rostrocaudal identities (by modulating signalling dynamics). Overall, I think it provides an important contribution to the field that will likely be of interest to the readers of Development.

Comments for the author

This is a solid paper that suggests the role of extrinsic FGF/Gdf11 signalling in regulating HOX gene tempo in axial progenitors and the subsequent rostrocaudal identity of MNs. I would encourage revision (some suggestions below) and would support its consideration for publication.

The authors firstly describe the HOX gene expression pattern and MN marker expression in in vivo human embryos from 6.3- and 7.5-weeks of gestation, which provides a useful resource for other researchers in this field. Having characterised this pattern of expression, and validated their antibodies, the authors use HOX gene expression as a proxy for rostrocaudal identity in their hPSC-derived MN differentiation protocols. Building on their previous methodology (Maury et. al., 2015), they suggest that delayed exposure to RA/SAG promotes caudalisation of their MN population. By varying the duration of RA/SAG exposure the authors show that it is the timing of exposure, rather than the duration, that leads to this effect. Using RNA-seq approaches, they characterise the molecular changes occurring prior to RA/SAG exposure and show the temporal activation of the HOX gene clusters that mirrors the rostrocaudal identity acquisition of the progenitors. By examining enrichment for significantly upregulated genes in this dataset, the authors identify FGF signalling as a putative regulator of this process. By experimentally inhibiting or activating this pathway in their MN protocol, they are able to show delay and anticipation of more caudal identities of MNs, respectively. This could be fine-tuned according to duration of FGF2 exposure and co-exposure to GDF11, indicating that such signalling might be acting as an extrinsic control of the HOX 'clock' and dynamically coordinating rostrocaudal identity.

Some comments and suggestions:

1. The authors main claim builds on the notion that their EB differentiation protocol produces axial progenitors, which undergo progressive HOX gene activation in culture prior to MN specification. However, I find the data comparing the EBs at day 3 to axial progenitors rather unconvincing. While a thorough investigation into the bipotent potential of these cells would be beyond the scope of this work I feel that some statements could be toned down or better supported. One important way to do so, would be to use a quantitative measure of transcriptomic similarity rather than just comparing enriched gene lists with published NMP signature genesets. This could also take into account the actual levels of gene expression observed, rather than just the fold-enriched gene sets.

2. In a similar vein, I would have liked to see the dynamic changes (including both up-regulation and down-regulation) in gene expression along the full developmental timecourse d0,2,3,4 rather than a simple pairwise comparison between d0-d2 (Fig 2B), d0-d3 (Fig2C) or d2-d3 (Fig2F). The different pairs in these panels also makes it difficult to assess comparatively. This

transcriptomic data showing how the early axial progenitor cells progressively acquire more caudal HOX expression states is perhaps the most interesting of the whole paper so it is a shame that the transcriptomic changes are analysed in quite a crude manner.

3. Finally, and probably most importantly, the main novelty of this paper is in showing that FGF signalling inhibition or activation leads to delay or anticipation of HOX gene signatures in axial progenitors. No data is presented that supports the molecular basis for this observation (presumably epigenetic), which would have enhanced the value of the work. Instead, their main claim relies on a few qPCR quantifications at d3-5, and antibody staining much later in d14 MNs. The data reported therefore only weakly support the conclusions drawn. Full transcriptomic profiles showing global similarities between timepoints in control FGF signalling inhibited and FGF signalling activated conditions would add support to this claim (is it only the HOX genes that are delayed/advanced for example?) as would epigenetic approaches to examine molecular mechanisms underpinning this observation.

Minor Comments

1. It seems, in Figure 3D, like the expression of HOXC6 and HOXC8 is actually decreased following MEKi rather than merely delayed or, as the authors say, “blocked [in its] increase” (pg7, line 196). Is this the case? I wonder if the authors could comment on this observation as it seems surprising.

2. In Supplementary Figure S2D, it appears that RA exposure on D3-8 increases the expression of HOXC6 compared to RA exposure on D3-5. Yet the text reads: “None of these shorter RA treatments promoted the birth of more caudal MNs. These results showed that the day at which progenitors are exposed to RA/SAG is the main trigger of caudalisation”. The figure seems to suggest that duration of exposure to RA/SAG does have an effect on the R/C identity of the cells - just not in the expected manner. Perhaps the example in the figure is misleading, in which case it would be good to show a quantification of the HOX expression across experimental replicates, similar to those shown in other panels, in order to support this claim.

3. The observed requirement for increased concentration of CHIR depending on different lines is an interesting observation. However, it is not clear from the figure legend of Fig S2, which cells are hiPSCs and which are hESCs. It would be good to show a comparison of both cell types with both concentrations. Do these line-to-line differences also exist between cells of the same type, or is it specific to iPS vs ES lines?

4. On page 5, line 131 the text says, “Pathway analysis of the genes enriched more than 2 fold ($p<0.05$) in the progenitors compared to hESC indicated an activation of the Wnt pathway paralleled by a transcriptional activation of HOX genes (not shown)”. It seems this is a critical point, partially shown in Fig 2E which should be made available for readers. Similarly, references are made to the loss of pluripotency markers, which is an additionally important observation that should perhaps be available in a Supplementary Figure, rather than just the supplementary data.

5. It is a minor point, but I’m not sure that the longer cultures of cells should be referred to as ‘aging’. Most of the experiments have the same final time-point and it is the signalling exposure that is modulated, so the cells (if we’re being literal) have the same age, even though they might have different rostrocaudal identities.

6. I noticed that the authors have included a list of nonspecific / not working antibodies in Supplementary Table 3 and wanted to commend them for this.
Many hours are often wasted when researchers try to use antibodies that others have already observed not to work, so it is a valuable resource for the community that these authors have not only catalogued the ‘good’ but also the ‘bad’ and communicated this to readers.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Mouilleau et al., “Dynamic extrinsic pacing of the HOX clock in human axial progenitors controls motor neuron subtype specification”, addresses the fundamental biological question of whether anteroposterior identity in the progenitors of spinal cord motor neurons is set intrinsically (by a cell-autonomous timing mechanism) or extrinsically (by signalling).

The authors use embryoid bodies (EB) generated in vitro from human pluripotent stem cells to generate spinal cord motor neurons via axial progenitor intermediates. The authors use a combination of HOX expression profiles and neural markers to determine the identity of the cells produced, by comparison with equivalent immunostains in human embryo samples. The authors report the interesting finding that the anteroposterior identity of spinal cord motor neurons can be adjusted by altering the concentration of FGF and GDF11 ligands, mirroring an increase in FGF signalling pathway components observed when cells are maintained in a progenitor state for successively longer periods. This suggests that previously-reported increases in mRNA encoding FGF signalling components in mouse trunk progenitors are a functional component of the mechanism by which cells adopt successively more posterior identities during axis elongation.

Comments for the author

Overall the study is well designed and executed, and the quality of the figures and writing is generally good. The comments below suggest improvements to the clarity of the manuscript and data presentation.

Major points

The central argument that timing of activation is extrinsically determined is not entirely proven. While it is clear that posterior Hox gene expression can be accelerated with FGF/GDF11, the experiments do not rule out an intrinsic element to Hox regulation. For example, longer exposure to these factors (48 versus 24 hours) results in higher expression of HOX PG6-10 mRNA (Figure 4F). Definitively ruling out cell-intrinsic activation of Hox genes would require selective inhibition of FGF and GDF11. Inhibition of MEK does inhibit the activation of Hox genes (Figure 3), but does it just delay it, as suggested by an apparent partial upregulation of HOXC6-HOXC9 expression between d4-5 of MEK inhibition (Figure 3D)?
What are the implications of the more severe phenotype of inhibiting MEK than FGFR (Figure 3B)? Are there any other signalling molecules acting via MEK whose expression is upregulated in this timeframe in vivo (data represented in Figure 2F)?

The authors find new conditions that activate more posterior Hox genes when compared to untreated controls. However, the cells produced in most conditions have a predominantly upper trunk rather than lumbar identity. When discussing the results through the text, it should be emphasised that the extent of caudalisation achieved is mainly within the posterior brachial/ anterior-mid thoracic region. The proportion of cells with a lumbar phenotype (HOXC10+) induced by GDF11 is low (Fig 4B, E). An additional slight worry is that in Fig. S1, stainings seem to show that ISL1 does not fully colocalise with other neural markers in 6.3GW 7.5GW embryos specially at the caudal-most regions. Since ISL1 is not completely specific for motor neurons, staining for HB9 (Fig 4B,E, and elsewhere in the manuscript) would strengthen the conclusion that the cells produced are motor neurons.

Although it is likely that the following point can be addressed by changes to the text, description of the experimental procedures lack clarity in places and need further information. In particular:

1. The authors state in M&M that for each condition, at least 4 independent EBs were imaged. How many sections per EB were imaged and used for quantification? Were the sections taken for quantification a representative sample of the EBs (i.e, “top”, “middle”, “bottom” sections)? Also n is missing from legends in all figures and quantification plots where statistics have been applied (p-values given).

2. The experimental scheme in Fig 1B shows looks like in RA exposure starts on day 3 (but not SAG, which is delayed one day). However, in the text (line 96) and throughout figure 1, the authors state that results of RA and SAG from D2 are shown. Could the authors clarify when RA/SAG is added and why SAG is delayed one day in one the culture conditions? This delay in addition of SAG is also shown in the schemes of figure 3 and figure 4.

3. The authors should clarify how co-localisation of HOXC6/8 and HOXC8/9 is quantified in ISL1+ cells on figures 1E, S2K, S3E and S4E. The images shown in the corresponding immunostain panels look like different sections and thus do not seem to show dual immunofluorescence.

Minor points

4. If external cues are the only factors controlling the "HOX clock" to generate progenies of particular identities, one could think that it would be possible to reverse the expression of Hox genes from posterior to anterior in vitro, a speculation in the discussion section (lines 261-267). However, the authors have only demonstrated acceleration of Hox expression rather than reversion- it would be good to make this clear.

5. Can the authors comment on whether RA in the context used here (ie after induction of an axial progenitor phenotype) triggers commitment to a specific anteroposterior identity? Does any of the data support the idea that RA shuts down the ability of cells to respond to FGF by inducing successively more posterior HOX genes?

6. The authors use human embryo tissue sections at 6.3 and 7.5 weeks of development to map the expression of HOX genes and neural markers. Please clarify the extent to which they represent the stages modelled in vitro.

7. In M&M, authors indicate that 2 iPSC cell-lines were used. Are the results presented in S2H-J a combination of both cell lines?

8. In line 123 it is stated that "The duration of the time window between Wnt and RA establishes the final positional identity of the progenies". However, CHIR is only used on d0-4 as stated in all the culture condition schemes. Could the authors clarify if CHIR and RA are present in cultures where RA is added at d2-4? Could the authors comment on the possibility of CHIR "priming" cells to RA response, independently of the duration of time window between Wnt and RA?

9. Adding RA to axial progenitors at day 4 causes cells to adopt a more posterior identity at the end of the culture than when adding RA at earlier days.
However results from the transcriptome analysis at day 4 are not presented. Does the transcriptome indicate caudalisation at the progenitor stage relative to day 2/3?

10. Fig2F (Reactome pathway analysis) shows FDR plotted against enrichment score. It's not clear whether this provides more useful information than a ranked list, nor whether the colour representing P value adds any further information.
Why is the enrichment score compared to the FDR? It's also not clear why the d4 axial progenitors are not also represented in this analysis.

11. In Figure 3, the authors state that addition of the two inhibitors of FGFR/MEK do not affect the proportion of MN (assessed by ISL1) but the quantification is not shown in Figure 3 (it seems to be missing by mistake as the legend exists). The stainings shown in Fig3B (which in the legend is referenced as 3C) seem to show an enhanced staining for ISL1 in cultures where RA and inhibitors are present.

Line 264: authors reference Wymeersch et al 2019 for observations on heterochronic grafting of "old" axial progenitors to "young" zones but the referenced article does not contain such experiments. Please update/remove reference as appropriate.

12. Please state the rationale for the concentration of GDF11 chosen.

13. The authors choose red and green colours in various bar graphs. I would strongly recommend that this combination is substituted for a colour-blind friendly one. Fig 1E is particularly hard to distinguish.

1. It would be very helpful for readers if the headings of FigS1A and S1D could be directly compared to the scheme in Fig1A. In S1A, vertebrae ID is used while in S1D it is more of a cervical-sacral general nomenclature (and there are 2 examples for some of the regions without stating what the difference is).
2. Fig1SB has arrowheads in all images but only 4 (iii,v,iv and vi) are explained in the legend.
3. In Fig3D what do the horizontal comparison lines between bars represent?
it is not clear what is the significant comparison.
Also, the legend includes p values for *, ** and *** but only 1 * is used through the figure.
4. Fig S3F typo in heading (FGF120)
5. Please check the figure referencing throughout the text. Examples:
a. FigS2F-G where authors say that RA/SAG addition at d6 or 7 is shown (line 113) but the figure states it is RA at day 4 or 7.
b. Line 144, S4B is referenced but the data in that plot does not correspond to this section.
c. Please revise figure referencing in lines 152-165.

Reviewer 3

Advance summary and potential significance to field

This work studies an important question — whether the well-known “HOX clock” is an intrinsic timing mechanism or it is regulated by external signals. To answer this question, the authors use an in vitro system to differentiate human ES or iPS cells into motor neurons (MNs) and show that the sequential activation of caudal HOX genes is paced by dynamically increased FGF signaling. This is a novel finding that is also relevant to another question surrounding HOX regulation, which is: how is HOX gene expression coordinated between different cells in a tissue? I support publication after some revisions.

Comments for the author

• The authors show that RA treatment at progressively later stages induces motoneurons with progressively more posterior identity. As RA treatment is necessary for motoneuron induction, this implies that in their system NMP-like cells are maintained for a long time in the cultures (ie up to Day7). It would be important to validate/clarify this point.
• RA has been shown, both in vivo and in vitro (Liu et al., Neuron, 2001; Narendra et al., Science, 2015), to be able to induce rostral HOX gene expression (HOX1 to HOX5). In this study, RA+SAG treatment seems, either directly or indirectly (by inducing neurogenesis), to fix the "HOX codes" expressed in the progenitors. Why does RA lose its ability to induce anterior genes in aged progenitors?
• Figure 1E is very confusing, especially, the colors of HOXC6 and C9 are quite close to each other on my computer screen.
• Figure 2E. The legend says “Temporal transcriptional changes in HOX genes coding for HOX regionally expressed in human MNs in vivo”. Please revise it to make it more readable? Also, this figure is not mentioned in the main text.
• In Figure 2E, HOXC9 is much higher on Day 4 than Day3, but why is it almost invisible in Figure 1C?
• Will the progenitor cells (before RA+SAG treatment) express more posterior genes if they are cultured longer than Day 4? Do Day 4 progenitor cells express all the anterior genes?
• Both FGF inhibition (Figure 3B) and addition (Figure 4B) show nicely that FGF signaling can bring forward more posterior HOX gene expression (i.e. HOXC8 and HOXC9). However, the progenitor cells can still express HOXC8 and HOXC9 on Day4, even if the FGF signaling is not manipulated. Do the authors think FGF is more important for regulating the “HOX clock” (temporal collinearity) or for helping cells to sequentially acquire a stable “HOX code”?

Minor points:
• In Summary, Line 9 “… accelerated the HOX clock”. As mentioned in point 6 above, the authors show that more posterior HOX genes are brought forward by FGF. If it is an acceleration, the time

interval between two neighboring HOX gene expression should be shortened, which is not shown in the paper.
• Some sentences are not well constructed, making them difficult to read. e.g. the last sentence in the Summary (Line 11-14)
• Line 17, remove “cn”
• Line70, what is “SCIP”?
• Line 121, “and t”=that?
• Line 174, the reference “Wymeersch et al., 2019” is a work done in mouse not chick.

First revision

Author response to reviewers' comments

We thank the reviewer for the positive and constructive comments on our manuscript. Their insightful feedbacks really helped improving the quality of the manuscript. The reviewer specific comments (shown in blue) are addressed below

We have colored in blue the major changes/additions made to the text of the article.

Reviewer 1

Advance Summary and Potential Significance to Field:

This paper describes the differentiation of hPSCs into Motor Neuron (MN) fates of different rostro-caudal identities, by adjusting signalling exposure. Importantly, it suggests that FGF2/GDF11 exposure acts as an extrinsic regulator of the dynamics of HOX gene progression, which provides some answer to a fundamental question of timing in developmental patterning; namely, whether HOX gene activation progresses as an intrinsic ‘clock’ or whether the tempo of the process is regulated extrinsically. As such, it is significant not only for providing new insights into an old question, but it also has implications for directed engineering of in vitro approaches towards MN of different rostro-caudal identities (by modulating signalling dynamics). Overall, I think it provides an important contribution to the field that will likely be of interest to the readers of Development.

Thank you for finding that our work is of interest for the field as well as for your different suggestions and comments to improve our manuscript.

1. The authors main claim builds on the notion that their EB differentiation protocol produces axial progenitors, which undergo progressive HOX gene activation in culture prior to MN specification. However, I find the data comparing the EBs at day 3 to axial progenitors rather unconvincing. While a thorough investigation into the bipotent potential of these cells would be beyond the scope of this work, I feel that some statements could be toned down or better supported. One important way to do so, would be to use a quantitative measure of transcriptomic similarity, rather than just comparing enriched gene lists with published NMP signature genesets. This could also take into account the actual levels of gene expression observed, rather than just the fold-enriched gene sets.

We apologize if the comparison between Day 3 EBs and axial progenitors was unconvincing. First, we wish to clarify why we called the hPSC-derived progenitors prior RA exposure “axial progenitors” and were careful at not calling them neuromesodermal progenitors (NMPs) for example which represent a subset of axial progenitors.

We qualified the progenitors as “axial progenitors” primarily on a functional assessment. “Axial progenitors” were identified in mouse and chick by grafting experiments and single cell lineage as caudally located progenitors producing differentiated derivatives along the antero-posterior axis in the somites and/or the spinal cord (Albors et al., 2018; Cambray and Wilson, 2002; Cambray and Wilson, 2007; Forlani et al., 2003; Frith et al., 2018; Gouti et al., 2014; Gouti et al., 2017; Henrique et al., 2015; Mathis and Nicolas, 2003; Metzis et al., 2018; Tzouanacou et al., 2009; Wymeersch et

al., 2019).
The progenitors we produced from hPSC are able to give rise to a large palette of the MN diversity found along the rostro-caudal axis of the spinal cord. We thus named them axial progenitors.

Then, in mouse and chick axial progenitors are in different states comprising multipotent progenitors giving rise to neural and different mesodermal progenies (lateral, axial, paraxial…), neuromesodermal progenitors (NMPs) that generate both spinal and paraxial mesoderm, as well as monofated progenitors that generate either paraxial mesoderm or spinal progenies (Edri et al., 2019a; Forlani et al., 2003; Guillot et al., 2020a; Henrique et al., 2015; Mathis and Nicolas, 2000; Nicolas et al., 1996; Tzouanacou et al., 2009; Wymeersch et al., 2016). Therefore, using the transcriptomic data and immunostaining we aimed at determining whether the reported axial progenitors shared molecular characteristics with axial progenitors found in the caudal region of the mouse embryo which comprise axial progenitors coexpressing SOX2 and TBXT some of these cells being NMPs (Gouti et al., 2014; Guillot et al., 2020b; Tzouanacou et al., 2009; Wymeersch et al., 2016; Wymeersch et al., 2019).

We reported in the previous version that SOX2 and TBXT are co-expressed in D2 and 3hPSC derived progenitors. By comparing our transcriptomic data to single cell transcriptomic data of NMPs from the Briscoe lab (Gouti et al., 2017), we could identify additional NMPs-like features harbored by the produced hPSC derived progenitors. Yet, we were careful at not naming the progenitors NMPs as it remains unclear even in mouse whether all Sox2/Tbxt-double positive cells are indeed bipotent and bifated progenitors. This is not trivial to demonstrate at a single cell level and we thought was out of the scope of our study as mentioned by the referee. Furthermore, the dynamic nature of axial progenitor states including NMPs that change over time (Gouti et al., 2017; Wymeersch et al., 2019) can render detailed quantitative comparative analysis complex and difficult to interpret. Finally; quantitative comparison of single cell RNA sequencing data of mouse cells with bulk RNA sequencing of human cells is not straight forward and could be potentially misleading.

For all these reasons, we did not perform a more in-depth quantitative comparison of the transcriptomic similarities. We rather decided to further tone down our conclusion as you proposed and only states line 234 to 240 that “D3 progenitors resemble cells of the mouse caudal lateral epiblast (in which NMPs reside)”. And, we have added that “the degree of similarity with the different states of mouse axial progenitors including early and late NMPs and whether individual cells are bipotent would require additional analysis and experiments.” (line 238-240).

In addition, we have now stated that the CLE genes are all among the most highly enriched genes compared to hESC as illustrated in Fig S4 G-J. We added immunostaining for SOX2, CDX2 and T on Day 4 progenitors that showed a further decrease in TBXT expression. These observations suggest that the progenitors transition toward an axial progenitor state seems to be more biased toward neural differentiation as reported in the anterior CLE (Wymeersch et al., 2016). In addition, we have also included a comparison with another recent study (Frith et al., 2018) which reported the generation of human axial progenitors derived from hPSCs. This comparative analysis showed a strong overlap between the two type of reported axial progenitors (Fig S4J). We have now included in the discussion a paragraph regarding axial progenitors, NMPs and the limitation of our study (line 377-388).

2- a similar vein, I would have liked to see the dynamic changes (including both up-regulation and down-regulation) in gene expression along the full developmental timecourse d0,2,3,4 rather than a simple pairwise comparison between d0-d2 (Fig 2B), d0-d3 (Fig2C) or d2-d3 (Fig2F). The different pairs in these panels also makes it difficult to assess comparatively. This transcriptomic data showing how the early axial progenitor cells progressively acquire more caudal HOX expression states is perhaps the most interesting of the whole paper, so it is a shame that the transcriptomic changes are analysed in quite a crude manner

We thank the reviewer for these suggestions which helped us improving the representation of the transcriptomic data. We are now providing:
i) a heat map showing the temporal dynamics of all genes presenting at least a significant differential expression between two time points (Fig S4D).
ii) ii) another heat map illustrating the temporal dynamic of all HOX genes which further highlight the temporal increase of the 5’*HOX* genes in axial progenitors (Fig. 2D).

iii) Finally, as suggested in minor comment 7, we have included graphic representation of the temporal changes of WNT and FGF pathway related genes, pluripotency genes and the most common CLE progenitor markers (Fig. 2C).

3. Finally, and probably most importantly, the main novelty of this paper is in showing that FGF signalling inhibition or activation leads to delay or anticipation of HOX gene signatures in axial progenitors. No data is presented that supports the molecular basis for this observation (presumably epigenetic), which would have enhanced the value of the work.Full transcriptomic profiles showing global similarities between timepoints in control, FGF signalling inhibited and FGF signalling activated conditions would add support to this claim (is it only the HOX genes that are delayed/advanced for example?) as would epigenetic approaches to examine molecular mechanisms underpinning this observation. Instead, their main claim relies on a few qPCR quantifications at d3-5, and antibody staining much later in d14 MNs. The data reported therefore only weakly support the conclusions drawn.

We fully agree that deciphering the molecular mechanisms linking signaling effectors, epigenetic changes and *HOX* activation is a very interesting and important question. It's actually a line of research we actively pursue. To convincingly demonstrate these links an entire new set of technics and experiments would be required. We thus think that these questions, while very exciting, are out of the scope of the current manuscript which aimed at reporting the dominant role of extrinsic cues in controlling the tempo of the *HOX* clock. Nevertheless, we have included in the discussion how our data fit with the current models of chromatin regulation in *HOX* clusters (line 398-409).
Our goal was to test whether extrinsic cues control the temporal activation of *HOX* genes that display collinear activation in axial progenitors. We thus focused our attention on HOX6-9 genes which increased in axial progenitors between D2 and D4 and for which we were able to determine functionally the consequences of a change in their regulation. We performed immunostaining only at D14 as HOX proteins (except HOXC9) are not expressed or at very low level before the motor neuron stage as in chick and mouse (Dasen et al., 2003). To broaden our analysis, we have now included analysis of other genes: *HOX*A7 which increases over time in axial progenitors and importantly *HOXA4, A5* and *C5* genes that are stable over time in the timeframe of our analysis. Importantly, we demonstrated that the expression of *HOX4-5* genes is not modified by inhibition of FGFR. These results further support the idea that FGF pathway is required to control the tempo of the clock rather than maintaining the HOX code.

Minor Comments

4. It seems, in Figure 3D, like the expression of HOXC6 and HOXC8 is actually decreased following MEKi rather than merely delayed or, as the authors say,"blocked [in its] increase" (pg7, line 196). Is this the case? I wonder if the authors could comment on this observation as it seems surprising.

When we mentioned that MEKi blocked the increase we referred to the fact that the observed increase of *HOXC6, 8, 9* mRNAs between D3 and 5 was prevented when MEK1/2 was inhibited.
We indeed see an initial decrease in the expression of *HOXC6* and *C8* and *C9* following MEK1/2 inhibition which return toward their D3 levels after 48h of inhibition.
A possible explanation is that blocking the increase of the genes in very actively dividing cells results in a dilution of the transcripts in the population and an apparent decrease in their expression. Please see also our response to reviewer 2 second comment regarding the effect of MEK inhibition.

However, importantly, this decrease in expression has no or little functional consequences of these as we did not observe a significant decrease of HOXC6 expression on Day 14 (Fig. 3D-F).

Of notes, we have included new data showing that inhibition of FGF receptors also prevents the temporal increase of *HOXC6-9* expression (see new results in Fig 3B, S5A B). However, we did not observe a significant reduction of *HOXs* compared to their D3 levels as observed following MEK1/2 inhibition.

This difference might be due to several reasons including a partial inhibition of FGFR or a stronger effect of inhibiting the terminal part of the pathway (Please see response to comment 3 of the

reviewer 2 for more details).

We need to mention that have modified the representation of the real time PCR data. We normalized the data to the maximum expression level rather than the fold changes compared to their D3 level. This representation conserves the information of the levels of the different HOXs relative to each other's. It thus provides a better representation of the temporal increase of *HOXs* over time as well as the impact of the different inhibitors. However, this new representation moderates the visualization of the decreased expression of *HOXC6* and *C8* and *C9* induced by MEKI. This is why we decided to maintain the fold change representation in FigS5 B-C to still provide this information to the readers.

5. In Supplementary Figure S2D, it appears that RA exposure on D3-8 increases the expression of HOXC6 compared to RA exposure on D3-5. Yet the text reads: "None of these shorter RA treatments promoted the birth of more caudal MNs. These results showed that the day at which progenitors are exposed to RA/SAG is the main trigger of caudalisation". The figure seems to suggest that duration of exposure to RA/SAG does have an effect on the R/C identity of the cells - just not in the expected manner. Perhaps the example in the figure is misleading, in which case it would be good to show a quantification of the HOX expression across experimental replicates, similar to those shown in other panels, in order to support this claim.

We wanted to state that the induction of more caudal MN subtypes is not promoted by shorter RA treatment as we did not observe the specification of HOXC8 or C9, D09 or C10 MNs when we reduced the duration of RA treatment. However, we indeed see a decrease in HOXC6 expression in the shortest RA treatment. Proper Hoxc6 expression in chick and mouse is dependent on RA ((Liu et al., 2001; Philippidou and Dasen, 2013). A 24h RA treatment might thus be sufficient for MNs generation but not for HOXC6 expression. We did not investigate further the role of RA duration in HOXC6 expression but concluded that reducing the duration of RA exposure is not responsible for the induction in more caudal HOXs.

6. The observed requirement for increased concentration of CHIR depending on different lines is an interesting observation. However, it is not clear from the figure legend of Fig S2, which cells are hiPSCs and which are hESCs. It would be good to show a comparison of both cell types with both concentrations. Do these line-to-line differences also exist between cells of the same type, or is it specific to iPS vs ES lines?

We took these different comments into consideration to revisit the Figure 2 and its associated supplementary figure (now S3, S4)
We have now specified in the legends the type of pluripotent stem cells (hiPSC or hESC). Differentiation data at d14 upon RA delays for two hiPSCs and two hESC lines are now included in Fig. S3A. Only the WTS2 iPSC line did not generate HOXC6, 8 or 9 MNs when RA was delayed upon 3 ¤M of CHIR. However, these MN subtypes can be generated from this cell line using 4 ¤M of CHIR (Fig S3C-E). We have included a comparison of CDX2 induction in hiPSC and hESC derived D3 progenitors at the two CHIR concentration (Fig; S3F). Overall, our data indicate that the difference in CHIR sensitivity is not linked to the type of pluripotent stem cells (hESC versus iPSC) but rather suggests a line to line difference. Similar observations for the response to WNT have been shown in a other type of differentiations (for example see (Moris et al., 2020)). However, revealing the extent of the variability between cell lines and the cause of these differences would require a systematic comparison of many more cell lines. We have maintained this technical aspect in the main text as it could be of critical importance for future studies aiming at studying *HOX* induction or MN subtype specification using hPSCs.

7. On page 5, line 131 the text says, "Pathway analysis of the genes enriched more than 2 fold ($p<0.05$) in the progenitors compared to hESC indicated an activation of the Wnt pathway paralleled by a transcriptional activation of HOX genes (not shown)". It seems this is a critical point, partially shown in Fig 2E, which should be made available for readers. Similarly, references are made to the loss of pluripotency markers, which is an additionally important observation that should perhaps be available in a Supplementary Figure, rather than just the supplementary data.

We have now included this information in Fig. 2 and S4. We have incorporated functional enrichment

analysis on upregulated genes at day 3 and day 4 which indicated an activation of *HOX* genes as well as the WNT pathway (no significant functional enrichment was detected when comparing day 2 and hESC). We have also included Heat maps showing the dynamic expression of pluripotency genes and of the main components of the Wnt pathway in Fig. 2C.

8. It is a minor point, but I'm not sure that the longer cultures of cells should be referred to as 'aging'. Most of the experiments have the same final time-point and it is the signalling exposure that is modulated, so the cells (if we're being literal) have the same age, even though they might have different rostro-caudal identities.

We referred to aging axial progenitors in the first part of the manuscript when axial progenitors are maintained for different periods of time prior exposure to RA (Fig. 1). The longest they are in culture prior RA the more 5' *HOX* genes they expressed and the more caudal the MN subtypes generated are. We agree that axial progenitors exposed to extrinsic factors are treated at the same time point (Day 3) and therefore are not aging. Nevertheless, we have removed the term "aging" in the new version of the manuscript.

9.I noticed that the authors have included a list of nonspecific / not working antibodies in Supplementary Table 3 and wanted to commend them for this. Many hours are often wasted when researchers try to use antibodies that others have already observed not to work, so it is a valuable resource for the community that these authors have not only catalogued the 'good' but also the 'bad' and communicated this to readers

We thank the reviewer for noticing this. We agree that it can be useful for the community.

Reviewer 2

Overall the study is well designed and executed, and the quality of the figures and writing is generally good. The comments below suggest improvements to the clarity of the manuscript and data presentation.

Thank you for finding the study well designed and executed as well as for all the thoughtful comments and suggestions that helped us improve the manuscript.

Major points

1 - The central argument that timing of activation is extrinsically determined is not entirely proven. While it is clear that posterior Hox gene expression can be accelerated with FGF/GDF11, the experiments do not rule out an intrinsic element to Hox regulation. For example, longer exposure to these factors (48 versus 24 hours) results in higher expression of HOX PG6-10 mRNA (Figure 4F).

Our main conclusion is that the pace at which *HOX* genes are activated (i.e. the tempo of the *HOX* clock) is largely controlled by the levels of extrinsic cues. We based this conclusion on the observation that

1) blocking FGF signaling with FGFR or MEK inhibitors while axial progenitors are maintained in culture stalls *HOX* activation despite the time passing by (Fig. 3B-C, S5A). It also full prevents caudal MN specification (data in Fig. 3). We think that this result demonstrate that the *HOX* clock can be blocked or largely slowed down by decreasing extrinsic signaling.

2) In contrast, increasing FGF and/or GDF11 levels on Day 3 promoted the precocious expression of 5' HOXC9, D9, C10 genes which shows that the temporal sequence of *HOX* activation can be greatly accelerated by exposure parameters to extrinsic cues.

We agree that we are not completely excluding intrinsic contributions to the tempo. Determining whether extrinsic factors are modulating the speed at which an intrinsic program is unfolding or whether they are entirely responsible of the temporal sequence will require additional experiments. As mention to the reviewer 1 we are currently approaching this question. Nonetheless, we think that in light of our results mentioning that the extrinsic factors are pacing the *HOX* clock is correct. We have modified the discussion to better reflect this view by adding line 370_372 a sentence saying "These results support the view that the HOX clock is largely paced by extrinsic factors rather than by an intrinsic timer". We also pointed out line 393-395 "the sequential changes in chromatin structure occurring along *HOX* complexes during their activation might be a consequence of dynamic

changes in these extrinsic signals rather than the main mechanism that would intrinsically control the timing of *HOX* gene expression.

2- Inhibition of MEK does inhibit the activation of Hox genes (Figure 3), but does it just delay it, as suggested by an apparent partial upregulation of HOXC6-HOXC9 expression between d4-5 of MEK inhibition (Figure 3D)? *

Indeed, there's an upregulation of *HOX* expression 48h post MEK1/2 treatment compared to 24h post- treatment. This apparent MEK independent temporal increase follows an initial drop in *HOX* expression 24h post treatment. Then the levels of the transcripts return toward their initial level after 48h of inhibition (while the inhibitor is maintained). However, at 48h the genes are still expressed at a lower level than in D3 progenitors and the upregulation, in presence of MEK inhibitor, do not result in the generation of HOXC8 or HOXC9 MNs. We cannot completely exclude that an upregulation of *HOX6- 9 mRNAs,* up to the levels observed in the control D4/5 progenitors, could be observed if we maintained the progenitors much longer. In agreement, we have proposed that the pace of the *HOX* clock is controlled by extrinsic factors, a proposition which we think is compatible with a delay or a full inhibition. Also, we want to stress that we did not observe this initial decrease and the secondary increase at 48h when axial progenitors were treated with FGFR inhibitors (new data in Figure 3B and S5A-B).

3- What are the implications of the more severe phenotype of inhibiting MEK than FGFR (Figure 3B)? Are there any other signalling molecules acting via MEK whose expression is upregulated in this timeframe in vivo (data represented in Figure 2F)?

We indeed observed a tendency of the MEK inhibitor to more severely inhibit HOX induction at day 14 than the FGFR inhibitor (Fig. 3C in the previous version now 3E). While the difference was not statistically significant, the stronger effect of MEKi was confirmed by our new results in which we investigated the impact of FGFR inhibitor on *HOX* gene induction between D3 and D4 (Fig. 3B-C). The FGFR inhibitor has a "weaker" effect than MEK inhibitor on *HOX* expression as it prevented the temporal increase of HOXC6, A7, C8 and C9 expression but did not induce a drop below their Day 3 levels as observed with the MEK inhibitor (this decrease is now presented in Fig. S5C as we modified the representation of the real time PCR data to better take into account the relative expression of the different *HOX*s). This differential effect might be due to residual FGF signaling through other FGFR than FGFR1 and 3. For example FGFR4 which is also expressed in the progenitors. Also, inhibition of FGFR might result in a weaker downregulation of MEK activity than directly inhibiting MEK1/2. Even though we cannot exclude that another signaling molecule actives MEK1/2 in our model and *in vivo*, we favor the idea that the difference between FGFR and MEK inhibition might be due to a partial inhibition of MAPK signaling with FGFR inhibitors. However, and most importantly, FGFR inhibition blocks with PD173074 the temporal increase of *HOX6-9* and the specification of C8 and C9 motor neurons.

4- The authors find new conditions that activate more posterior Hox genes when compared to untreated controls. However, the cells produced in most conditions have a predominantly upper trunk rather than lumbar identity. When discussing the results through the text, it should be emphasised that the extent of caudalisation achieved is mainly within the posterior brachial/ anterior-mid thoracic region. The proportion of cells with a lumbar phenotype (HOXC10+) induced by GDF11 is low (Fig 4B, E).

In the previous version we showed that FGF2 +GDF11 added for 48h together with RA resulted in the relatively efficient specification of HOXD9 MNs (58% of the MNs) which are located in the caudal thoracic region. However, we completely agree that the proportion of HOXC10 MNs was low. In this revised version, we are now showing that applying FGF2 and GDF11 prior RA induces a much stronger caudalization with now 81 % of the MNs expressing HOXC10 (Fig. S7G-I). While it further demonstrates that D3 progenitors are competent to induce a lumbar identity, it also supports the idea that RA antagonizes FGF/GDF11 induced caudalization. RA might either repress FGFs expression as previously demonstrated (Del Corral and Storey, 2004; Del Corral et al., 2003; Kumar and Duester, 2014) and/or induce a differentiation stage in which *HOX* expression is not plastic anymore as supported by previous experiments (Ensini et al., 1998).

5- An additional slight worry is that in Fig. S1, stainings seem to show that ISL1 does not fully

colocalise with other neural markers in 6.3GW 7.5GW embryos specially at the caudal-most regions. Since ISL1 is not completely specific for motor neurons, staining for HB9 (Fig 4B,E, and elsewhere in the manuscript) would strengthen the conclusion that the cells produced are motor neurons.

We have now included co-staining for HB9 and ISL1 for the different reported conditions that demonstrated that the majority of the cells indeed co-express the two markers.

Although it is likely that the following point can be addressed by changes to the text, description of the experimental procedures lack clarity in places and need further information. In particular:

6.The authors state in M&M that for each condition, at least 4 independent EBs were imaged. How many sections per EB were imaged and used for quantification? Were the sections taken for quantification a representative sample of the EBs (i.e, "top", "middle", "bottom" sections)? Also n is missing from legends in all figures and quantification plots where statistics have been applied (p-values given).

One section per EB was imaged and quantified but several independent EBs were quantified for each replicate. While we mentioned at least 4 EBs (4 correspond to the number of EBs quantified in one of the replicates of the D3-7 FGFRI condition) in most experiments 6 to 9 EBs were quantified which represent thousands of cells. As EBs are small, round and embedded "floating" in OCT before freezing, we cannot select or determine the level at which a given EB is sectioned. The sections are automatically imaged and the pictures selected for quantification randomly picked. We do not see much variability between EBs in a given experiment. The main variability that we observed and that is presented in the different graphs is mostly between biological replicates (i.e. independent differentiations). In consequence, in each graph a dot represents one biological replicate. We have now included the minimum number of biological replicates in the legends of each figure.

7 .The experimental scheme in Fig. 1B shows looks like in RA exposure starts on day 3 (but not SAG, which is delayed one day). However, in the text (line 96) and throughout figure 1, the authors state that results of RA and SAG from D2 are shown. Could the authors clarify when RA/SAG is added and why SAG is delayed one day in one the culture conditions? This delay in addition of SAG is also shown in the schemes of figure 3 and figure 4.

SAG is indeed added at day 4 as depicted in the schemes. We previously showed that addition of SAG together with RA or in a 48 h-window following RA was not affecting MN (Maury et al., 2015). However, when SAG is further delayed or precedes RA the efficiency of MN generation decreases. The day at which SAG is added does not change MN subtype identity. We therefore decided to fix one-time point for SAG addition. As if SAG precedes RA MN number decreases, when RA was delayed (D5, 6,
7) SAG addition was delayed as well. We have clarified the text to better reflect the protocol (Line 158).

8 .The authors should clarify how co-localisation of HOXC6/8 and HOXC8/9 is quantified in ISL1+ cells on figures 1E, S2K, S3E and S4E. The images shown in the corresponding immunostain panels look like different sections and thus do not seem to show dual immunofluorescence.

We added a sentence line 591-592 to clarify the quantification of colocalization. To provide more information: we always perform at least triple staining (Two HOXs and ISL1, except for the data presented in Fig. S2G for which we performed quadruple stainings for ISL1 HOXC8 HOXC9 together with FOXP1 or SCIP). The sections are then imaged in the three or four channels and the percentage of colocalization are quantified automatically as described in the methods. Costaining are better illustrated with merged images as in Fig. S2B, F, G, but some stainings are sometime difficult to see (when they decrease for example). We thus chose to display black and white images of individual channels for the HOXs to make sure that the different profile would be clearly visible. To better illustrate co- localizations, we have now make sure that at least 2 photos most of the time 3 are coming from the same section (ISL1 and two HOXs or SCIP and FOXP1 for example). The other pictures are coming from sections of other EBs. To simplify the figures, we did not display multiple times a given staining (for example in most cases, HOXC8 is costained with HOXC6 as well as with HOXC9).

Minor points

9 .If external cues are the only factors controlling the "HOX clock" to generate progenies of particular identities, one could think that it would be possible to reverse the expression of Hox genes from posterior to anterior in vitro, a speculation in the discussion section (lines 261-267). However, the authors have only demonstrated acceleration of Hox expression rather than reversion- it would be good to make this clear.

As mention in our response first comment, in light of our results, we propose that FGFs and GDF11 are pacing the tempo of the *HOX* clock. Whether they are the only factors controlling the clock will require further experiments. Concerning the reversion, we apologize for the lack of clarity in the previous version. The fact that sequence of activation from 3' to 5' genes is maintained under different experimental conditions suggests the extrinsic cues might modulate the speed but not directionality at which a repressive chromatin state is cleared from *HOX* clusters. We have included this remark in the discussion (line 398-401). Furthermore, in contrast to the reversion of the HOX profile described in McGrew et al, our results argue that inhibiting the FGF pathway is not sufficient to observe such an effect. Indeed, we observe that MEK and FGFR inhibition on day3 blocked the specification of HOXC8 and HOXC9 MNs but HOXC6 MNs were maintained. Furthermore, we have included new results (Fig. 3G-I) in which we show that MEK inhibition a day later, at day 4, followed by RA on day 5 blocked HOXC9 MNs specification but not HOXC8 MNs generation. Hence, blocking FGF signaling a day later blocked the progression toward C9 MNs but is not preventing HOXC8 MNs generation. Hence, FGF inhibition is not reverting the HOX profile to a more anterior HOX profile than the one expected. The results suggest that the FGF pathway is important for the progression of the *HOX* clock but not the maintenance of the HOX profile.

10- Can the authors comment on whether RA in the context used here (ie after induction of an axial progenitor phenotype) triggers commitment to a specific anteroposterior identity? Does any of the data support the idea that RA shuts down the ability of cells to respond to FGF by inducing successively more posterior HOX genes?

Considering that applying RA on different days leads to neurons of different RC identity RA doesn't seem to commit cells to a specific antero-posterior identity. We believe that RA rather promotes neural induction and that the *HOX* clock is stopped during the differentiation toward a neural progenitor stage. Our new result showing that a pretreatment with FGF/ GDF11 prior RA leads to a stronger caudalization than when FGF/GDF11 is combined with RA supports the view of an antagonistic relationship between caudalizing factors and RA. This might be due to a differentiation toward a stage at which FGF is not able to induce more 5' HOX genes and/or to a direct downregulation of FGF ligands by RA receptors as previously shown (Del Corral and Storey, 2004; Del Corral et al., 2003; Kumar and Duester, 2014; Patel et al., 2013).

11- The authors use human embryo tissue sections at 6.3 and 7.5 weeks of development to map the expression of HOX genes and neural markers. Please clarify the extent to which they represent the stages modelled in vitro.

Assigning a precise correspondence between in vivo and in vitro timing is difficult. Motor neurons are progressively generated in vivo, both along the rostro-caudal axis and at a given rostro-caudal level. Caudal MNs are born later in development than rostral ones. Furthermore, MN progenitors are not synchronously generated but progressively differentiate over time giving rise to early and late born MNs. At cervical/anterior brachial level MN specification seem to occur between the 4th and the end of the 6th week of gestation (Marklund et al., 2014; Rayon et al., 2020). Acquisition of columnar and pool identity occurs concomitant to the settlement of cell bodies at specific location within the ventral horn (Catela et al., 2015; Dasen, 2009). We observed column and pool markers in 6.3-week embryos as well as in vitro (expression of FOXP1 and SCIP). Therefore, the 6.3-week stage might correspond to the stage analyzed at day14 or to a slightly more advanced stage in development as columns and pools are already well organized. A longitudinal analysis of progenitor, columnar and pool markers between the 5th and 6th week of development at brachial and lumbar level would be required to avoid mistakes and provide a proper assessment. Our *in vivo* analysis was mostly aiming at testing whether HOX expression profile are regionalized in human as observed in

mouse and validating HOX antibodies in human.

12- In M&M, authors indicate that 2 iPSC cell-lines were used. Are the results presented in S2H-J a combination of both cell lines?

The results presented were obtained with one iPSC line (the WTS2 line). The WTS8 line was not used in this study. We apologize for this typo in the method section. In this new version of our manuscript, we have now included, in addition of the WTS2 data, data obtained with another iPSC line and a second hESC line (Fig S3A)

13- In line 123 it is stated that "The duration of the time window between Wnt and RA establishes the final positional identity of the progenies". However, CHIR is only used on d0-4 as stated in all the culture condition schemes. Could the authors clarify if CHIR and RA are present in cultures where RA is added at d2-4? Could the authors comment on the possibility of CHIR "priming" cells to RA response, independently of the duration of time window between Wnt and RA?

We were referring to the fact that the duration between the initial addition of CHIR and the addition of RA sets the final position identity. We have clarified the sentence in the new version of the manuscript: "The time window between the initial exposure to WNT agonist and RA defines the positional identity of the generated progenies" (line 189-191) CHIR is maintained between day 0 and day 4 and is thus present when cells receive RA on day 2 or 3 or 4. CHIR (WNT) is priming the cells to respond to RA in the sense that it induces the axial progenitor state and the collinear activation of *HOX6-10* (Neijts et al., 2016) necessary for the generation of caudal motor neurons. High RA together with SAG in absence of CHIR specify cervical MNs as previously shown (Amoroso et al., 2013).

14. Adding RA to axial progenitors at day 4 causes cells to adopt a more posterior identity at the end of the culture than when adding RA at earlier days. However, results from the transcriptome analysis at day 4 are not presented. Does the transcriptome indicate caudalisation at the progenitor stage relative to day 2/3?

We apologize if the figures were not clear enough. We showed in the previous Fig2E and G data from the transcriptiomic analysis describing an increase in HOXC9 mRNA level at day 4 compare to the previous days. Figure 2 has now been largely modified to better illustrate the increase in HOX gene expression over time (now a heatmap for all *HOX*s is included in Fig 2D in which the increase of caudal *HOX* genes is more pronounced on day 4). Furthermore, the realtime PCR analysis in 3D (now Fig. 3B-
C) confirmed this temporal increase. Hence day 4 and day 5 progenitors express more caudal HOX genes compared to day 3. In agreement they generate more caudal MN subtypes.

15. Fig2F (Reactome pathway analysis) shows FDR plotted against enrichment score. It's not clear whether this provides more useful information than a ranked list, nor whether the colour representing P value adds any further information. Why is the enrichment score compared to the FDR? It's also not clear why the d4 axial progenitors are not also represented in this analysis

We think that representing the FDR plotted against enrichment score provides additional meaningful information. The ordinate axis (-log10(FDR)) provide an information similar to a ranked list, the highest annotations corresponding to the most significant ones. However, FDR do not account for non- uniformity in the number of genes annotated to individual functions or the number of functions associated with individual genes. It tends to over-estimates significance if a gene set has an unusually high number of annotations. The enrichment score considers this potential size effect (i.e. the proportion of genes in the list of interest with an annotation X compared to the total number of genes in the human genome with the same X function).
We agree that the p-value gradient in the previous version was unnecessary. We thus removed this information. Functional enrichment for genes enriched in D4 axial progenitor has been also added in Fig S3F.

16 .In Figure 3, the authors state that addition of the two inhibitors of FGFR/MEK do not affect the proportion of MN (assessed by ISL1) but the quantification is not shown in Figure 3 (it seems to be

missing by mistake as the legend exists). The stainings shown in Fig3B (which in the legend is referenced as 3C) seem to show an enhanced staining for ISL1 in cultures where RA and inhibitors are present

We have now included the quantification in Fig. S5F. We do observe a trend toward an increase in the percentage of MNs. However, the difference was not statistically significant. We therefore concluded that the inhibitors do not impact on the percentage of motor neurons. As FGF has been implicated in neural induction in several studies we wanted to stress out that the specification of neurons is not affected when FGF signaling is inhibited in these axial progenitors.

17- Line 264: authors reference Wymeersch et al 2019 for observations on heterochronic grafting of "old" axial progenitors to "young" zones but the referenced article does not contain such experiments. Please update/remove reference as appropriate.

We have removed the reference

18. Please state the rationale for the concentration of GDF11 chosen.

The concentration of GDF11 was chosen based on previous studies (Liu, 2006; Liu et al., 2001; Shi and Liu, 2011).

19 .The authors choose red and green colours in various bar graphs. I would strongly recommend that this combination is substituted for a colour-blind friendly one. Fig 1E is particularly hard to distinguish.

We apologize for this issue and fully agree with the reviewer. We have now modified the color code of all the figures using a color blind friendly palettes.

19 .It would be very helpful for readers if the headings of FigS1A and S1D could be directly compared to the scheme in Fig1A. In S1A, vertebrae ID is used, while in S1D it is more of a cervical-sacral general nomenclature (and there are 2 examples for some of the regions without stating what the difference is). 2.Fig1SB has arrowheads in all images but only 4 (iii,v,iv and vi) are explained in the legend.

We have homogenized the headings for FigS1.A and S1D with the one of Fig. 1. However, we decided to keep the information concerning the level of the section in Fig. S1.A which define more precisely the position of each section.

20 .In Fig3D what do the horizontal comparison lines between bars represent? it is not clear what is the significant comparison. Also, the legend includes p values for *, ** and *** but only 1 * is used through the figure.

We modified the figure and legend accordingly. We did not represent all the possible combination of statistical comparison in the graph to simplify the figures.

21.Fig S3F typo in heading
(FGF120) We have corrected this.
5.Please check the figure referencing throughout the text. Examples: a.FigS2F-G where authors say that RA/SAG addition at d6 or 7 is shown (line 113) but the figure states it is RA at day 4 or 7.

b.Line 144, S4B is referenced but the data in that plot does not correspond to this section. c.Please revise figure referencing in lines 152-165.
We have checked the referencing.

Reviewer 3

This work studies an important question — whether the well-known "HOX clock" is an intrinsic timing mechanism or it is regulated by external signals. To answer this question, the authors use an in vitro system to differentiate human ES or iPS cells into motor neurons (MNs) and show that the sequential

activation of caudal HOX genes is paced by dynamically increased FGF signaling. This is a novel finding that is also relevant to another question surrounding HOX regulation, which is: how is HOX gene expression coordinated between different cells in a tissue? I support publication after some revisions.

Thank you for your overall positive evaluation of our work as well as for your comments and suggestions that we are addressing below.

Reviewer 3 Comments for the Author:

1- The authors show that RA treatment at progressively later stages induces motoneurons with progressively more posterior identity. As RA treatment is necessary for motoneuron induction, this implies that in their system NMP-like cells are maintained for a long time in the cultures (ie up to Day7). It would be important to validate/clarify this point.

While we showed that the reported progenitors share similarities with NMPs we tried not to refer to them as NMP-like cells as we never demonstrated that the reported progenitors are bipotent cells (please see also our response to reviwer 1 first comment). We instead named them “axial progenitors” based on their ability to give rise to progenies found at distinct level of the rostro-caudal axis as described in mouse and chick.(Edri et al., 2019a; Forlani et al., 2003; Gouti et al., 2015; Gouti et al., 2017; Guillot et al., 2020a; Henrique et al., 2015; Mathis and Nicolas, 2000; Nicolas et al., 1996; Tzouanacou et al., 2009; Wymeersch et al., 2016). Determining whether they are NMP-like cells would require additional experiments which we considered not absolutely required for this study which aimed at studying HOX induction mechanisms. Indeed, there are different states of axial progenitors that are unlikely to be all bipotents and it is unlikely as well that NMPs are the sole site of *HOX* activation as not all spinal cells derive from them (Henrique et al., 2015). Hence we do not claim that we are maintaining an NMP-like state in our model. It actually unlikely to be the case as TBXT is decreasing over time in the progenitors (Fig. 2B and S4C) which argue against the maintenance of a stable progenitor NMP-like state. It rather suggests that the cells progressively transition toward a more neural fated progenitor state, a type of axial progenitor that might be similar to ones observed in the anterior part of the caudal lateral epiblast or the region just anterior to the node (Wymeersch et al., 2016). A transition toward more neural fated progenitors was already observed with mouse NMPs (Edri et al., 2019b). However, and critically, the hPSC-derived axial progenitors display a temporal collinear activation of *HOX* genes from day 2 to day 5 (transcriptomic data and real time PCR in Figs 3B-C) and are able to differentiation in motor neurons at all these stages. Finally, and very importantly, to test the impact of extrinsic cues on the temporal activation of *HOX* genes we focused our study on Day 3 progenitors to demonstrate that the *HOX* clock can be entrained by extrinsic cues in a given axial progenitor state and thus that the maintenance over time of a specific axial progenitor state, including an NMP site, is not a prerequisite for the *HOX* clock to proceed.

2- RA has been shown, both in vivo and in vitro (Liu et al., Neuron, 2001; Narendra et al., Science, 2015), to be able to induce rostral HOX gene expression (HOX1 to HOX5). In this study, RA+SAG treatment seems, either directly or indirectly (by inducing neurogenesis), to fix the "HOX codes" expressed in the progenitors. Why does RA lose its ability to induce anterior genes in aged progenitors?

We are now providing a better representation of *HOX* expression (Heat map of *HOX* expression levels derived from in Fig. 2D and real time PCR analysis of multiple HOXs in Figs. 3B, C). Anterior HOX genes are actually expressed in the reported axial progenitors. WNT pathway have been shown to induce collinear activation of HOX complexes starting from the 3’ side (Neijts et al., 2016; Neijts et al., 2017) Accordingly, we see early expression (day 2) of most of the HOXB complex which is expressed earlier in development (and not differentially expressed in the spinal cord) as well as most of the 3’ genes of the A, C, and D complexes. Hence, RA is added on progenitors already expressing anterior HOXs together with more caudal HOXs. However, spinal anterior HOXs (A4/5, C4/5) are expressed at much lower level than the more 5’ *HOX* genes (*HOXC6 or HOXC8)*. Defining whether higher RA concentration could induce more potently these genes or whether they are less or non-responsive to RA in axial progenitors would be interesting.

3- Figure 1E is very confusing, especially, the colors of HOXC6 and C9 are quite close to each other

on my computer screen.

Thank you for suggesting the changes in color as it clearly improved the manuscript.

4- In Figure 2E, HOXC9 is much higher on Day 4 than Day3, but why is it almost invisible in Figure 1C?

Indeed, *HOXC9* mRNA is expressed at higher level on day 4 than on day 3 in the transcriptomic (Fig. 2D, now presented as a heat map) and real-time PCR data in Fig. 3B-C (the new graph provides a better presentation of the relative level of expression of the different *HOX* mRNAs). However, *HOXC9* is still expressed at lower level than C6 and C8 and further increase at Day 5 (Fig. 3B-C). In agreement, there is very few HOXC9 MNs generated when D3 progenitors are exposed to RA a little more from day 4 progenitors and much more from day 5 (quantification in Fig. 1F). Hence, the number of HOXC9 MNs appear to correlate with the relative level of expression of *HOXC9* in the D3, 4 and 5 progenitors.

5- Will the progenitor cells (before RA+SAG treatment) express more posterior genes if they are cultured longer than Day 4? Do Day 4 progenitor cells express all the anterior genes?

They do express more posterior HOX genes on day 5 than on day 4 as shown by the increase in *HOXC9* mRNA expression (Fig. 3B-C) but they do not express *HOXD9* and *HOXC10 (Fig 4F)*. Either we did not maintain the progenitors long enough or we favor the idea that this reflects the requirement for GDF11 signaling to induce these genes. As illustrated in Fig. 2D and real-time pCR data in Fig. 3B-C, the progenitors do express most of the anterior *HOX* genes as mentioned above.

6- Both FGF inhibition (Figure 3B) and addition (Figure 4B) show nicely that FGF signaling can bring forward more posterior HOX gene expression (i.e. HOXC8 and HOXC9). However, the progenitor cells can still express HOXC8 and HOXC9 on Day4, even if the FGF signaling is not manipulated. Do the authors think FGF is more important for regulating the "HOX clock" (temporal collinearity) or for helping cells to sequentially acquire a stable "HOX code"?

The increase in expression of *HOXC8* and *HOXC9* mRNAs in day 4 progenitors in absence of extrinsic manipulation of the FGF pathway is triggered by endogenous autocrine or paracrine FGF signaling as inhibiting FGFR or MEK1/2 on day 3 blocks the increase of these genes as well as the specification of MNs expressing HOXC8 or HOXC9. Furthermore, when we inhibit MEK1/2 on day 4, we block the generation of HOXC9 MNs but not HOXC8 MNs (new result in Fig 3H-I). We thus think that FGF is more important to regulate the clock than to maintain a stable HOX code.

<u>Minor points:</u>

- In Summary, Line 9 "… accelerated the HOX clock". As mentioned in point 6 above, the authors show that more posterior HOX genes are brought forward by FGF. If it is an acceleration, the time interval between two neighboring HOX gene expression should be shortened, which is not shown in the paper.

- Some sentences are not well constructed, making them difficult to read. e.g. the last sentence in the Summary (Line 11-14)

We have now tried to improve the text and make it easier to read.

- Line 17, remove "cn"

Amended

- Line70, what is "SCIP"?

We have modified the text to explain what is SCIP

- Line 121, “and t”=that?

We have modified the text

- Line 174, the reference “Wymeersch et al., 2019” is a work done in mouse not chick.

We have changed the text

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Second decision letter

MS ID#: DEVELOP/2020/194514

MS TITLE: Dynamic extrinsic pacing of the *HOX* clock in human axial progenitors controls motor neuron subtype specification

AUTHORS: Vincent Mouilleau, Celia Vaslin, RÃ©mi Robert, Simona Gribaudo, Nour Nicolas, Margot Jarrige, Angelique Terray, Lea Lesueur, Mackenzie W Mathis, Gist Croft, Mathieu Daynac, Virginie ROUILLER-FABRE, Hynek Wichterle, Vanessa Ribes, Cecile Martinat, and Stephane Nedelec

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 reviewers' evaluation is positive and we would like to publish a revised manuscript in Development, provided that you address the remaining minor suggestions of referee 2. Please attend to these comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of them, please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper has been clearly improved by the work of the authors following the previous round of comments. I am satisfied that the authors adequately addressed my comments and suggestions, and those of the other reviewers. I would gladly recommend this work for publication.

Comments for the author

I have no further recommendations for the authors.

Reviewer 2

Advance summary and potential significance to field

The authors have significantly improved the quality of the manuscript and answered the reviewers' criticisms well. With the additional data that includes new timings/ combinations of factors, it is now clear that more caudal Hox genes are induced upon addition of FGF and GDF11. The extended RNA seq analysis now gives important further information, e.g. presence of CLE-like cells in culture, the sequence of Hox activation at different days of their cultures, and the involvement of FGF/MAPK signalling in progenitors during the differentiation protocol. The manuscript now documents the extrinsic timing of activation of Hox cluster genes, a significant finding for the field of axial patterning.

Comments for the author

Minor points It would be helpful if Table S2 added a column denoting fold change, rather than just including genes with FC>= 2 in alphabetical order.
A mainly aesthetic point: Is the double helix representation of DNA necessary in Fig 2G? It stands out more than the gene names. By contrast, the dotted lines representing positive or negative regulation are almost invisible.
Also mainly aesthetic, in Fig 3B, is it possible to reduce the size of the circles representing replicate points (or make them transparent)? The narrow columns make the data points obtrusive and their overlap means it's hard to see how many replicates there are.
In fig S3B, the circle symbol seems to be missing in the second barplot (D7-9) and the key.

Reviewer 3

Advance summary and potential significance to field

My comments have been appropriately addressed and the paper is now suitable for publication in the journal

Comments for the author

My comments have been appropriately addressed and the paper is now suitable for publication in the journal

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Second revision

Author response to reviewers' comments

Point to point response to the reviewers

We deeply thank the three reviewers for their positive evaluation of our revised manuscript. The reviewer specific comments (shown in blue) are addressed below
We have colored in blue the slight changes made to the text to improve the clarity and readability of few sentences.
Reviewer 2 Comments for the Author:

We thank the reviewer for the minor comments which are again very helpful. We have modified the manuscript accordingly.

Minor points

It would be helpful if Table S2 added a column denoting fold change, rather than just including genes with FC>= 2 in alphabetical order.

We have included the fold changes in the Table S2

A mainly aesthetic point: Is the double helix representation of DNA necessary in Fig 2G? It stands out more than the gene names. By contrast, the dotted lines representing positive or negative regulation are almost invisible.

We have reduced the size of the DNA helix and modified the dotted lines to make them more visible

Also mainly aesthetic, in Fig 3B, is it possible to reduce the size of the circles representing replicate points (or make them transparent)? The narrow columns make the data points obtrusive and their overlap means it's hard to see how many replicates there are.

We have reduced the size of the dots. The number of replicates is now visible.

In fig S3B, the circle symbol seems to be missing in the second barplot (D7-9) and the key.

We have corrected this error.

Third decision letter

MS ID#: DEVELOP/2020/194514

MS TITLE: Dynamic extrinsic pacing of the *HOX* clock in human axial progenitors controls motor neuron subtype specification

AUTHORS: Vincent Mouilleau, Célia Vaslin, Rémi Robert, Simona Gribaudo, Nour Nicolas, Margot Jarrige, Angélique Terray, Lea Lesueur, Mackenzie W Mathis, Gist Croft, Mathieu Daynac, Virginie Rouiller-Fabre, Hynek Wichterle, Vanessa Ribes, Cécile Martinat, and Stéphane Nedelec
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.