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

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ABSTRACT

Retinoic acid (RA) signaling is essential for multiple developmental processes, including appropriate pancreas formation from the foregut endoderm. RA is also required to generate pancreatic progenitors from human pluripotent stem cells. However, the role of RA signaling during endocrine specification has not been fully explored. In this study, we demonstrate that the disruption of RA signaling within the NEUROG3-expressing endocrine progenitor population impairs mouse β cell differentiation and induces ectopic expression of crucial δ cell genes, including somatostatin. In addition, the inhibition of the RA pathway in hESC-derived pancreatic progenitors downstream of NEUROG3 induction impacts insulin expression. We further determine that RA-mediated regulation of endocrine cell differentiation occurs through Wnt pathway components. Together, these data demonstrate the importance of RA signaling in endocrine specification and identify conserved mechanisms by which RA signaling directs pancreatic endocrine cell fate.

KEY WORDS: β cell differentiation, Retinoic acid signaling, Wnt signaling, Pancreas development, Diabetes, δ cell specification

INTRODUCTION

Cell signaling pathways are used continuously throughout development and adulthood to mediate tissue interactions and precisely control gene expression. In particular, retinoic acid (RA) signaling plays crucial roles in a wide range of developmental processes at multiple stages during embryogenesis (Ghyselinck and Duester, 2019). In vertebrates, the basic mechanisms of RA function are conserved: vitamin A is converted into RA through a series of enzymatic reactions and enters the nucleus to interact with the transcriptional effectors of the pathway, RA receptor (RAR) and retinoid X receptor (RXR), as well as co-activators and co-repressors to regulate context-specific target genes (Fig. S1A).

In the pancreas, RA signaling is necessary for the onset of pancreagenesis; previous studies have demonstrated that the inhibition of this pathway leads to pancreas agenesis (Arregui et al., 2016; Oström et al., 2008; Molotkov et al., 2005; Martin et al., 2005; Stafford and Prince, 2002). Based on these studies, exogenous RA is included in human pluripotent stem cell (hPSC) β-like cell differentiation protocols to facilitate the earliest stages of pancreas development. Although high levels of RA are crucial for initial pancreas specification, RA has been shown to subsequently inhibit endocrine differentiation in several model systems (Cardenas-Diaz et al., 2019; Pagliuca et al., 2014; Huang et al., 2014; Rezania et al., 2012; Rovini et al., 2011); therefore, most protocols use progressively lower doses of RA after pancreatic endoderm specification. Despite the reduction or exclusion of exogenous sources of RA from endocrine differentiation media at these later differentiation stages, it has been shown that a cell-autonomous source of RA might still exist (Huang et al., 2014). This is also supported by gene expression analyses in mice demonstrating the presence of many key RA pathway genes in the embryonic pancreas (Krentz et al., 2018). The function of RA signaling during vertebrate islet endocrine cell differentiation, however, has not been fully explored.

To define the role of RA signaling during pancreas endocrine development, we inhibited RA signaling specifically in endocrine progenitors by expressing the RARdn under the regulation of the Neurog3:cre allele. These studies demonstrated that RA signaling is required for both β cell specification and the inhibition of δ cell gene transcripts, including Hhex, Rbp4 and Sat. The inhibition of RA in the NEUROG3δ endocrine progenitor population resulted in reduced numbers of insulin-producing β cells, and contributed to impaired blood glucose regulation in postnatal and adult mice. Similarly, the chemical inhibition of RA specifically after NEUROG3 induction in hPSC β-like cell differentiations decreased insulin production. At the molecular level, we demonstrate that RA-mediated repression of Wnt signaling allows for proper endocrine cell differentiation. Together, these results establish the importance of RA signaling in the endocrine progenitor population for appropriate mouse and human β cell specification.

RESULTS AND DISCUSSION

The RARdn efficiently disrupts pancreas development in mice

To disrupt RA signaling in a cell-specific manner, we used the previously described dominant-negative human RAR allele that had been inserted into the R26R locus downstream of a flox-stop-flox cassette (RARdnlox; Fig. S1B; Rosselot et al., 2010). To validate that the RARdn allele functioned appropriately, we generated
**RARdnflox/flox; Pdx1:Cre** mice to broadly inhibit RA signaling in pancreatic progenitors. Similar to previous studies (Öström et al., 2008), the disruption of RA signaling in all pancreatic progenitors led to the formation of a smaller pancreas that contained fewer β [insulin (INS)], α [glucagon (GCG)] and δ cells [somatostatin (SST)] at embryonic day (E)16.5 and E18.5 (Fig. S1C). The disruption of RA signaling using the tamoxifen-inducible Pdx1:creEr1 allele (Gu et al., 2002) at E9.5, a slightly later stage of development after the pancreatic progenitor population has been established, also resulted in the formation of a smaller pancreas and a significant reduction in islet cluster formation, and fewer hormone-producing cells (Fig. S1D-G).

**Endocrine-specific RA inhibition impairs the formation of insulin-producing β cells and causes ectopic somatostatin RNA expression**

To determine whether RA signaling was also required during endocrine cell differentiation downstream of endocrine progenitor formation, we disrupted RA signaling specifically within the NEUROG3 endocrine progenitor population using RARdnflox/flox; Neurog3:cre mice. Remarkably, this disruption of RA signaling in the endocrine progenitor population resulted in significantly fewer β cells as early as E16.5, without notable changes in the other endocrine cell types (Fig. 1A,B). The timing of reduced β cell numbers, combined with no apparent changes in β cell death or proliferation, suggests the inhibition of RA signaling in endocrine progenitor cells impairs β cell differentiation (Fig. 1C,D).

Consistent with the reduced number of β cells in the RARdnflox/flox; Neurog3:cre mice, there was also a reduction of INS1 and INS2 RNA expression (Fig. 1E). Interestingly, however, there was also a significant increase in SST RNA that did not correspond to an increase in δ cell numbers (Fig. 1E compared with Fig. 1B). To determine the explanation for discordant expression between SST RNA and δ cell numbers, we performed RNAscope combined with immunofluorescence on E16.5 pancreatic tissue sections. This analysis verified an increase in cells expressing SST RNA, many of which were not SST-producing δ cells (Fig. 1F,G, all arrows). SST transcripts could be detected in other endocrine cell types, including insulin-producing β cells (Fig. 1G, yellow arrows). We also observed an additional class of SST+/δ cells that did not co-express INS, SST or GCG (Fig. 1G, white arrows), which could represent a population of β cells that no longer express INS. However, the observed percentage of SST+/SST− cells did not correlate with the more substantial decrease in δ cell numbers in RA mutants (Fig. 1B,F), suggesting the β cell loss is at least partially independent of the upregulation of the δ cell transcriptional program.

**Postnatal hormone expression and blood glucose homeostasis are disrupted in RA mutants**

To determine whether the endocrine cell defects persisted postnatally, we assessed hormone expression in postnatal day (P)2 mice. Consistent with the defects observed at E16.5, the reduction in the number of INS+ β cells and ins transcripts was maintained in neonates, and was again accompanied by an increase in SST RNA expression without an impact on δ cell numbers (Fig. 2A-C). Furthermore, the RARdnflox/flox; Neurog3:cre neonatal mice were overtly hyperglycemic compared with littermate Neurog3:cre controls (Fig. 2D). Although there was a significant reduction in β cell numbers, the observed decrease is not usually sufficient to cause hyperglycemia (Bonner-Weir et al., 1983; Yasugi et al., 1976), suggesting that the remaining β cells in RARdnflox/flox; Neurog3:cre mice are dysfunctional, which could at least partially be explained by the ectopic expression of δ cell transcripts within the INS+ β cell population. This is also consistent with the glucose intolerance phenotype observed when RA signaling was specifically inhibited in adult mouse β cells (Brun et al., 2015). RARdnflox/flox; Neurog3:cre mice had a normal lifespan; however, impaired glucose homeostasis persisted into adulthood (Fig. 2E,F).

**RA signaling regulates human β cell differentiation after pancreas progenitor specification**

To determine whether the role of RA signaling in murine pancreatic endocrine specification was conserved during human β cell differentiation, we inhibited RA signaling at stage 4 [pancreatic progenitor stage 1 (PP1) day 11] of the human β cell differentiation protocol (Fig. 3A) (Cardenas-Diaz et al., 2019; Tyaboonchai et al., 2017). The differentiation of human pluripotent stem cells (hPSCs) into β-like cells requires the addition of RA from stages 1 to 2 (days 3 to 8) to generate PDX1-expressing posterior foregut-like cells; as well as between stages 2 and 5 (days 8 to 13), during which time NEUROG3 begins to be expressed (Fig. 3A,B). To simulate our murine *in vivo* experiments, we inhibited RA signaling after NEUROG3 induction by excluding exogenous RA from the media starting at stage 4 (PP1, day 11) and adding the high-affinity pan-RAR inhibitor AGN193108 to the culture media (RAi, Fig. 3A,B) (Johnson et al., 1999). To ensure that we had not altered pancreatic progenitor differentiation, we confirmed the presence of PDX1/NKX6.1 double-positive cells at day 13, 2 days after treatment with RAi (Fig. 3C). Following an additional 2 weeks of culture in RAi-supplemented media to inhibit any potential role of autocrine RA signaling during endocrine specification, we observed a significant decrease in INS RNA, with no statistically significant changes in either GCG or SST RNA expression (Fig. 3D,F). Analysis of endocrine cell numbers at day 28 showed no significant effect on GCG+ or SST+ cells, but there was a decrease in C-PeptidE+ cells, although this difference was not significant (Fig. 3G-I). These findings are in contrast to a report by Huang et al. (2014) that demonstrated that RA inhibition in human endocrine cell differentiation led to increased INS, GCG and SST. These differences might be due to the method of RA inhibition, as Huang et al. (2014) employed an ALDH inhibitor that acts upstream of RA and does not inhibit all ALDH enzymes, raising the possibility that RA signaling could continue via other ALDH homologs (Morgan et al., 2015; Dueter, 2001). Inhibition of the RA pathway at the RAR/RRXR level effectively avoids potential compensatory mechanisms that could reduce the efficacy of upstream inhibitors. Overall, our results suggest that, in humans, RA signaling is important for achieving optimal INS production and, to a lesser extent, β cell differentiation (Fig. 3D,G compared with Fig. 2A,B and 1B,E).

**Wnt signaling is derepressed in RARdnflox/flox; Neurog3:cre mutants during murine endocrine specification**

To identify pathways regulated by RA signaling in the endocrine progenitor lineage, we performed transcriptome analysis of whole-mouse pancreata at E16.5 and found 1392 significantly changed genes (padj≤0.05) in RARdnflox/flox; Neurog3:cre mutant mice, including Ins1, Ins2 and Sst (Fig. 4A,B, Table S1). In addition to the increase in Sst transcript, we also identified several other upregulated δ cell genes, including Hhex, Rhp4 and Crhr2 (Fig. 4C) (DiGruccio et al., 2016), suggesting that RA signaling in endocrine progenitors is necessary to repress ectopic expression of the δ cell transcriptional program. Although there were also changes in several RA-associated genes, classic RA targets, such as HoxA1 (Marshall et al., 1996) and Cyp26A1 (Loudig et al., 2000),
that have been described in other developmental contexts, were not significantly altered (Fig. 4A,B, Table S1). This is probably because of the absence of these genes in the NEUROG3+ endocrine progenitor population (Krentz et al., 2018), and demonstrates that RA targets are highly context dependent. Interestingly, we did observe a modest reduction ($P=0.057$, $P_{adj}=0.253$) in $Mnx1$, a known RA-regulated pancreatic development factor (Dalgin et al., 2011). Endocrine deletion of $Mnx1$ resulted in reduced $\beta$ cells and...
Fig. 1. Endocrine-specific RA inhibition disrupts β cell development by E16.5 and increases Sst transcript expression. (A) Representative immunofluorescence images of E16.5 INS, SST, GCG and GHRL in RARdnflox/flox; Neurog3:cre mutants. (B) Quantification of A (n=4 control, n=5 mutant; statistical analysis was completed for multiple t-tests using the Holm–Sidak method to correct for multiple comparisons; *Padj<0.05 is significant). (C) TUNEL+INS staining at E16.5. DNase1(+) sample is a positive control (n=3). (D) β cell proliferation reported as a percentage of proliferating versus non-proliferating β cells (n=3, statistical analysis was performed using an unpaired parametric Student’s t-test; P=0.05, not significant). (E) RT-qPCR gene expression analysis of E16.5 whole pancreata from RARdnflox/flox; Neurog3:cre mutants (n=3, statistical analysis was completed by the Benjamini, Krieger and Yekutieli two-stage step method to correct for multiple comparisons using Student’s t-tests; *P<0.05. **P<0.005 are significant). (F) Quantification of the percentage of cells expressing Sst RNA but not SST protein (relative to all Sst RNA+ cells) in Neurog3:cre alone or RARdnflox/flox; Neurog3:cre mutants at E16.5 (n=3, statistical analysis was completed by an unpaired parametric Student’s t-test; *P<0.05 is significant). Representative images can be seen in Fig. 1G. (G) Dual RNA-protein visualization using RNAscope and immunofluorescence analyses (n=3). White arrows indicate Sst RNA+ cells, SST protein+ cells; yellow arrows indicate Sst RNA−/SST protein+ cells. All n values represent biological replicates. Data are mean±s.d. ns, not significant.

increased δ and α cells (Pan et al., 2015). Although we did not observe an α cell phenotype in RARdnflox/flox; Neurog3:cre mutants, this may be because of the lesser reduction of Mnx1 expression. To better define the molecular mechanisms by which RA signaling in the murine endocrine lineage affects the development of hormone-producing cells, we completed a gene ontology (GO) term analysis (Mi et al., 2019) of significantly changed genes at E16.5. These GO terms revealed that Wnt signaling components were significantly affected by RA inhibition in NEUROG3+ mouse and human endocrine cells. Interestingly, we did not observe an increase in SST transcript expression in the human β cell differentiation platform. This could be because of the fact that the in vitro hPSC model has been engineered to optimally generate β cells, rather than the other endocrine cells. Alternatively, the unperturbed human SST RNA levels could suggest that the disruption of β cell differentiation and the upregulation of the δ cell transcriptional program are separable events, as indicated by the lack of direct correlation between the two phenotypes in the RARdnflox/flox; Neurog3:cre mutants (Fig. 1B,F,G), with only the RA regulation of β cell differentiation being conserved in humans. Finally, it remains possible that this discrepancy between mice and humans could be attributed to the artificial nature of the in vitro β cell differentiation system.

The ability to generate β cells in vitro from hPSCs has greatly improved during the last decade; however, despite significant progress, direct differentiation of functionally mature human β cells in culture remains a challenge. As these differentiation protocols have been extensively informed by developmental studies in rodent models, we examined RA signaling, a crucial mediator of early pancreas formation, in the differentiation of pancreatic endocrine cells in both mice and in the differentiation of human β-like cells. Inhibition of the RA pathway in NEUROG3+ mouse and human pancreatic endocrine progenitors resulted in defective β cell production and Ins expression, phenotypes that are at least partially due to the derepression of the Wnt signaling pathway. This study also identified a novel role for RA signaling in the repression of δ cell gene expression. Taken together, these data demonstrate a conserved and previously unappreciated role of RA signaling during pancreatic endocrine development. These results will inform β cell differentiation conditions to facilitate the generation of functionally mature human β cells in vitro, and advance our understanding of pancreatic endocrine development.

MATERIALS AND METHODS

Animal models

Mice were maintained under protocol 00045 as approved by the University of Colorado Denver Institutional Animal Care and Use Committee. All animals were bred on a mixed C57Bl6/129SV genetic background and group housed by sex with up to five siblings in each cage with constant access to food and water at room temperature (22°C). Cages were changed once every 2 weeks and regularly monitored for virus and parasite infection, which were never present during this study. Euthanasia was performed by CO2 inhalation and by cervical dislocation after asphyxiation as a secondary method of euthanasia. For timed matings, the identification of a vaginal plug in the morning was defined as E0.5. The male was removed from the cage and the female was monitored to ensure pregnancy until sacrifice. All mice and embryos were genotyped with primers listed in Table S4 using standard PCR with Go Taq DNA Polymerase MasterMix (Promega). All mice used are available from Jackson Laboratories: Tg(Neurog3-cre)C1Able1/J (005667; RRID:IMSR_JAX:005667), B6.FVB-Tg(Pdx1-cre)6Tuv/J (014647; RRID:IMSR_JAX:014647), Gt(Rosa26Sor/Sm22Cre)Kve7/HsvJ (029852; RRID:IMSR_JAX:029852) and Tg(Pdx1-cre;Esr1*)#Dam1/J (024968; RRID:IMSR_JAX:024968).

hPSC culture

All hPSC studies were performed using the authenticated H1 human embryonic stem cell line (hESCs) line (which is routinely checked for contamination) (Thomson et al., 1998). hPSC cell lines were cultured on 0.1% gelatin and irradiated mouse embryonic fibroblast feeder cells in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 supplemented with 2 mM of glutamine, and 15% KnockOut Serum Replacement [1× non-essential amino acids, penicillin/streptomycin, 0.1 mM β-mercaptoethanol and 10 ng/ml of basic fibroblast growth factor (bFGF)]. This hPSC medium was changed every day. Cells were passaged when they reached 80% confluence, approximately every 4 days, using TrypLE at a 1:6 ratio. In all hPSC cultures, 5 μM Rho-associated protein kinase (ROCK) inhibitor
Y-27632 (Selleck Chemicals, S1049) was only added into the culture media for ~18 h when passaging or thawing hPSCs.

**Embryoid body generation**

hPSCs were incubated with Accutase solution for 7 min at 37°C, transferred to a 50 ml Falcon tube and washed twice using 40 ml of DMEM-F12. Then, 5.5 million cells were resuspended in 5 ml of hPSC medium with 1 μM of ROCK inhibitor and plated in one well of an ultra-low attachment six-well cell culture plate. The plate was placed on a 100 rpm orbital shaker inside a 37°C incubator with 5% CO2. The cells form embryoid bodies overnight and were fed for 2 days using hPSC medium. After 2 days, the medium was removed and replaced with pancreatic differentiation medium for day 0.

**Pancreatic differentiation from hPSCs**

Pancreas differentiation was initiated on the hPSC embryoid bodies with day 0 media containing Roswell Park Memorial Institute growth media (RPMI).
supplemented with 3 μM Chir99021 and 100 μg/ml activin A. On day 1, the media were changed to RPMI with 100 μg/ml activin A, 0.3 μM Chir99021 and 5 μg/ml bFGF. On day 2, the media were changed to serum-free defined medium with 100 μg/ml activin A. From days 3 to 5, cells were fed with DMEM-F12 containing 0.25 mM ascorbic acid, 50 ng/ml FGF7 and 1.25 μM IWP2. Day 6-8 media contained DMEM high glucose (5 g/l) supplemented with 1:100 B27 without RA, 1× GlutaMAX, 0.25 mM ascorbic acid, 100 nM LDN-193189 and 500 nM phorbol. The media for days 9 to 11 consisted of DMEM high glucose (5 g/l) supplemented with 20 mM glucose, 2% fetal bovine serum (FBS), 1× GlutaMAX, 1:200 ITS-X, 10 μg/ml heparin, 10 μM zinc sulfate, 0.5 μM SANT-1, 0.05 μM RA, 200 nM LDN-193189, 1 μM T3 and 10 μM ALK5i II. For experiments with the pan-RAR inhibitor, 2 μM of AGN193108 was added to the media from day 11 onwards. Cells were harvested on day 13 for flow cytometry analysis and RNA collection using 0.25% Trypsin for 5 min. From day 13 to day 28, cells were fed every other day with media that contained MCDB131 with 20 mM glucose, 2% FBS, 1× GlutaMAX, 1:200 ITS-X, 10 μg/ml heparin, 10 μM zinc sulfate, 200 nM LDN-193189, 1 μM T3, 10 μM ALK5i II and 100 nM GSIS XX. Cells were harvested on day 28 (β-like stage) for flow cytometry analysis and RNA collection.

Fig. 3. Human β cell differentiation requires RA signaling after posterior foregut formation. (A) Summary of human β cell differentiations with RA addition in red. Highlighted area (yellow) indicates when exogenous RA was removed from the media and RA inhibitor (RAi) was added. (B) RT-qPCR gene expression analysis of NEUROG3 expression, normalized to TBP. (C) Percentage of PDX1+/NKX6.1* cells at stage 5 (ST5) of the differentiation with and without RAi treatment (n=3 per treatment, statistical analysis was performed using an unpaired parametric Student’s t-test method; P>0.05 not significant). (D-F) RT-qPCR gene expression analysis of INS, GCG and SST expression at day 28, normalized to TBP for total RNA and CHGB for endocrine cells to control for differentiation efficiency (n=3 per treatment, statistical analysis was performed using a paired parametric Student’s t-test method; *P<0.05 is significant). (G-I) Percentage of cells positive for C-peptide, glucagon and somatostatin by flow cytometric analysis (n=3 per treatment, statistical analysis was performed using an unpaired parametric Student’s t-test method; P>0.05, not significant). All n values represent biological replicates. ns, not significant. Data are mean±s.d.
**Immunofluorescence**

Tissues were fixed in 4% paraformaldehyde (PFA) for 4 h at 4°C, washed in ice-cold 1× PBS, and incubated in 30% sucrose overnight at 4°C. The next day, samples were incubated in 50% optimum cutting temperature compound (OCT) (in sucrose) for 15 min, 100% OCT for 15 min, and frozen on dry ice. Blocks were sectioned (10-12 μm) for immunofluorescence. All antibodies used in this study are listed in Table S3. In brief, samples were blocked in 2% normal donkey serum for 30 min and incubated with primary antibodies overnight at 4°C. The next day, they were washed in PBS-T and incubated in secondary antibodies for 1-3 h, washed in PBS-T, incubated in DAPI for 10 min, washed in PBS-T and mounted using hard-set Vectashield. For prolonged storage for more than 2 weeks, nail polish was used to seal the edges of coverslips and slides were stored at 4°C. For TUNEL staining, the TMR red in situ Cell Death Detection Kit (Millipore, 12156792910) was used following the manufacturer’s instructions, prior to antibody staining for insulin.

**Morphometric analysis**

For protein quantification, the pancreas was completely sectioned and every 10th slide was stained and quantified (five slides for E16.5 and ten slides for P2). All E16.5 embryos were sectioned without further dissection of the

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**Fig. 4. RA signaling inhibits the Wnt pathway to promote β cell specification and inhibit the δ cell transcriptional program.** (A) Volcano plot of differentially expressed genes from whole-transcriptome analysis by RNA-seq of RARdnfflox; Neurog3:cre mutants (n=3 biological replicates). (B) A selection of genes from A with Log2 fold change and P-adjusted value (Padj<0.05 is significant). (C) A selection of upregulated δ cell genes compared with Ins1/2 (Crhr2, Padj=0.053). (D) Significantly upregulated GO terms via PANTHER analysis of genes reported in A and Table S1. (E) Significantly enriched Wnt signaling components (GO:0017147). (F) Model demonstrating that during endocrine differentiation, RA signaling represses Wnt to promote β cell differentiation and repress δ-cell genes.
pancreas. At P2, the pancreas, stomach, duodenum and intestines were isolated. Images were obtained at 20× magnification using a Leica DM5500 B microscope. For both stages, DAPI-positive pancreas area was measured and was not significantly different between control and mutant samples. At E16.5, individual hormone-positive cells were counted manually using Fiji or Adobe Photoshop counting tools. P2 samples were quantified by hormone-positive area/DAPI-positive area using a Matlab program to detect positively stained pixels in each fluorescent channel. Antibody information can be found in Table S3. All quantification was performed blinded to genotype.

**RNA extraction and RT-qPCR in mice**

Whole pancreata were collected and total RNA extractions were completed using the RNA Easy Mini Kit and eluted in 30 μl RNase-free H2O. RNA (200 ng) was used to generate cDNA via the iScript cDNA Synthesis Kit (Bio-Rad). The cDNA was then diluted in RNase free H2O to 1 ng/200 ng was used to generate cDNA via the iScript cDNA Synthesis Kit (Bio-Rad). Expression levels were normalized to Actb

**Flow cytometry**

Single cell suspensions were prepared by treating cells with 0.25% Trypsin/ EDTA for 3 to 5 min. For intracellular staining, cells were fixed with 1.6% PFA (Electron Microscopy Sciences) for 30 min at 37°C. Cells were washed, permeabilized and stained with 1× saponin buffer (BioLegend). Primary antibodies were diluted to the appropriate concentrations in 100 μl of saponin buffer and cells were stained for 30 min at room temperature. Samples were washed twice using 100 μl saponin buffer and incubated for 30 min using the appropriate secondary antibody. Following staining, cells were resuspended in fluorescence-activated cell sorting buffer (DPBS with 0.1% bovine serum albumin and 0.1% sodium azide). All samples were run on a FACS Canto II or CytoFLEX flow cytometer (Becton Dickinson) and analyzed using the FlowJo (Treestar) software program.

**RNA extraction and RT-qPCR in human cells**

Cells were lysed using the lysis buffer provided with the PureLink RNA Micro Kit (Invitrogen, 12183-016) and stored at −80°C. To harvest RNA, samples were thawed at 4°C and RNA was extracted using the PureLink RNA Nano Kit following the manufacturer’s instructions. RNAase free water (14 μl) was used to resuspend the isolated RNA. cDNA was produced using the SuperScript III First-Strand Synthesis System kit (Invitrogen). qPCR was carried out using a LightCycler 480 II with SYBR Select Master (Roche) platform that reports both P-value and adjusted value q-value, which are detailed in the figure legends. For mouse analyses, each n value represents a different animal. Analyses were performed with littermate controls, when available. Gender was not assessed for any embryonic studies but all adult mouse glucose tolerance tests were completed on male mice. The E16.5 RNA-seq experiments were analyzed by the DESeq2 platform that reports both P-value and P-adjusted, with the latter being used as a significance cutoff at Padj≤0.05 as defined by the Benjamini-Hochberg procedure, which corrects for multiple hypothesis testing.

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Huang, W., Wang, G., Delaspre, F., Viteri, M. C., Beer, R. L. and Parsons, M. J. (2002). Direct evidence for the...HUMAN DEVELOPMENT


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Data availability

Sequencing data have been deposited in GEO under accession number GSE144953. The MatLab program used for analyses of hormone expression at P2 is available upon request.

Supplementary information

Supplementary information available online at https://dev.biologists.org/lookup/doi/10.1242/dev.189977.supplemental.

References


