E2A regulates neural ectoderm fate specification in human embryonic stem cells

Siqi Yi, Xiaotian Huang, Shixin Zhou, Yuan Zhou, Michele K. Anderson, Juan Carlos Zúñiga-Pflücker, Qingxian Luan and Yang Li
DOI: 10.1242/dev.190298

Editor: Gordon Keller

Review timeline
Original submission: 6 March 2020
Editorial decision: 8 June 2020
First revision received: 19 August 2020
Editorial decision: 23 October 2020
Second revision received: 27 October 2020
Accepted: 27 October 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/190298

MS TITLE: E2A regulates neural ectoderm fate specification in human embryonic stem cells

AUTHORS: Yang Li, Siqi Yi, Xiaotian Huang, Yuan Zhou, Shixin Zhou, Michele K Anderson, Juan Carlos Zuniga-Pflucker, and Qingxian Luan

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

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

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

The manuscript entitled “E2A regulates neural ectoderm fate specification in human embryonic stem cells” by Yi S. et al., investigates the role of the E2A protein in hESC differentiation, specifically mesoderm and neural differentiation. The authors combined RNA- Seq and Chip-Seq analysis and identified E2A direct target genes in hESC neuronal differentiation. Their results provide evidence that E2A regulates the expression of several neuronal specific genes as well as that of genes involved in the Nodal pathway, including the Nodal obligate coreceptor Cripto. Despite the fact that E2A interacts with PRC2 complex, E2A KO has little effect on the epigenetic landscape of hESCs during neuronal differentiation. Finally, the authors investigate the impact of E2A KO on hESC differentiation, and provide molecular and phenotypic evidence that neuronal differentiation is altered in E2A KO hESCs. Mechanistically, they propose that this is due to the persistent upregulation of the mesoderm-inducing gene Cripto and to the lack of proper induction of neuronal-specific genes.

Comments for the author

This is a potentially interesting study and the manuscript reads well. However, authors’ conclusions are not fully supported by the results. In particular, authors' conclusion that "the absence of E2A results in a profound block in neural development" is an over interpretation of their results. There are several points that should be addressed before the manuscript could be considered for publication. Statistical details are often missing and the overall quality of the pictures is poor and should be improved.

Major points:
1. Figure 1B. The authors state that E2A KO hESCs colony morphology is indistinguishable from that of WT hESCs; however, the images show that E2A KO colonies are much smaller compared to WT. This is also in contrast with CCK8 results that E2A KO hESCs display a higher proliferation rate. How do the authors explain this discrepancy? Are the images representative? Scale bar is missing.
2. Figure 2C. Densitometric analysis of the WB should be provided. How many replicates have been performed? Statistical analysis should also be provided.
In general, Western Blot analysis are often of poor quality; see for instance GADPH in Supplementary Figure 1C. This should be improved throughout the manuscript.
3. Figure 2D. The quality of the IFs is poor and should be improved, also by providing larger magnifications. Scale bars are hardly visible.
4. Page 6, line 117-121. If the expression of the pluripotency markers is comparable between WT and E2A KO, the authors should assess the self-renewal capacity of these cells by performing colony assays. This is highly relevant to support authors’ conclusion that E2A KO specifically affects hESC differentiation. Is Nodal/Cripto upregulated in undifferentiated E2A KO hESCs? Is Nodal/Smad signalling iper activated? This should be easily evaluated by WB analysis of P-Smad2 5. Figure 2E. The data on the tumor volumes should be reported, at least endpoint analysis. How many animals have been used for the analysis? How fast WT and E2A teratomas grow? E2A KO hESCs have a higher proliferation rate, does it alters the growth of teratomas. Besides H&E staining, teratomas composition should be analysed by IHC with specific markers of the different germ layers. This is particularly relevant since E2A KO teratomas are enriched in neuronal tissue (Fig. 4A). βIII tubulin staining should be included in the analysis to assess if terminally differentiated neurons also increased. Does neuronal differentiation increases at the expense of mesoderm - derived tissues?? This is relevant since authors conclude that "loss of E2A promotes mesendoderm differentiation in hESCs in vitro (see page 8, line 159).
6. Figure 2D and 4E RNA-Seq data lack statistical details. Fold change and p-value of deregulated genes should be reported.
7. page 7 line 132. RNA-Seq analysis revealed upregulation of mesendodermal genes and downregulation of neuroectodermal genes in E2A KO hESCs. How do authors explain these results? Is the analysis performed on undifferentiated hESCs? Although mesodermal genes are expressed in primed hESCs, neuroectodermal markers should not be expressed. Also, it is not clear if Cripto is upregulated in E2A KO undifferentiated hESCs, this would be expected being Cripto a target of E2A

as shown here. The authors should clarify this point and comment. Furthermore, the level of Cripto/Nodal signalling should be evaluated in E2A KO undifferentiated hESCs by WB analysis.
8. Fig. 2. The quality of the Figure is very poor and it is difficult to read. The size of the panels and the letters font in the panels should be increased.
9. Page 8, line 161; and page 12, line266. Authors' conclusion that E2A blocks hESC neural differentiation is overstated, and is not supported by the results. Lack of evidence include: i) Poor description/characterization of the cell types generated in the neuronal differentiation protocol used, which has not been properly described. The authors refer to the use of "commercial medium" and analyse two different time points, i.e day3 and day6 of differentiation. Which types of cells are generated in those culture conditions? Which is the expression profile of E2A in the neuronal differentiation of WT hESCs under these culture conditions? Furthermore, how can the authors conclude that neuronal differentiation is blocked rather than delayed? Later time points should be included in the analysis. Furthermore, given the key role of Cripto/Nodal in maintaining undifferentiated hESCs, upregulation of Cripto/Nodal and sustained Nodal signalling will keep hESCs undifferentiated and/or delay exit from pluripotency. This likely results in a delayed differentiation, which well explain the phenotype described. Delay of neuronal differentiation may also be further due to lack of expression/reduced induction of the E2A target neuronal genes.
- The role of E2A in neural differentiation must be deeper analysed. i) Time course expression profile of E2A mRNA and protein should be analysed. ii) The effect of E2A KO should be analysed at later time points of neural differentiation. Do E2A KO hESCs show a delayed differentiation capacity compared to WT? iii) Do neural precursors (SOX1 and PAX6 positive cells) show increased proliferation compared to WT. The authors should analyse the proliferation rate of WT and E2A KO hESCs at an early time point during neural differentiation, by using for instance EdU or BrdU incorporation.
10. Figure 4G: The observation that E2A KO cells retain high levels of OCT4 and NANOG is not convincing. Quantification of OCT4 and/or NANOG positive cells should be performed by IFs analysis. Do the authors suggest that pluripotent cells persist during neural differentiation of E2A cells? This is highly relevant as it may explain at least in part the mutant phenotype, i.e. delayed neuronal differentiation
11. Figure 5E, F. Both RNA-Seq and Chip-Seq data should be validated by performing q-RT PCR and Chip analysis of selected genes, respectively. For instance, data in panel G are FPKM frgmts from RNA-Seq. These should be replaced by q-RT PCR data to validate the RNA-Seq results.
12. Figure 5D. GO analysis of upregulated genes reveal a significant enrichment of gense involved in glicolitic metabolism- and hESCs are glycolitic. The authors should comment on that 13. Page 8, line 171. In the RNA- Seq analysis of 3days-neuronal differentiation, the authors focused only on the downregulated genes although they found more than 3000 upregulated genes. Gene ontology of upregulated gene should be reported and discussed. Is there any enrichment of pluripotent or mesoderm-specific markers?
14. Page 9, line 182. The rescued cells should be better characterised. For instance, exogenous E2A levels are much higher than those of WT cells (WB in Suppl Figure 2B), this should be taken into consideration, as these are E2A overexpressing rather than rescued cells.
15. Pharmacological inhibition of Nodal signalling. The authors show that SB does not exert any effect of WT hESCs (Suppl figure 3). This is puzzling as i) SB4315442 inhibits ALKs receptors, and thus blocks both Activin and Nodal, ii) hESCs are Activin/Nodal- dependent. It is not clear when SB4315442 is added to the cells and for how long. WB analysis of P-Smad2 should be performed to assess the level of Nodal/Activin signalling. The authors should clarify this issue and the non-specific effect of SB4315442 on Nodal should be indicated.
16. Page 10, line 222: The authors refer to neural progenitor cells (NPCs). This is misleading and should be replaced throughout the ms with 3d neuronal differentiation. There is no evidence that these are population of neural progenitors (see point 9 above).
17. Figure 6 A, B. Panel size and letter fonts should increase. Difficult to read.
18. Figure 6C: The quality of the IFs is poor. Co-localization of E2A with H3K27me3 is not evident at all. The quality of the images should be improved and IFs performed on cytospinned cells in order to quantify the number of cells co-expressing both markers.
19. Figure 6E: Interaction of E2A with EZH2, SUZ12, AEBP2 and JARID2 is not convincing. Figure 6 lacks statistical analysis. How many replicates were performed?
20. Page 11, lines 236-244: Description of the results is misleading. Is not clear if there are differences in the binding peaks between WT and E2A KO; however the authors conclude that binding of E2A to PRC2 complex does not change H3K4me3 and H3K27me3 distributions in

NPCs (????) from WT and KO cells. The authors should at least speculate on how E2A binds PRC2 and several promoter genes, but it regulates the epigenetic landscape only of a specific set.
21. Figure 7A: GAPDH of DKO cells is almost absent.
22. Figure 7E: Nestin staining is mostly nuclear. This is very puzzling as Nestin does not have a nuclear localization.
23. The observation that E2A/HEB double knockout (DKO) hESCs show a more severe neural differentiation defect is not convincing. The cells are poorly described and results do not add much to the story.

Minor points:
1. Generation of E2A KO hESCs. The manuscript lack a detailed description of the mutations obtained using two gRNAs targeting exon 1 of E2A. By observing the genome sequences of the clones utilized throughout the study, they show different mutations. Clone KO1 have different mutations on the two alleles, while KO4 show similar mutations on both alleles, with exception of one nucleotide. Describe in detail the resulting mutations obtained, and the impact of such deletions on the amino acid sequences.
2. Figure 2G: from the figure, the genes decreased in the absence of E2A are 10 and not 28 as stated on page 7, line141.
3. Page 7, line 152: For CD117+ CD184+, I would add double positive cells, in order to distinguish cases in which positive cells for one marker only are analysed, as in page 8, line168, SOX1+ cells and PAX6+ cells.
4. Figure 3E: MESP1 graph lacks KO.
5. Figure 3F: Please clarify how CD34+ cells were analysed. Immunofluorescence or FACS? Please show a representative picture.
6. Figure 4D-E and G: Is not clear which E2A KO clones are analysed. Please clarify in the figure legend.
7. Figure S2D: E2A ectopic expression rescues the defect of the neural differentiation of E2A KO hESCs. The qPCR analysis should show statistical significance between E2A KO + OE and E2A KO samples.
8. Page 10, line 219: Please add a description of the mSigDB database in the Materials and Methods.
9. Figure S1 C and D: Clarify H1- WT hESCs?
10. Fig. S 2C: Pax6 and not Pax61

Reviewer 2

Advance summary and potential significance to field

This paper shows that knockout hESCs for the E protein E2A have a neural differentiation defect without impacting self-renewal properties. Based on gene expression and ChiP-Seq studies it looks like E2A acts both by directly regulating neural-related genes and by suppressing Nodal signaling components including Nodal itself and Cripto. Conversely, KO for E2A enhances mes-endodermal differentiation. The authors contrast the results with their past work in knockout hESCs for the E protein HEB/TCF12 which resulted in a mesendodermal defect. In addition to opposing roles on mesendoderm induction the authors shown partially overlapping role of the two E proteins for neural diff where both factors upon KO impair neural differentiation and the two factors combined (double KO) may have more severe phenotype.

Overall, the study supports an early role for E2A in neural specification and presents complimentary data to recent work from the Lowell lab (Rao et al., Development 2020) showing a similar role for E2A during mouse ESC differentiation in promoting neural gene expression and suppressing nodal signaling. The work also complements their own previous work on HEB, showing both opposing (mes-endoderm) and complementary (neuroectoderm) roles during hESC differentiation.

Comments for the author

Main points:
1. Neural differentiation assays. It is important to clarify whether the authors are using in their kit for neural differentiation (Stem cell technologies) conditions that already include TGFb

inhibitor SB431542, as this will impact interpretation of the neural differentiation results. If it already does include SB431542, would this argue for dose-dependent effects such as in the rescue experiments (see point #2 below)?

2. Rescue experiments. The authors use rescue experiments to conclude effects both directly via E2A-mediated effects on neural gene expression and indirectly via modulation of nodal signaling. Knockout hESCs were rescued either by SB431542 exposure (see issue above) or by overexpression of E2A. As overexpression of E2A alone, acting as homo-dimer, triggers neural differentiation (e.g. see Rao et al., Development 2020), it would be important to see whether the such over-expression further enhances (e.g. accelerates) neural differentiation in WT cells. A more elegant overall experimental approach would have been to have a degron based LOF of E2A that could be easily induced and would be reversible without the need for overexpression. However, I understand that this would be likely beyond the scope of the current study. Accordingly, I would propose to include GOF in WT cells and to determine that levels of E2A are in physiological range.

3. NPC experiments. Neural precursors (NPC) seem to be defined by authors as day 3 cells during neural diff. However, at day 3 of differentiation, the cell composition, i.e. the ratio of neural cells versus remaining pluripotent cells (Figure 4) seems to be very different between WT and E2A KO cells. This makes interpretation of data (e.g. ChIP-Seq) challenging as binding of E2A in pluripotent cells vs neural cells is likely very distinct. Therefore, the findings may not really define differences in NPCs (with/w.o E2A) but rather changes in cell composition. Furthermore, the term NPC is usually applied to cells that are at a later stage, post neural commitment, when neural cells are maintained as precursors (hence NPCs).

4. All the data seem to be generated from a single hESC line (H1) and from a maximum of 2 clones. As there can be variability across lines, it would be valuable to confirm the result using at least one independent line.

5. Clarifying data presentation. There seems to be several inconsistencies that should be addressed.

a. In Figure 2B, there seems to be no segregation of the hESC data by PCA comparing E2A-KO vs WT but there seems to be segregation between the groups in the heat map in Figure 2C. While subtle in either case, please explain this discrepancy.
b. Comparing overlap of ChiP seq and differential gene expression, they report: “28 genes decreased in the absence of E2A” however, Figure 2G seems to indicate that 10 genes are overlapping rather than 28? – please clarify.
c. In Figure 6A , EED targets are found in both up and in the downregulated genes?

6. Figure 4F is not very informative listing terms such as system development, structure development, organismal development…and so on.. – may be GSEA for more specific developmental functions would be more useful.

7. In Figure 1B, it would be helpful to clearly state (e.g. in Figure legend) how many replicates (clones, independent experiments) were performed to obtain this result.

8. The authors should use references more carefully, as they use incorrect references at several places in the manuscript such as at the very start of the manuscript (line 46/47): “Human embryonic stem cells (hESCs) can be expanded indefinitely and differentiate into all human cell types (Evans and Kaufman 1981; Ying et al., 2008)” --- those are both classic mouse rather than human ES cell references ---. “As such, they hold great promise for developmental studies, drug screening, cell-based therapy and disease modeling (Yamanaka 2009)”. --- which is primarily an iPSC reference

9. I could not find the raw supplementary data for review which would have been helpful. For example, the authors report regulation of neural differentiation markers including ASIC2, BOC, GLI3, LEF1, LMX1A, NRP2, PREX2 and SEMA3E (Figure 5E). Those are not necessarily the most

canonical neural related genes, and I was wondering the data also showed downregulation of factors such as PAX6 and SOX1 which would be expected based on the results shown in Figure 4.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript entitled “E2A regulates neural ectoderm fate specification in human embryonic stem cells” by Yi S. et al., investigates the role of the E2A protein in hESC differentiation, specifically mesoderm and neural differentiation. The authors combined RNA- Seq and Chip-Seq analysis and identified E2A direct target genes in hESC neuronal differentiation. Their results provide evidence that E2A regulates the expression of several neuronal specific genes as well as that of genes involved in the Nodal pathway, including the Nodal obligate coreceptor Cripto. Despite the fact that E2A interacts with PRC2 complex, E2A KO has little effect on the epigenetic landscape of hESCs during neuronal differentiation. Finally, the authors investigate the impact of E2A KO on hESC differentiation, and provide molecular and phenotypic evidence that neuronal differentiation is altered in E2A KO hESCs. Mechanistically, they propose that this is due to the persistent upregulation of the mesoderm-inducing gene Cripto and to the lack of proper induction of neuronal-specific genes.

Reviewer 1 Comments for the Author:
This is a potentially interesting study and the manuscript reads well. However, authors’ conclusions are not fully supported by the results. In particular, authors' conclusion that "the absence of E2A results in a profound block in neural development" is an over interpretation of their results. There are several points that should be addressed before the manuscript could be considered for publication. Statistical details are often missing and the overall quality of the pictures is poor and should be improved.
Thanks for your careful review. We agree with you that “the absence of E2A results in a profound block in neural development” is an over interpretation, as we focused mainly on the function of E2A in early neural ectoderm specification of human ESCs. We may also note that this study focuses on the process by which pluripotent cells induced into neural progenitor cells instead of mature neurons (“neuronal differentiation” judgement below). Moreover, we performed new experiments to answer the block/delay question. Firstly, the IHC analysis of ß-III-tubulin in teratoma demonstrated that limited neural tissues formed in the E2A KO group after two months (Fig. 1G). Secondly, we repeated the flow cytometry staining of SOX1 and PAX6 after 9 days of induction and got consistent results (Supplementary Figure 3C). Thirdly, we employed EdU staining to assess the proliferation of WT and E2A KO cells during neural induction (Supplementary Figure 3D). The Edu results demonstrated lower proliferation rate in E2A KO cells, providing strong evidence against the possibility of proliferation-induced delayed differentiation.

Major points:
1.Figure 1B. The authors state that E2A KO hESCs colony morphology is indistinguishable from that of WT hESCs; however, the images show that E2A KO colonies are much smaller compared to WT. This is also in contrast with CCK8 results that E2A KO hESCs display a higher proliferation rate. How do the authors explain this discrepancy? Are the images representative? Scale bar is missing.
Thanks for your suggestion. The colonies images showed representative morphology of wild type and E2A KO cells. However, the cells weren’t seeded at the same timepoint in the same density, so, the size of these colonies do not represent the proliferation rate of WT and E2A KO hESCs. This has now been clarified in the Figure Legend. In addition, a scale bar has been added in Figure 1A.

2.Figure 2C. Densitometric analysis of the WB should be provided. How many replicates have been performed? Statistical analysis should also be provided. In general, Western Blot analysis are often of poor quality; see for instance GADPH in Supplementary Figure 1C. This should be improved throughout the manuscript.

I think you mean Figure 1D rather than Figure 2C. WB analysis in Figure 1D were performed in three replicates. Densitometric analysis of the WB was demonstrated in Figure 1E. The Western Blot analysis have been improved throughout the manuscript.

3. Figure 2D. The quality of the IFs is poor and should be improved, also by providing larger magnifications. Scale bars are hardly visible.
Thanks for your suggestions, I think you mean Figure 1C rather than Figure 2D. We have replaced the IFs pictures with higher resolution.

4. Page 6, line 117-121. If the expression of the pluripotency markers is comparable between WT and E2A KO, the authors should assess the self-renewal capacity of these cells by performing colony assays. This is highly relevant to support authors' conclusion that E2A KO specifically affects hESC differentiation. Is Nodal/Cripto upregulated in undifferentiated E2A KO hESCs? Is Nodal/Smad signalling iper activated? This should be easily evaluated by WB analysis of P-Smad2
Thanks for your suggestions. The colony forming assay was performed in WT and E2A KO hESCs, the results demonstrated similar colony morphology with more colony numbers in E2A KO hESCs (Supplementary 2A). The Nodal/Smad signaling was decreased in E2A KO hESCs which is demonstrated in Supplemental Figure 2B.

5. Figure 2E. The data on the tumor volumes should be reported, at least endpoint analysis. How many animals have been used for the analysis? How fast WT and E2A teratomas grow? E2A KO hESCs have a higher proliferation rate, does it alters the growth of teratomas. Besides H&E staining, teratomas composition should be analysed by IHC with specific markers of the different germ layers. This is particularly relevant since E2A KO teratomas are enriched in neuronal tissue (Fig. 4A). βIII tubulin staining should be included in the analysis to assess if terminally differentiated neurons also increased. Does neuronal differentiation increases at the expense of mesoderm - derived tissues?? This is relevant since authors conclude that "loss of E2A promotes mesendoderm differentiation in hESCs in vitro (see page 8, line 159).
Thanks for your suggestions. We repeated the teratoma experiments by collecting and injecting 2*106 cells into NOD/SCID mice (each group with six mice). After 4 weeks, 12 mice were sacrificed and teratomas were harvested. The size of teratomas formed by E2A KO cells were larger than WT on average (Figure 1F). IHC staining analysis of three germ layers' markers βIII-tubulin, α-SMA and pan-CK were tested in teratoma sections (Fig. 1G). The IHC analysis reveals that neural marker βIII-tubulin expression was dramatically decreased in E2A KO teratomas, whereas expression level of mesendodermal markers α-SMA and pan-CK were slightly increased in E2A KO teratomas.

6. Figure 2D and 4E RNA-Seq data lack statistical details. Fold change and p-value of deregulated genes should be reported.
Thanks. Three replicates of WT and E2A KO-1 hESCs were collected for RNA-seq. We have uploaded the unprocessed and processed RNA-seq data in GEO dataset (GSE154625) and Supplementary Data; you can find the representative DEGs in the Supplemental Data 1.

7. page 7 line 132. RNA-Seq analysis revealed upregulation of mesendodermal genes and downregulation of neuroectodermal genes in E2A KO hESCs. How do authors explain these results? Is the analysis performed on undifferentiated hESCs? Although mesodermal genes are expressed in primed hESCs, neuroectodermal markers should not be expressed. Also, it is not clear if Cripto is upregulated in E2A KO undifferentiated hESCs, this would be expected being Cripto a target of E2A as shown here. The authors should clarify this point and comment. Furthermore, the level of Cripto/Nodal signalling should be evaluated in E2A KO undifferentiated hESCs by WB analysis.
Thanks for your suggestions. The bulk RNA-seq was performed on undifferentiated hESCs. Compared with high expression level of pluripotency markers including OCT4, NANOG, SOX2 et al, the expression level of mesodermal genes and neuroectodermal markers was relatively low, which led to its sensitivity to a loss of E2A. The relevant expression data can be found in Supplementary Data 1.
As we showed in Figure 2H, E2A could regulate the expression level of neuroectodermal markers SOX5 and FGF1 by directly binding to their promoter regions, which may lead to downregulation of neuroectodermal genes. Considering the counterbalance between neuroectoderm and mesendoderm differentiation, the declined expression of neuroectodermal genes may lead to upregulation of mesendodermal genes. As for Cripto in hESCs, the combination of E2A ChIP-seq and

RNA-seq data had demonstrated Cripto was not a target of E2A in undifferentiated hESC. The different results between hESCs and NPCs may due to the different culture medium (see next Question). The level of Cripto/Nodal signaling was evaluated by p-Smad2 WB analysis, described in our answer to Question 4.

8. Fig. 2. The quality of the Figure is very poor and it is difficult to read. The size of the panels and the letters font in the panels should be increased.
Thanks for your suggestion. We have improved the Figures with larger panels and letters font.

9. Page 8, line 161; and page 12, line266. Authors' conclusion that E2A blocks hESC neural differentiation is overstated, and is not supported by the results. Lack of evidence include: i) Poor description/characterization of the cell types generated in the neuronal differentiation protocol used, which has not been properly described. The authors refer to the use of "commercial medium" and analyse two different time points, i.e day3 and day6 of differentiation. Which types of cells are generated in those culture conditions? Which is the expression profile of E2A in the neuronal differentiation of WT hESCs under these culture conditions? Furthermore, how can the authors conclude that neuronal differentiation is blocked rather than delayed? Later time points should be included in the analysis. Furthermore, given the key role of Cripto/Nodal in maintaining undifferentiated hESCs, upregulation of Cripto/Nodal and sustained Nodal signalling will keep hESCs undifferentiated and/or delay exit from pluripotency. This likely results in a delayed differentiation, which well explain the phenotype described. Delay of neuronal differentiation may also be further due to lack of expression/reduced induction of the E2A target neuronal genes.
- The role of E2A in neural differentiation must be deeper analysed. i) Time course expression profile of E2A mRNA and protein should be analysed. ii) The effect of E2A KO should be analysed at later time points of neural differentiation. Do E2A KO hESCs show a delayed differentiation capacity compared to WT? iii) Do neural precursors (SOX1 and PAX6 positive cells) show increased proliferation compared to WT. The authors should analyse the proliferation rate of WT and E2A KO hESCs at an early time point during neural differentiation, by using for instance EdU or BrdU incorporation.
Thank you for advices. The commercial medium from STEM CELL Technologies was called STEMdiff™ Neural Induction Medium (#05835) which enables high efficient generation of neural progenitor cells using either embryoid body or monolayer culture-based protocols. The kit combines neural induction medium and supplement, which directs differentiation by blocking Tgf-ßand BMP-dependent SMAD signaling, resulting in efficient neural induction of pluripotent stem cells(Saini et al., 2017). After 6-9 days monolayer induction, nearly all cells can express CNS-type NPC markers PAX6, SOX1 and NESTIN according to the manufacture's protocol. However, for following downstream differentiation, it's better to culture neural progenitor cells for 18-21 days (Figure Below). In our study, approximately 90% and 70% cells were SOX1 positive at day 6 and day 3, respectively, and PAX6 was also expressed. (Fig. 4B, C).

We have removed unpublished data provided for the referees in confidence.

We also tested the expression level of E2A during neural differentiation via WB analysis and qPCR analysis. The results demonstrated the expression level of E2A gradually increased from day 2 to day 6 (Supplementary Figure 3A-B). The decreased expression of E2A from day 0 to day 2 may due to transition from E8 medium to neural induction medium.
We also analyzed the expression level of neural differentiation markers after 9 day induction. The E2A KO group demonstrated consistent decreases compare to the WT group (Supplemental Figure 3C).
Moreover, the proliferation rates of WT and E2A KO cells were analyzed by EdU assay. The results demonstrated that the proliferation rate of E2A KO cells was slightly lower than the WT cells during neural differentiation (Supplemental Figure 3D), ruling out the possibility of proliferation-induced block of neural differentiation.

10. Figure 4G: The observation that E2A KO cells retain high levels of OCT4 and NANOG is not convincing. Quantification of OCT4 and/or NANOG positive cells should be performed by IFs analysis. Do the authors suggest that pluripotent cells persist during neural differentiation of E2A cells? This is highly relevant as it may explain at least in part the mutant phenotype, i.e. delayed neuronal differentiation

Thanks for your advice. We performed the experiments with IFs analysis in 3 days post neural differentiation, the results demonstrated slightly higher expression level of OCT4 in E2A KO cells compared to WT cells (Supplementary Figure 4B). In order to verify the results, we also repeated the results with WB analysis, the results also demonstrated slightly higher expression level of OCT4 and NANOG in E2A KO cells during neural differentiation (Supplementary Figure 4A), which may partially explain the blocked neural differentiation.

11. Figure 5E, F. Both RNA-Seq and Chip-Seq data should be validated by performing q-RT PCR and Chip analysis of selected genes, respectively. For instance, data in panel G are FPKM frgmts from RNA-Seq. These should be replaced by q-RT PCR data to validate the RNA-Seq results.
Thanks for your suggestion. We have replaced Figure 5E, F with qRT-PCR and ChIP-PCR analysis (Figure 5E-F). The relevant qRT-PCR and ChIP-PCR primers were listed in Table 1.

12. Figure 5D. GO analysis of upregulated genes reveal a significant enrichment of gense involved in glicolitic metabolism- and hESCs are glycolitic. The authors should comment on that
The GO analysis of upregulated genes are derived from combination of E2A ChIP-seq and RNA-seq data performed in 3 days neural induction of hESCs. The upregulated glycolytic metabolism related genes in E2A group may be the causes which led to a block of neural induction in E2A knockout hESCs, ie the high flux of glycolytic metabolism maintained E2A knockout hESCs in a pluripotent state during neural induction process (Moussaieff et al., 2015; Ryall et al., 2015).

13. Page 8, line 171. In the RNA- Seq analysis of 3days-neuronal differentiation, the authors focused only on the downregulated genes although they found more than 3000 upregulated genes. Gene ontology of upregulated gene should be reported and discussed. Is there any enrichment of pluripotent or mesoderm-specific markers?
Thanks for your suggestions. Although 3000 genes were upregulated in E2A KO neural differentiation period, only 57 upregulated genes were direct target gene analyzed by combination of RNA-seq and ChIP-seq. We focused on these genes and found most of the genes were related to glycolysis process.
Moreover, the 3347 up-regulated and 2009 down-regulated genes were enriched in Figure 4F. We didn't find any obvious enrichment of pluripotent or mesoderm-specific markers. However, the expression level of mesendodermal genes higher in E2A KO cells than WT cells during neural differentiation period although at quite low expression level (Supplementary Data4).

14. Page 9, line 182. The rescued cells should be better characterised. For instance, exogenous E2A levels are much higher than those of WT cells (WB in Suppl Figure 2B), this should be taken into consideration, as these are E2A overexpressing rather than rescued cells.
Thanks for your advice. We have run out of E2A expressing lentivirus and couldn't buy from commercial company due to coronavirus pandemic. Hence, we sorted and collected infected GFP positive embryonic stem cells by flow cytometry. WB analysis was performed in WT, E2A KO, E2A KO + CON, E2A KO + E2A cells. The results demonstrated that the expression level of E2A were comparable in WT and E2A KO + E2A cells, which eliminated the effects of E2A overexpression in E2A KO cells (Supplementary Figure 6A-B).

15. Pharmacological inhibition of Nodal signalling. The authors show that SB does not exert any effect of WT hESCs (Suppl figure 3). This is puzzling as i) SB4315442 inhibits ALKs receptors, and thus blocks both Activin and Nodal, ii) hESCs are Activin/Nodal- dependent. It is not clear when SB4315442 is added to the cells and for how long. WB analysis of P-Smad2 should be performed to assess the level of Nodal/Activin signalling. The authors should clarify this issue and the non-specific effect of SB4315442 on Nodal should be indicated.
Thanks for your kind advice. The neural induction medium was added with 100nM LDN193189 and 10uM SB431542 to drive efficient neural differentiation(Saini et al., 2017). Therefore, 10uM SB431542 didn't exert much more effect to WT hESCs. Moreover, various concentration of SB431542 was added into neural induction medium on the very beginning timepoint of neural induction period. The results demonstrated that 10uM SB431542 could partially reverse the neural differentiation defects in E2A KO cells. In addition, SB431542 reduced neural induction efficiency in WT cells when the final concentration up to 20uM and 50uM. We also tested the expression level of P-SMAD2 in WT and E2A KO cells during neural induction period, the results demonstrated slightly increased P-smad2 expression level in E2A KO group (Supplemental Figure 4A).

SB431542 is a relatively specific inhibitor of Tgf-ßsignaling and we agree with the reviewer that we can't exclude other effects though other members of Tgf-ßsignaling superfamily. However, considering the low Tgf-ßsignaling activities in neural differentiation culture medium (supplemented with LDN-193189 and SB431542), the non-specific effect of SB431542 are likely minimal.

16. Page 10, line 222: The authors refer to neural progenitor cells (NPCs). This is misleading and should be replaced throughout the ms with 3d neuronal differentiation. There is no evidence that these are population of neural progenitors (see point 9 above).
Thanks for your suggestions. We have made corrections accordingly.

17. Figure 6 A, B. Panel size and letter fonts should increase. Difficult to read.
Thank you, we have uploaded modified figure according to your recommendations.

18. Figure 6C: The quality of the IFs is poor. Co-localization of E2A with H3K27me3 is not evident at all. The quality of the images should be improved and IFs performed on cytospinned cells in order to quantify the number of cells co-expressing both markers.
Thank you, we have uploaded modified figure according to your recommendations.

19. Figure 6E: Interaction of E2A with EZH2, SUZ12, AEBP2 and JARID2 is not convincing. Figure 6 lacks statistical analysis. How many replicates were performed?
Thanks, we repeated the co-ip experiments and uploaded modified image in Figure 6E. In addition, for the western blot analysis, immunofluorescence analysis and co-IP analysis are repeated three times in 3 days' neural differentiated cells. Quantitative analysis of EZH2, H3K27me3 and H3K4me3 western blot results was placed in Supplementary Figure 8.

20. Page 11, lines 236-244: Description of the results is misleading. Is not clear if there are differences in the binding peaks between WT and E2A KO; however the authors conclude that binding of E2A to PRC2 complex does not change H3K4me3 and H3K27me3 distributions in NPCs (????) from WT and KO cells. The authors should at least speculate on how E2A binds PRC2 and several promoter genes, but it regulates the epigenetic landscape only of a specific set.
Thanks for your advice. In Figure 6D-E, we have demonstrated E2A can bind to PRC2 complex subunit EZH1, EZH2, SUZ12 during neural differentiation period. However, we didn't find neural related genes upregulated directly by E2A/PRC2 complex. In the meantime, we have analyzed the promoter region of E2A directly downregulated genes and found no significant difference in epigenetic landscape (Figure 6F-G). Therefore, we concluded that the binding of E2A to PRC2 doesn't change H3K4me3 and H3K27me3 distributions in neural progenitor cells (now has been corrected into neural differentiation period). Moreover, we have uploaded the unprocessed and processed high throughput data into GEO datasets (GSE 154625) and Supplemental Data 7-8 respectively. We also speculate that E2A/PRC2 complexes act at certain genes, not globally, due to the need for additional complex components that provide specificity.

21. Figure 7A: GAPDH of DKO cells is almost absent.
Thank you, we have uploaded modified figure according to your recommendations.

22. Figure 7E: Nestin staining is mostly nuclear. This is very puzzling as Nestin does not have a nuclear localization.
Sorry for mistaking the PAX6 staining to NESTIN staining in Figure 7E. Moreover, we also performed NESTIN staining in WT and E2A KO neural progenitor cells and obtained consistent results (Supplementary Figure 9B).

23. The observation that E2A/HEB double knockout (DKO) hESCs show a more severe neural differentiation defect is not convincing. The cells are poorly described and results do not add much to the story.
The E2A/HEB double knockout cells were generated on the basis of HEB KO cells, which was a kind gift from Professor Juan Carlos Zúñiga-Pflücker's Lab. The genotype was added in Supplemental Figure 9A, and the western blot analysis of E2A and HEB was demonstrated in Figure 7A. Moreover, we repeated neural induction in WT, E2A KO, HEB KO and DKO hESCs and got consistent results. The immunofluorescent staining of PAX6 and NESTIN also demonstrated the same results.

The relatively low neural differentiation efficiency was mainly due to the neural induction time of 3 days. The shorten neural induction time was due to the low proliferation rate of double KO cells in neural induction medium.
E2A was reported to function in concert with HEB to regulate B cell development (Welinder et al., 2011). In addition, HEB and E2A were reported to work together to maintain DP (CD4(+)CD8(+) double-positive) fate and to control the DP to SP(CD4(+)CD8(+) single-positive) transition during T lymphocyte development(Jones and Zhuang, 2007). These observations provided our rationale for creating the DKO hESCs in order to reveal the function of E2A in neural differentiation and alleviate functional compensation between E2A and HEB.

Minor points:
1. Generation of E2A KO hESCs. The manuscript lack a detailed description of the mutations obtained using two gRNAs targeting exon 1 of E2A. By observing the genome sequences of the clones utilized throughout the study, they show different mutations. Clone KO1 have different mutations on the two alleles, while KO4 show similar mutations on both alleles, with exception of one nucleotide. Describe in detail the resulting mutations obtained, and the impact of such deletions on the amino acid sequences.
Thanks for your advice. We added the detailed description of mutations in Supplemental Figure 1A-B. The E2A KO clone 1 exhibited a 29bp deletion in one allele, while two deletions (26bp deletion and 29bp deletion) in another allele. The E2A KO clone 4 exhibited two deletions (4bp deletion and 2bp deletion) in one allele and another two deletions (4bp deletion and 3bp deletion) in the other allele. Upon deletion, installation of a premature termination codon from a frameshift-inducing INDEL that elicits nonsense-mediated decay of the mutant mRNA, which leading to selective gene ablation(Tuladhar et al., 2019).

2. Figure 2G: from the figure, the genes decreased in the absence of E2A are 10 and not 28 as stated on page 7, line141.
Sorry to mistake the wrong numbers of overlapped genes. In the analysis, we found 10 genes were directly bound by E2A in their promoter region. However, 28 genes bound by E2A in all regions excluding intergenic regions. We have made modifications accordingly in the manuscript.

3. Page 7, line 152: For CD117+ CD184+, I would add double positive cells, in order to distinguish cases in which positive cells for one marker only are analysed, as in page 8, line168, SOX1+ cells and PAX6+ cells.
Thanks. We have made modification accordingly.

4. Figure 3E: MESP1 graph lacks KO.
Thanks for your suggestions. We have added the “KO” in MESP1 graph.

5. Figure 3F: Please clarify how CD34+ cells were analysed. Immunofluorescence or FACS? Please show a representative picture.
Thank you. We have added the representative FACS image of CD34 in Figure 3E.

6. Figure 4D-E and G: Is not clear which E2A KO clones are analysed. Please clarify in the figure legend.
Thanks for your suggestions. For RNA-seq analysis in Figure 4D-4E, wild type, E2A KO-1 clones and E2A-4 KO clones were used in the experiments. For real-time qPCR analysis in Figure 4G, wild type and E2A KO-1 clones were used in the experiments. We have clarified the information in the figure legend.

7. Figure S2D: E2A ectopic expression rescues the defect of the neural differentiation of E2A KO hESCs. The qPCR analysis should show statistical significance between E2A KO + OE and E2A KO samples.
Thanks for your advice, we have made modification accordingly.

8. Page 10, line 219: Please add a description of the mSigDB database in the Materials and Methods.
Thanks, we have added description of mSigDB database in Material and Methods accordingly.

9. Figure S1 C and D: Clarify H1- WT hESCs?

Thanks for your suggestions, we have replaced “H1” with “WT” in supplemental Figure 1C and 1D, indicating negative control in the experiments.

10. Fig. S 2C: Pax6 and not Pax61
Thanks for catching that typo, we have made the correction.

Reviewer 2 Advance Summary and Potential Significance to Field:
This paper shows that knockout hESCs for the E protein E2A have a neural differentiation defect without impacting self-renewal properties. Based on gene expression and ChiP-Seq studies it looks like E2A acts both by directly regulating neural-related genes and by suppressing Nodal signaling components including Nodal itself and Cripto. Conversely, KO for E2A enhances mes-endodermal differentiation. The authors contrast the results with their past work in knockout hESCs for the E protein HEB/TCF12 which resulted in a mesendodermal defect. In addition to opposing roles on mesendoderm induction, the authors shown partially overlapping role of the two E proteins for neural diff where both factors upon KO impair neural differentiation and the two factors combined (double KO) may have more severe phenotype. Overall, the study supports an early role for E2A in neural specification and presents complimentary data to recent work from the Lowell lab (Rao et al., Development 2020) showing a similar role for E2A during mouse ESC differentiation in promoting neural gene expression and suppressing nodal signaling. The work also complements their own previous work on HEB, showing both opposing (mes-endoderm) and complementary (neuroectoderm) roles during hESC differentiation.

Reviewer 2 Comments for the Author:

Main points:
1.Neural differentiation assays. It is important to clarify whether the authors are using in their kit for neural differentiation (Stem cell technologies) conditions that already include TGFb inhibitor SB431542, as this
will impact interpretation of the neural differentiation results. If it already does include SB431542, would this argue for dose-dependent effects such as in the rescue experiments (see point #2 below)?
Thanks for your suggestions. The commercial medium from STEM CELL Technologies was called STEMdiff™ Neural Induction Medium (#05835) which enables high efficient generation of neural progenitor cells using either embryoid body or monolayer culture-based protocols. The kit combines neural induction medium and supplement, which directs differentiation by blocking Tgf-βand BMP-dependent SMAD signaling, resulting in efficient neural induction of pluripotent stem cells. After 6-9 day’s monolayer induction, nearly all cells can express CNS-type NPC markers PAX6, SOX1 and NESTIN according to the manufacture’s protocol. The neural induction medium was added with 100nM LDN193189 and 10uM SB431542 to drive efficient neural differentiation(Saini et al., 2017). Moreover, various concentration of SB431542 was added into neural induction medium on the very beginning timepoint of neural induction period. The results demonstrated that 10uM SB431542 could partially reverse the neural differentiation defects in E2A KO cells. In addition, SB431542 may reduce neural induction efficiency in WT cells when the final concentration raise to 20uM and 50uM (Supplementary Figure 7D). So yes, it does appear to be dose-dependent, and we have added that point to the text.

2. Rescue experiments. The authors use rescue experiments to conclude effects both directly via E2A-mediated effects on neural gene expression and indirectly via modulation of nodal signaling. Knockout hESCs were rescued either by SB431542 exposure (see issue above) or by overexpression of E2A. As
overexpression of E2A alone, acting as homo-dimer, triggers neural differentiation (e.g. see Rao et al., Development 2020), it would be important to see whether the such over-expression further enhances (e.g. accelerates) neural differentiation in WT cells. A more elegant overall experimental approach would have been to have a degron based LOF of E2A that could be easily induced and would be reversible without the need for overexpression. However, I understand that this would be likely beyond the scope of the current study. Accordingly, I would propose to include GOF in WT cells and to determine that levels of E2A are in physiological range.

Thanks for your advice. We have run out of E2A over-expressing lentivirus and couldn't buy from commercial company due to coronavirus pandemic. So we could not perform E2A over-expression in WT cells. Hence, we sorted and collected infected GFP positive embryonic stem cells by flow cytometry. WB analysis was performed in WT, E2A KO, E2A KO + CON, E2A KO + E2A cells. The results demonstrated that the expression level of E2A were comparable in WT and E2A KO + E2A cells, which demonstrated the levels of E2A were in physiological range (Supplementary Figure 6B).

3. NPC experiments. Neural precursors (NPC) seem to be defined by authors.as day 3 cells during neural diff. However, at day 3 of differentiation, the cell composition, i.e. the ratio of neural cells versus remaining pluripotent cells (Figure 4) seems to be very different between WT and E2A KO cells. This makes interpretation of data (e.g. ChIP-Seq) challenging as binding of E2A in pluripotent cells vs neural cells is likely very distinct. Therefore, the findings may not really define differences in NPCs (with/w.o E2A) but rather changes in cell composition. Furthermore, the term NPC is usually applied to cells that are at a later stage, post neural commitment, when neural cells are maintained as precursors (hence NPCs).
Thanks for your suggestions. This is always a challenge when working with small cell numbers. As ChIP-seq for E2A binding was performed on WT cells after 3 days of neural differentiation, data is likely to be representative of a largely homogeneous population. After 6-9 day's monolayer induction, nearly all cells can express CNS-type NPC markers PAX6, SOX1 and NESTIN using this method. However, for following downstream differentiation, it's better to culture neural progenitor cells for 18-21 days (Figure Below). In our study, approximately 70% and 90% cells were SOX1 positive at day 3 and day 6, respectively. We agree therefore that the differences in RNAseq in WT versus E2A KO could reflect changes in cell composition, especially for those genes that are not directly bound by E2A in the WT cells.

We have removed unpublished data provided for the referees in confidence.

4. All the data seem to be generated from a single hESC line (H1) and from a maximum of 2 clones. As there can be variability across lines, it would be valuable to confirm the result using at least one independent line.
Thanks for your advice. We generated E2A KO in another hESC line (H9) and repeated the neural differentiation experiments in corresponding cells. The genotype and western blot analysis of E2A KO H9 cells were demonstrated in Supplemental Figure 5 A-B. The consistent neural differentiation results were demonstrated in Supplementary Figure 5 C.

5. Clarifying data presentation. There seems to be several inconsistencies that should be addressed.
a. In Figure 2B, there seems to be no segregation of the hESC data by PCA comparing E2A-KO vs WT but there seems to be segregation between the groups in the heat map in Figure 2C. While subtle in either case, please explain this discrepancy.
Thanks, we have replaced the correct one with old PCA image in Figure 2B.
b. Comparing overlap of ChiP seq and differential gene expression, they report: "28 genes decreased in the absence of E2A" however, Figure 2G seems to indicate that 10 genes are overlapping rather than 28? – please clarify.
Sorry to mistake the wrong numbers of overlapped genes. In the analysis, we found 10 genes were directly bound by E2A in their promoter region. However, 28 genes bound by E2A in all regions excluding intergenic regions. We have made modifications accordingly in the manuscript.
c. In Figure 6A , EED targets are found in both up and in the downregulated genes?
We indeed found EED targets are found in both up and downregulated genes (Supplementary Data 6), which may indicating some genes are upregulated by E2A/PRC2 complex. However, the directly upregulated genes were less correlated with neural differentiation, thus we focused on the downregulated genes in this paper.

6. Figure 4F is not very informative listing terms such as system development, structure development, organismal development…and so on.. – may be GSEA for more specific developmental functions would be more useful.
Thanks for your suggestions, we have analyzed the RNA-seq data with GO FAT and got more specific results. The results image was placed in Figure 4F.

7. In Figure 1B, it would be helpful to clearly state (e.g. in Figure legend) how many replicates (clones, independent experiments) were performed to obtain this result.
Thanks for your advice. Three times of 5 replicates of WT and E2A-1 KO clones were performed to obtain the CCK8 results. Moreover, the colony forming assay results also demonstrated higher proliferation rate in E2A KO embryonic stem cells.

8. The authors should use references more carefully, as they use incorrect references at several places in the manuscript such as at the very start of the manuscript (line 46/47): “Human embryonic stem cells (hESCs) can be expanded indefinitely and differentiate into all human cell types (Evans and Kaufman, 1981; Ying et al., 2008)” --- those are both classic mouse rather than human ES cell references ---. “As such, they hold great promise for developmental studies, drug screening, cell-based therapy and disease modeling (Yamanaka, 2009)”. --- which is primarily an iPSC reference
Thanks for your kind and careful suggestion. We have made modification accordingly in the manuscript.

9. I could not find the raw supplementary data for review which would have been helpful. For example, the authors report regulation of neural differentiation markers including ASIC2, BOC, GLI3, LEF1, LMX1A, NRP2, PREX2 and SEMA3E (Figure 5E). Those are not necessarily the most canonical neural related
genes, and I was wondering the data also showed downregulation of factors such as PAX6 and SOX1 which would be expected based on the results shown in Figure 4.
Thanks for your suggestions. We have uploaded the high throughput sequencing date in GEO dataset (GSE154625) and processed sequencing data in Supplemental files. Unfortunately, we didn’t find PAX6 and SOX1 as direct targets of E2A. However, the expression level of PAX6 and SOX1 were dramatically decreased in E2A KO cells compared to WT cells during neural differentiation process, which is likely reflective of the shift in cell populations.

Reference:

Jones, M. E. and Zhuang, Y. (2007). Acquisition of a functional T cell receptor during T lymphocyte development is enforced by HEB and E2A transcription factors, *Immunity* 27(6): 860-70.
Moussaieff, A., Rouleau, M., Kitsberg, D., Cohen, M., Levy, G., Barasch, D., Nemirovski, A., Shen-Orr, S., Laevsky, I., Amit, M. et al. (2015). Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells, *Cell Metab* 21(3): 392-402.
Ryall, J. G., Cliff, T., Dalton, S. and Sartorelli, V. (2015). Metabolic Reprogramming of Stem Cell Epigenetics, *Cell Stem Cell* 17(6): 651-662.
Saini, J. S., Corneo, B., Miller, J. D., Kiehl, T. R., Wang, Q., Boles, N. C., Blenkinsop, T. A., Stern, J. H. and Temple, S. (2017). Nicotinamide Ameliorates Disease Phenotypes in a Human iPSC Model of Age-Related Macular Degeneration, *Cell Stem Cell* 20(5): 635-647 e7.
Tuladhar, R., Yeu, Y., Tyler Piazza, J., Tan, Z., Rene Clemenceau, J., Wu, X., Barrett, Q., Herbert, J., Mathews, D. H., Kim, J. et al. (2019). CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation, *Nat Commun* 10(1): 4056.
Welinder, E., Mansson, R., Mercer, E. M., Bryder, D., Sigvardsson, M. and Murre, C. (2011). The transcription factors E2A and HEB act in concert to induce the expression of FOXO1 in the common lymphoid progenitor, *Proc Natl Acad Sci U S A* 108(42): 17402-7.

Second decision letter

MS ID#: DEVELOP/2020/190298

MS TITLE: E2A regulates neural ectoderm fate specification in human embryonic stem cells

AUTHORS: Yang Li, Siqi Yi, Xiaotian Huang, Yuan Zhou, Shixin Zhou, Michele K Anderson, Juan Carlos Zuniga-Pflucker, and Qingxian Luan

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development. The reviewer has asked for several minor revisions. Please address these as soon as possible and get the manuscript back to us for acceptance for publication.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed most of the comments and the manuscript is improved.
However, there remain a few issues to be addressed.
Page 19: E2A loss of function blocks hESC neural ectoderm differentiation.
This conclusion is not fully supported by the data, and should be tempered.
Overall authors' findings point to a delay rather than a block of neural differentiation. For instance:
... the results also demonstrated slightly higher expression level of OCT4 and NANOG in E2A KO cells during neural differentiation (Supplementary Figure 4A), which may partially explain the blocked neural differentiation.
This may be well explained by a delay rather than a block.

1.The colonies images showed representative morphology of wild type and E2A KO cells. However, the cells weren't seeded at the same timepoint in the same density, so, the size of these colonies do not represent the proliferation rate of WT and E2A KO hESCs. This has now been clarified in the Figure Legend.
This clarification is missing in the Figure legend and should be included to avoid confusion.

Comments for the author

Page 19: E2A loss of function blocks hESC neural ectoderm differentiation.
This conclusion is not fully supported by the data, and should be tempered.
Overall authors' findings point to a delay rather than a block of neural differentiation. For instance:
... the results also demonstrated slightly higher expression level of OCT4 and NANOG in E2A KO cells during neural differentiation (Supplementary Figure 4A), which may partially explain the blocked neural differentiation.
This may be well explained by a delay rather than a block.

1.The colonies images showed representative morphology of wild type and E2A KO cells. However, the cells weren't seeded at the same timepoint in the same density, so, the size of these colonies do not represent the proliferation rate of WT and E2A KO hESCs. This has now been clarified in the Figure Legend.
This clarification is missing in the Figure legend and should be included to avoid confusion.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author...

Page 19: E2A loss of function blocks hESC neural ectoderm differentiation.
This conclusion is not fully supported by the data, and should be tempered.
Overall authors' findings point to a delay rather than a block of neural differentiation. For instance:

... the results also demonstrated slightly higher expression level of OCT4 and NANOG in E2A KO cells during neural differentiation (Supplementary Figure 4A), which may partially explain the blocked neural differentiation.
This may be well explained by a delay rather than a block.

Thank you for this constructive suggestion. After carefully looking back to data in manuscript, we agree that E2A loss of function delays hESC neural ectoderm differentiation. We replaced “block” with “delay” in the manuscript, which were marked in blue. Thanks again for careful and prudent comments.

1. The colonies images showed representative morphology of wild type and E2A KO cells. However, the cells weren’t seeded at the same timepoint in the same density, so, the size of these colonies do not represent the proliferation rate of WT and E2A KO hESCs. This has now been clarified in the Figure Legend.
This clarification is missing in the Figure legend and should be included to avoid confusion.

Thanks for your suggestion. We have made clarification about morphology of wild type and E2A KO cells to avoid confusion in Figure legend, which were marked in blue.

Third decision letter

MS ID#: DEVELOP/2020/190298

MS TITLE: E2A regulates neural ectoderm fate specification in human embryonic stem cells

AUTHORS: Yang Li, Siqi Yi, Xiaotian Huang, Yuan Zhou, Shixin Zhou, Michele K Anderson, Juan Carlos Zuniga-Pflucker, and Qingxian Luan
ARTICLE TYPE: Research Article

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