Retinoic acid signaling within pancreatic endocrine progenitors regulates mouse and human β cell specification

David S. Lorberbaum, Siddharth Kishore, Carolina Rosselot, Dylan Sarbaugh, Elliott P. Brooks, Eloise Aragon, Shouhong Xuan, Olivier Simon, Debashis Ghosh, Cathy Mendelsohn, Paul Gadue and Lori Sussel
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Original submission

First decision letter

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MS TITLE: Retinoic acid signaling within pancreatic endocrine progenitors regulates mouse and human islet cell specification

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

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

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.

Reviewer 1

Advance summary and potential significance to field

In this study Lorberbaum et al reports the involvement of retinoic acid (RA) signaling during endocrine subtype differentiation. Using genetic mouse models and a human pluripotent stem cell

differentiation system, the authors demonstrate that RA signaling is important for proper beta cell specification and differentiation in mouse and human. Mechanistically, RA signaling regulates delta cell factors and likely inhibits Wnt signaling. Furthermore, they showed that impairment in this signaling during pancreas development results in expression of delta cell markers at transcriptional level in beta cells. This consequently, leads to generation of dysfunctional beta cells that are not able to properly regulate blood glucose homeostasis in adult mice. Overall the findings of this study are novel and important. A better understand of the mechanisms underlying endocrine subtype specification are urgently needed to better direct stem cells into alpha, beta and delta cell fates. Therefore, I recommend publishing this study in Development after the authors have addressed the following comments:

Comments for the author

Major points:
-The authors claimed that partial expression of delta cell genes in RARdn mutants results in the observed dysfunctional beta cell phenotype. It was suggested that upregulation of Wnt components in RARdn pancreas impairs the endocrine and beta cell specification. However, this claim is solely based on the higher expression of some Wnt components in the mutant pancreas that might not be a direct effect. Some RA associated genes are also dysregulated. To dissect how disturbed RA signaling in endocrine progenitors leads to the observed gene expression changes, it would be useful to perform a bioinfomatic analysis for RAR cis-regulatory binding in the differential expressed genes to see which are potential direct target genes. Can the authors suggest a regulatory model of beta/delta fate choice in Ngn3 progenitors depending on these direct RAR target genes? Is it RAR and Wnt/beta-catenin target gene activation/repression of let's say Hhex (important for delta cell fate) which drives beta vs delta fate allocation?

Other points:
-As the RARdn mutation mainly impair beta cell formation and does not affect other endocrine cell formation the title might be more specific?
-Figure 1F; the merge pictures are not informative. I would recommend to remove the DAPI channel from the merge picture.
-Figure 1; the authors showed that the number of insulin positive cells are reduced in RARdn pancreas while the number of other endocrine cells was not changed. In addition, they showed the appearance of a population of cell expressing Sst at transcriptional but not protein levels. It is necessary to quantify the number of this later population to indicate whether this population are quantitatively the exact replacement for the lost beta cells.
-Whenever possible, the graphs in Figure 3 could include a representative picture.
-How do the authors explain the reduced beta cell phenotype in human system, while they did not observe any increase in delta cell program?

Reviewer 2

Advance summary and potential significance to field

Retinoic Acid (RA) signaling has long been established in model systems to be indispensable for the specification of pancreatic progenitors. These findings have been repeatedly substantiated in vitro: All modern directed differentiation protocols for the production of human pluripotent stem cell (hPSC)-derived pancreatic progenitors require RA supplementation. However, the role of RA between the specification of the pancreatic progenitor and the fully functional beta cell has not been explored to date experimentally. To interrogate RA function beyond the Pdx1+ pancreatic progenitor, the authors developed a number of mouse genetic models combining previously described Cre drivers in combination with a previously described R26R targeted allele that conditionally expresses a dominant negative version of the Retinoic Acid Receptor (RARdn) (Rosselot et al., 2010). They additionally investigate the role of RA signaling in well-established platforms for the production of beta-like cells from the direct differentiation of hPSCs.

Supplementary Figure 1 establishes the validity of the RARdn model using well-established Cre deleter strains (e.g., Pdx1:Cre and Pdx1:creEsr1) that express Cre recombinase soon after the acquisition of `pancreatic progenitor fate. Both genetic combinations expectedly result in

pancreatic hypoplasia and consequently fewer hormone-containing cells. In Figure 1, the authors employ an Ngn3:Cre deleter line that activates expression of RARdn specifically in endocrine progenitors. This results in a decrease in Ins-positive cells at embryonic day (E) 16.5, with a curious, though modest, three-fold increase in Sst mRNA transcripts (Fig. 1E) but not Sst protein by IF (Fig. 1F). Next, the authors turn their attention to day 2 neonates, and find similar results—that is, reduced Ins2 mRNA transcripts alongside slightly higher blood glucose levels (Fig. 2D), which suggest that those beta cells that perdure are dysfunctional. (This Reviewer presumes that these animals live a long life.)
Next, the authors model their mouse results using the directed differentiation of hPSC into beta-like cells using established protocols that universally incorporate RA (at different concentrations) at requisite stages. In this series of experiments, RA is not supplemented to the differentiation medium while a potent small molecule inhibitor is added. The authors argue that between stages 4 and 5 (Fig. 3A), there is no significant impact on the emergence of PDX1+:NKX6-1+ endocrine progenitors in the presence of the RAi (Fig. 3C). This Reviewer notes that most modern beta cell differentiation protocols yield significantly greater numbers of PDX1+:NKX6-1+ cells (e.g., the most recent iterations of the Rezania and Hebrok protocols cf. Fig. 3C).
Modifying the differentiation protocol in this fashion has a tiny, borderline significant effect on INSULIN gene expression (Fig. 3D), and consequently an insignificant impact on the number of C-PEPTIDE-positive cells (Fig. 3G). Most significantly, however, in this Reviewer's opinion, the authors return to the mouse model and evaluate the consequences of RA inhibition on pancreatic development using comparative RNA seq at E16.5.
These data strongly argue that RA signaling is necessary to repress the delta-cell transcriptional program evidence for which emerged in Figure 1 with conventional qRT-PCR analyses (Fig. 1E). Employing GO analysis the most significantly changed genes segregate to the WNT signaling pathway, which is a novel finding, but in line with recent work from Sharon et al. (2019).

In summary, this study indisputably demonstrates genetically that RA signaling participates in the beta cell lineage beyond its perhaps most noteworthy function in assigning pancreatic fate (Pdx1+) in the first instance. Admittedly, the effects are modest at best, and the results presented in this manuscript do "rub up" against prior work (Arregi et al., (2016) and Ostrom et al. (2008)), which perhaps diminish novelty and invoke an "incremental" versus "significant" advance. In this Reviewer's opinion the present study nevertheless merits further consideration at Development.

Comments for the author

Please see above.

First revision

Author response to reviewers' comments

Response to reviewers (also available in the supplementary information)

We appreciate the reviewers' positive and constructive feedback. Please find below a complete list of our detailed responses to the reviewers' comments.

Reviewer 1
Major Points:

"…To dissect how disturbed RA signaling in endocrine progenitors leads to the observed gene expression changes, it would be useful to perform a bioinfomatic analysis for RAR cis-regulatory binding in the differential expressed genes to see which are potential direct target genes. Can the authors suggest a regulatory model of beta/delta fate choice in Ngn3 progenitors depending on these direct RAR target genes? Is it RAR and Wnt/beta-catenin target gene activation/repression of let's say Hhex (important for delta cell fate) which drives beta vs delta fate allocation?"

As suggested, we have conducted a bioinformatic analysis to identify putative RARα binding sites within the promoter proximal regions (defined as < 500bp from the transcriptional start site) of all significantly changed genes in the RARdnflox/flox; Neurog3:cre e16.5 transcriptome analysis. This identified two putative direct RA targets in the WNT pathway, Ror1 and Smo. The remaining differentially regulated WNT components did not contain predicted motifs in their promoter regions, however, they could be regulated through enhancer elements. Unfortunately, relevant distal enhancers are difficult to identify computationally, and currently good ChIP antibodies for RAR proteins do not exist. We have developed a mycRARalpha knockin mouse for precisely this reason, but these experiments are on hold until we regain lab access. We have added the computational analysis to the manuscript and indicate there could be both direct and indirect regulation of the WNT pathway by RA signaling. We did not find any predicted binding sites in the promoters of the upregulated delta cell genes; however, there is evidence that Hhex is regulated, at least indirectly by Wnt/beta-catenin. We have added this information to the manuscript. Based on this new analysis, which is included in a new Table S2, we more clearly discuss the possibility that RA directly and indirectly regulates WNT components, which could contribute to the upregulation of the delta cell gene program in lines 217-227.

Minor Points:

“As the RARdn mutation mainly impair beta cell formation and does not affect other endocrine cell formation, the title might be more specific?

We have updated the title from “Retinoic acid signaling within pancreatic endocrine progenitors regulates mouse and human islet cell specification” to “Retinoic acid signaling within pancreatic endocrine progenitors regulates mouse and human beta cell specification”.

“Figure 1F; the merge pictures are not informative. I would recommend to remove the DAPI channel from the merge picture.”

The DAPI channel has been removed from the relevant merged panels of Figure 1G (formerly Figure 1F) to improve visualization.

“Figure 1; the authors showed that the number of insulin positive cells are reduced in RARdn pancreas while the number of other endocrine cells was not changed. In addition, they showed the appearance of a population of cell expressing Sst at transcriptional but not protein levels. It is necessary to quantify the number of this later population to indicate whether this population are quantitatively the exact replacement for the lost beta cells.”

We have quantified the percentage of Sst+/SST- cells (relative to all Sst+ cells) in e16.5 RARdnflox/flox; Neurog3:cre pancreata, which demonstrates that while this population of cells is significantly increased in the mutants, there are not enough of these cells to be an exact replacement for the lost α cells in these animals. These data are presented as updated Figure 1F and the manuscript now includes discussion of these new data in lines 132-136. We have also included additional discussion in lines 242-259 to clarify that we believe the α cell phenotype is at least partially separable from the upregulation of the delta cell transcriptional program. This is supported by the results in the human beta cell differentiation experiments demonstrating a reduction of INS, but no change in SST RNA expression.

“Whenever possible, the graphs in Figure 3 could include a representative picture”

These graphs represent the FACs plots and not immunofluorescence images. We are happy to include the FACs plots as supplemental files, but they do not add new information.

“How do the authors explain the reduced beta cell phenotype in human system, while they did not observe any increase in delta cell program?”

We have expanded our discussion of this point. We interpret the absence of an altered δ cell program in the human differentiations as additional evidence that inhibition of RA causes potentially two independent defects during endocrine progenitor specification. One is an

impairment in beta cell differentiation and the other is a de-repression of the delta cell gene program. In addition, we acknowledge that the in vitro hPSC model is engineered to specifically generate beta cells and is not optimized for delta cell specification, which also may influence our ability to skew the fate choice more towards delta cells in this in vitro system. This is more thoroughly discussed in lines 242-259 of the updated manuscript.

Reviewer 2

Reviewer 2 provided a small number of comments that we have addressed.

This Reviewer presumes that these animals live a long life.
We have added that the mice live a normal lifespan in the text, lines 153-154.

The results presented in this manuscript do "rub up" against prior work (Arregi et al., (2016) and Ostrom et al. (2008)), which perhaps diminish novelty and invoke an "incremental" versus "significant" advance. In this Reviewer's opinion the present study nevertheless merits further consideration at Development.

Although we include some data that replicates the Arregi and Ostrom studies, this was only to validate our new experimental system. It should be noted that both of these previous studies use mutations that affect the first and earliest wave of endocrine differentiation using either Foxa2-cre or Pdx1-promoter elements, and do not specifically target the later stages of endocrine cell specification as we show in our study. The Ostrom study does suggest that RA is needed at different stages of beta cell differentiation using an ex vivo approach, but the analysis is perfunctory and does not include any mechanistic information. As the reviewer indicates, we believe our study provides sufficient novel information with our in vivo murine analyses that resulted in the identification of novel stage-specific RA-WNT interactions that contribute to a loss of beta cells and a disruption to the beta cell transcriptional program. We agree with the reviewer that these new advances merit publication at Development.

Second decision letter

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ARTICLE TYPE: Research Report

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

Reviewer 1

Advance summary and potential significance to field

All my comments have been addressed during the revision.

Comments for the author

Minor comment:I did not know the Smoothened is a Wnt pathway component, but rather thought it is a central 7TM receptor essential for Shh signal transduction. But very likely these pathways cross-regulate each other.

Reviewer 2

Advance summary and potential significance to field

The authors have responded satisfactorily to this Reviewer's initial comments and suggestions. The paper provides new insight into the role of retinoic acid signaling in pancreatic endocrine progenitors and their further differentiation into insulin-secreting cells.

Comments for the author

No further suggestions.