Murine Perinatal β-Cell Proliferation and the Differentiation of Human Stem Cell–Derived Insulin-Expressing Cells Require NEUROD1

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Inactivation of the β-cell transcription factor NEUROD1 causes diabetes in mice and humans. In this study, we uncovered novel functions of NEUROD1 during murine islet cell development and during the differentiation of human embryonic stem cells (HESCs) into insulin-producing cells. In mice, we determined that Neurod1 is required for perinatal proliferation of α- and β-cells. Surprisingly, apoptosis only makes a minor contribution to β-cell loss when *Neurod1* is deleted. Inactivation of *NEUROD1* in HESCs severely impaired their differentiation from pancreatic progenitors into insulin-expressing (HESC-β) cells; however, survival or proliferation was not affected at the time points analyzed. NEUROD1 was also required in HESC-β cells for the full activation of an essential β-cell transcription factor network. These data reveal conserved and distinct functions of NEUROD1 during mouse and human β-cell development and maturation, with important implications about the function of NEUROD1 in diabetes.

Studies in rodents have identified networks of transcription factors that are essential for the differentiation and maintenance of functionally mature pancreatic islet cells (reviewed in Romer and Sussel [1]). To date, most monogenic forms of diabetes have been linked to disruptive mutations in transcription factors essential for the development of pancreatic islet cells (2). Accordingly, homozygous and heterozygous inactivating mutations in the basic helix-loop-helix (bHLH) transcription factor NEUROD1 can cause permanent neonatal diabetes mellitus and maturity-onset diabetes of the young 6, respectively (3,4). NEUROD1 was initially identified as a transactivator of the insulin gene (5), and subsequent studies demonstrated that NEUROD1 has a broader role in regulating β-cell function through the direct activation of genes important for β-cell maturation and function (6–10).

Loss-of-function studies in mice have revealed distinct cell type– and age-dependent requirements for NEUROD1 in the development of functional α- and β-cells. During murine pancreas development, NEUROD1 is expressed by differentiating endocrine cells and is subsequently restricted to insulin-producing β-cells and a subset of glucagon-expressing α-cells (11–13). *Neurod1* knockout (KO) mice develop lethal neonatal diabetes that is at least partly due to a severe reduction in the numbers of α- and β-cells (12). Despite the transactivating functions of NEUROD1 for β-cell–specific gene expression, loss of Neurod1 in mice did not appear to affect the formation of the normal complement of insulin-expressing cells (12). Rather, the lack of α- and β-cells in *Neurod1* KO mice was reported to result from perinatal loss due to apoptosis (12). Unexpectedly, while the *Neurod1* KO phenotype suggested that NEUROD1 was a β-cell survival factor, β-cell–specific deletion of *Neurod1* did not affect β-cell numbers (11,14). However, β-cell deletion of *Neurod1* did impair the functional maturation of β-cells and blunted glucose-stimulated insulin secretion (14). Recent clinical studies as well as functional studies using the differentiation of human embryonic stem cells (HESCs) to model human pancreas development have demonstrated both conserved and divergent functions for several islet transcription factors between mice and humans (15–18). For example, NEUROG3, another bHLH transcription factor

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closely related to NEUROD1, is absolutely essential for the differentiation of endocrine cells in the mouse pancreas while only being partially required for the differentiation of human β-cells (16,17).

In this study, we revisited the embryonic phenotype of the *Neurod1* KO mice to determine whether the discrepant phenotypes associated with global versus β-cell–specific deletion of *Neurod1* were due to *1*) timing of *Neurod1* inactivation, *2*) previously unappreciated functions of NEUROD1, and/or *3*) cell nonautonomous effects. In addition, we performed detailed phenotypic analysis of *NEUROD1* deletion in mouse and human model systems to discover novel requirements for NEUROD1 during the development of murine islet cells and during the differentiation of HESC-derived insulin-producing (HESC-β) cells. We discovered that *Neurod1* KO mice develop fewer α- and β-cells due to major defects in embryonic α- and β-cell proliferation prior to any appreciable cell loss from apoptosis. Alternatively, disruption of *NEUROD1* in HESCs severely impaired the differentiation of pancreatic progenitors into HESC-β cells, without impacting proliferation or survival, revealing the importance of NEUROD1 for the differentiation of human insulin-producing cells. On the other hand, NEUROD1 appears to be essential for maintaining the maturation state of both mouse and human β-cells.

RESEARCH DESIGN AND METHODS

Mice

Details about the $Neurod1^{LacZ}$ (mouse genomics informatics [MGI]: 2385826) (19), $Neurod1^{Floxed}$ (MGI: 2385826) (20), $Neurog3^{Cre}$ (MGI: 3052639) (21), Ins^{Cre} (MGI: 2387567) (22), and $R26R^{Tomato}$ (MGI: 3809523) (23) mice and genotyping protocols used in these experiments have been previously published (14,19,21,24). All mice were maintained on a C57BL/J background. All experimental procedures and husbandry of mice were performed according to Columbia University–approved Institutional Animal Care and Use Committee protocols. Embryonic stages were counted as days after verification of insemination. Blood glucose of perinatal mice was measured using a glucometer (Freestyle Lite). Perinatal mouse studies did not consider sex as a factor in the statistical analysis of the data.

Culturing of HESCs

The HESCs used in these experiments are a National Institutes of Health–approved male line (MEL-1) that was previously modified to have a GFP expression cassette knocked into the insulin locus $INS^{GFP/wt}$ (research resource identifier [RRID] MEL-1 INSGFP/wt) (25). Cells of passage number 13–20 were maintained as previously described (26).

CRISPR Mutagenesis of HESCs

To target frameshift indels to the bHLH coding sequence of *NEUROD1*, a single guide (sg)RNA 5′-ACGCATGAA GGCTAACGCCC-3′ was designed with minimal off targets using the online CRISPR design tool (crispr.mit .edu). An sgRNA expression fragment was designed and synthesized using a previously published protocol (27). HESCs were nucleofected with this sgRNA fragment along with a Cas9-GFP plasmid (catalog no. 44719; Addgene) using a Human Stem Cell H9 Nucleofector kit (catalog no. VPH-5012; Lonza). GFP^+ cells were sorted 48 h post-nucleofection using a BD FACSAria II cell sorter and plated at 5,000 cells per 10-cm dish. Individual colonies were isolated by microdissection. QuickExtract DNA Extraction Solution (catalog no. QE09050; Epicentre) was used to isolate genomic DNA. Clonal lines were genotyped by Sanger sequencing of PCR products amplified using the following primers: forward 5′-CCAAGCTGGACAGAC-GAGTG-3′ and reverse 5′-AGTGTCGCTGCAGGATAGTG-3′. The genotypes of clonal lines with indels were then verified by cloning of PCR products using a TOPO TA cloning kit (catalog no. K4575J10; Invitrogen) followed by Sanger sequencing of at least four individual bacterial clones. Karyotyping was performed by Cell Line Genetics.

Differentiation of HESC-β Cells

After CRISPR mutagenesis, four clonal HESC lines homozygous with NEUROD1 bHLH–disrupting indels and four NEUROD1 wild-type control lines were differentiated into HESC-β cells using a published protocol (26).

Measurements of Insulin Secretion and Content

Insulin secretion and content were measured using 20 HESC-β cell clusters. The clusters were first rinsed in 0.5 mL Krebs-Ringer buffer (KRB) and then preincubated for 90 min in 0.2 mL 2.8 mmol/L glucose KRB inside a 1.5-mL tube with the cap open in a 37°C cell culture incubator. The clusters were then sequentially incubated in 200 μL of 2.8 mmol/L glucose KRB and then with 20 mmol/L glucose KRB for 30 min each. The insulin secreted by the clusters was measured using samples of media after each incubation. Insulin content was extracted by sonicating cells in 50 μL Tris EDTA buffer with 0.1% SDS followed by mixing the lysate in a 1:3 ratio with 0.18 mol/L HCl 96% ethanol. Insulin secretion and content were measured using a human insulin ELISA kit (catalog no. 10-1113-01; Mercodia).

Immunofluorescence Analysis

HESC-derived cells and frozen sections of mouse pancreas were prepared and stained for immunofluorescence (IF) analysis using previously described protocols (26,28). Primary and secondary antibodies are listed in Supplementary Table 1. Apoptotic cells were labeled using a TUNEL Assay Apoptosis Detection Kit (catalog no. 30064; Biotium). Entire sections of mouse pancreas or day 27 HESC clusters were imaged using a Zeiss LSM 710 confocal scope. HESC day 12 pancreatic progenitors were imaged using an Olympus IX53 fluorescent microscope. Fluorescently labeled cells and pancreas area were manually quantified using ImageJ software. Cells were quantified on at least three sections of mouse pancreas spaced 200 μm apart and on four sections of different day 27 HESC–derived clusters.

Pancreatic progenitors were quantified on four random images in the well.

Flow Cytometry Analysis

Cells were prepared and stained for flow cytometry analysis using previously described protocols (26). Primary and secondary antibodies used for flow cytometry analysis are listed in Supplementary Table 1. To measure 5-ethynyl-2′-deoxyuridine (EDU) incorporation, cells were incubated in media with 10 μmol/L EDU 4 h prior to their dissociation. A Click-iT EDU Cell Proliferation Kit (catalog no. C10338; Thermo Fisher Scientific) was used to stain cells that had incorporated EDU. Automated cell counting was performed using a BD Biosciences LSRFortessa cytometer, and data were analyzed using FCS Express 6 software.

RNA Sequencing

Single-cell suspensions of dissected embryonic day 17 (E17) mouse pancreas and HESC-β cell clusters were prepared by a 30-min 37°C digestion with Accutase (catalog no. A6964; Sigma-Aldrich) supplemented with 25 μg/mL DNASE1 (catalog no. 10104159001; Sigma-Aldrich) and TrypLE Express (catalog no. 12605036; Gibco) for 10 min at room temperature, respectively, followed by dissociation with pipetting. The mouse pancreas and HESC-β cell digestions were quenched by adding 2- and 10-fold the volume of RPMI 1640 media (catalog no. 11875119; Gibco) supplemented with 10% FBS (catalog no. 900-108; Gemini Bioproducts) and 10 μmol/L Y27632 rock inhibitor (catalog no. S1049; Selleckchem), respectively, filtered through a 40-μm mesh, pelleted by centrifugation, and suspended in quench media. Human INS-GFP^{+} and E17 mouse *Neurog3:Cre; R26R:Tomato*$^{+}$ lineage–labeled cells were sorted using a BD FACSAria II cell sorter. Pancreatic islets were isolated from *Neurod1*$^{fl/fl}$;*Ins*$^{Cre+}$ and control (*Neurod1*$^{fl/fl}$ or *Ins*$^{Cre+}$) 12-week-old female mice by the perfusion and digestion of pancreata using collagenase P (catalog no. 11 213 857 001; Roche), followed by the separation of islets using a Histopaque (catalog no. 10831; Sigma-Aldrich) gradient as previously described (14). RNA was purified using RNAeasy Micro Kits (catalog no. 74004; QIAGEN). RNA quality and concentration were determined using an Agilent Bioanlyzer. Due to low yield of E17 mouse *Neurog3:Cre; R26R:Tomato*$^{+}$ lineage–labeled cells, 2.315 ng RNA (RNA integrity number >8) was converted to cDNA and amplified using an Ovation RNA Amplification System V2 (catalog no. 3100-12; NuGen) according to the manufacture's protocol. Other cDNA libraries were prepared from ~70–700 ng total RNA (RNA integrity number >8) using a TruSeq RNA prep kit (catalog no. RS-122-2001; Illumina). RNA Sequencing (RNA-Seq) and data processing were performed by the Columbia Genome Center. Multiplexed libraries of cDNA were sequenced using an Illumina HiSeq2000 to yield ~30 million single-end 100-bp reads. Base calling was performed using RTA (Illumina) software, and bcl2fastq (version 1.8.4) coupled with adaptor trimming was used to convert binary base call format to fastq format. Tophat (version 2.1.0) was used to map reads to the reference genome (human: NCBI/build37.2; mouse: UCSC/mm9), allowing for four mismatches and 10 maximum multiple hits (29). Cufflinks (version 2.0.2) with default settings was used to estimate the relative expression level of genes and splice isoforms (30). Statistics for differential gene expression were calculated using the R package DEseq (31).

Real-Time Quantitative PCR

Total RNA was extracted from cells at days 13, 15, 17, 20, and 27 of differentiation using an RNeasy Mini Kit (catalog no. 74106; QIAGEN). Total RNA was reverse transcribed into cDNA using iScript Reverse Transcription Supermix (catalog no. 170-8841; Bio-Rad). Quantitative PCR reactions were set up by mixing SsoFast EvaGreen Supermix (catalog no. 172-5202; Bio-Rad) with primers and cDNA templates. Quantitative PCR was performed using a CFX96 Touch Real-Time PCR Detection System. Primer sequences were as follows: *INSULIN* (*INS*) (TTCTACACACCCAAGACCCG and CAATGCCACGCTTCTGC), *GLUCAGON* (*GCG*) (AAGTTCCCAAAGAGGGCTTG and AGCTGCCTTGTACCAGCATT), and *TBP* (TGTGCACAGGAGCCAAGAGT and ATTTTCTTGCTGCCAGTCTGG). *NEUROD1* primer sequences were published by Zengrong et al. (17).

Determination of Genes With Nearby Neurod1 Binding Sites

The identification of genes with nearby NEUROD1 binding sites in adult mouse islets was performed by the Herbert Irving Comprehensive Cancer Center Bioinformatics Shared Resource. ChIP-Seq data from Tennant et al. (6) for NEUROD1 binding in adult mouse islets was obtained as a BAM file from GSE30298 (32). Peaks were identified with MACS 14.0 (33) using default parameters and associated with genes 3 kb in either direction with ChipPeakAnno (34).

Statistics

The *P* value of significance for data sets of indicated genotypes was calculated using two-tailed, unequal variance Student *t* tests. Adjusted *P* values were calculated as false discovery rate using the Benjamini-Hochberg procedure.

Data and Resource Availability

RNA-Seq data sets are available at the National Center for Biotechnology Information's Gene Expression Omnibus database under the accession number GSE30298. All other data sets generated and/or analyzed for this study are available from the corresponding author upon reasonable request. *NEUROD1*$^{mut/mut}$ HESC lines [RRID: *MEL1INSGFP/wt NEUROD1*$(^{-/-})$] are available from the corresponding author upon reasonable request.

RESULTS

Murine NEUROD1 Is Required in the NEUROG3 Lineage for the Proliferative Expansion of Embryonic α- and β-Cells

To elucidate the Neurod1-dependent mechanisms that are important for the development of fully functional

complements of α- and β-cells, we first needed to precisely determine the developmental stage and cell type in which NEUROD1 is required for α- and β-cell development. Previous studies reported that NEUROD1 was dispensable for the differentiation of endocrine islet cells but was subsequently required for their perinatal survival and maturation (12,14). Specific deletion of *Neurod1* in β-cells, however, had no effect on β-cell numbers, suggesting that NEUROD1 has stage-specific functions and/or is required prior to endocrine cell differentiation to establish and/or maintain normal numbers of islet cells (11,14).

Consistent with previous reports, we found that *Neurod1* KO pancreata develop a nearly 3.0- to 4.5-fold reduction in the numbers of insulin-expressing β-cells and glucagon-expressing α-cells between E17 and birth (P0) (Supplementary Fig. 1*A* and *B*). There were no detectable defects in the formation of Neurog3$^+$ endocrine progenitor cells (Supplementary Fig. 1*C*), and the reduction in α- and β-cell numbers occurred after the majority of endocrine islet cell differentiation was completed, suggesting that loss of Neurod1 did not disrupt endocrine progenitor differentiation. This finding was supported by deletion of *Neurod1* in the NEUROG3 endocrine lineage using *Neurod1$^{fl/fl}$; Neurog3:Cre* mice (*Neurod1$^{\Delta endo}$*); these mice phenocopied *Neurod1* KO mice, resulting in a similar reduction in the numbers of Ins$^+$ and Gcg$^+$ cells in the pancreas by birth, as well as lethal perinatal hyperglycemia (11) (Supplementary Fig. 2*A*–*C*).

We also wanted to confirm that the observed reduction in β-cell numbers was due primarily to apoptosis, as previously described (12). Surprisingly, although *Neurod1* KO pancreata had significantly elevated numbers of apoptotic TUNEL$^+$ insulin-expressing cells at E18, these apoptotic cells were extremely rare (0.25% of insulin$^+$ cells) and were unlikely to explain the 3.0- to 4.5-fold reduction in the numbers of α- and β-cells that occurred between E17 and birth (Fig. 1*A* and *B*). We also did not detect measurable apoptosis at earlier developmental time points, suggesting that we had not missed an earlier wave of cell death that would account for the loss of α- and β-cells in *Neurod1* KO pancreata. Since the reduction in α- and β-cells could not be explained by defects in differentiation or elevated cell death, we examined the proliferation of α- and β-cells in *Neurod1* KO mice at E15, E17, and E18, prior to and during the stages of robust endocrine cell proliferation. Beginning at E17, *Neurod1* KO pancreata had a severe reduction of insulin$^+$ and glucagon$^+$ cells expressing the proliferation marker Ki67 (Fig. 1*A*, *C*, and *D*). This analysis suggests that the perinatal reduction of α- and β-cells in the *Neurod1* KO pancreas is primarily due to defects in their proliferative expansion, with only minor contribution from apoptosis.

Murine NEUROD1 Regulates a β-Cell Differentiation and Proliferation Program

The surprising discovery that α- and β-cell loss in the *Neurod1* KO mice was largely due to proliferation defects prompted us to revisit the molecular pathways regulated by NEUROD1 in the endocrine lineage. We performed RNA-Seq transcriptome analysis on sorted pancreatic *Neurod1$^{fl/fl}$; Neurog3:Cre; R26R:Tomato*$^+$ lineage–labeled endocrine cells and compared them to littermate controls at E17, prior to the reported major α- and β-cell deficiency caused by *Neurod1* ablation (Supplementary Table 2). As expected from previous studies, gene ontology analysis indicated that a significant number of genes downregulated by *Neurod1* depletion are involved in the regulation of insulin secretion, hormone secretion, and hormone levels (Supplementary Table 3). Importantly, the β-cells maintained expression of definitive β-cell transcription factors, including *Nkx6-1*, *Pdx1*, and *Nkx2-2*, suggesting that β identity was not compromised (Fig. 2*B* and Supplementary Fig. 3). However, *Neurod1*-deficient endocrine cells had reduced expression of genes that encoded proteins specifically important for the maturation and function of β-cells, including *Ins1* and *Ins2*, the β-cell maturation transcription factor gene *Mafa*, as well as several regulators of insulin secretion and insulin processing (Fig. 2*A*–*C*). In addition, the expression of *Gcg* and *Gast* was reduced (Fig. 2), agreeing with previous reports showing that pancreatic mouse *Gcg* and *Gast* expression is dependent on NEUROD1 regulation (24,35).

The expression profile of cell cycle regulators in *Neurod1*-deficient cells was consistent with the severe proliferation defects we observed in the endocrine cells. Notably, *Neurod1*-deficient cells had elevated expression of *Ccng2*, a negative regulator of cell cycle progression, and reduced expression of *Ccnb2, Ccnd2, Cdc25b*, and *Cdk1,* which promote cell cycle progression (Fig. 2*D*). In addition, *Neurod1*-deficient cells had reduced expression of *Prlr* and *Ret,* which are receptors for the potent secreted β-cell mitogens PROLACTIN and GDNF, respectively (36–39) (Fig. 2*D*). Both Prlr lactogens and GDNF have been shown to be elevated during embryogenesis at the stage where we observe β-cell proliferation defects in the *Neurod1* KO and *Neurod1$^{\Delta endo}$* mice (40,41). Furthermore, Prlr is required for the proliferation of β-cells specifically during the perinatal stages (42). These data support a role for Neurod1 in inducing a program essential for the proliferative expansion of embryonic α- and β-cells.

Interestingly, transcriptome analysis also indicated that *Neurod1*-deficient E17 endocrine cells had a significant number of downregulated genes that are involved in the unfolded protein response (UPR) and endoplasmic reticulum (ER)–associated degradation (Fig. 2*D* and Supplementary Table 3). UPR and ER-associated degradation genes remediate ER stress by facilitating the folding and degradation of misfolded proteins, respectively. Prolonged ER stress can blunt insulin secretion and induce β-cell apoptosis via induction of TXNIP, which had elevated expression in *Neurod1* KO cells (43,44) (Fig. 2*D*). These data suggest that in addition to its proliferation and maturation functions, NEUROD1 regulates genes important for the remediation of ER stress.

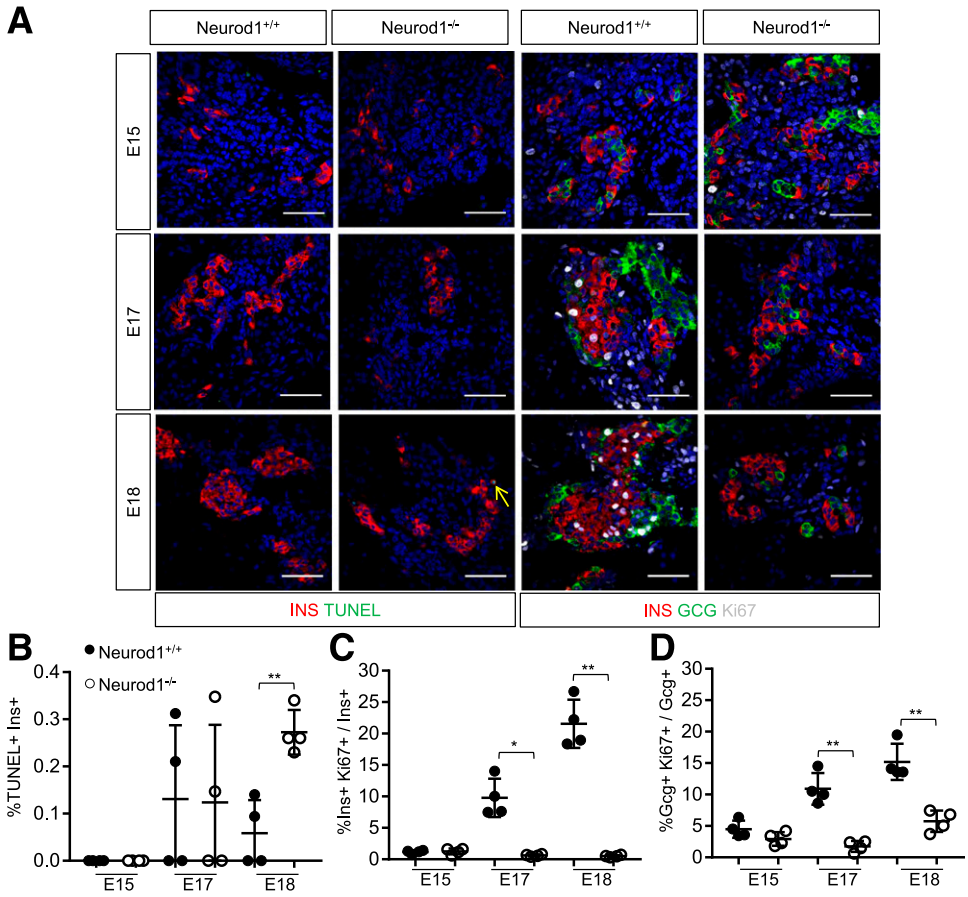


Figure 1—Murine NEUROD1 is required for the late fetal expansion of pancreatic INSULIN$^+$ and GLUCAGON$^+$ cells. *A*: IF expression analysis of indicated markers and TUNEL staining performed on sections of E15, E17, and E18 pancreata. Scale bar = 50 μm. *B*: Quantification of the percent of insulin$^+$ cells that are TUNEL positive. *C*: Quantification of INSULIN$^+$ cells expressing Ki67. The total number of Ins$^+$ cells counted per *Neurod1*$^{+/+}$ sample was E18 = 554 ± 162, E17 = 347 ± 184, and E15 = 308 ± 161 and per *Neurod1*$^{-/-}$ was E18 = 548 ± 102, E17 = 344 ± 100, and E15 = 293 ± 36. *D*: Quantification of GLUCAGON$^+$ cells expressing Ki67. The total number of GCG$^+$ cells counted per *Neurod1*$^{+/+}$ sample was E18 = 207 ± 89, E17 = 94 ± 49, and E15 = 220 ± 121 and per *Neurod1*$^{-/-}$ was E18 = 112 ± 45, E17 = 108 ± 42, and E15 = 111 ± 57. All error bars indicate SD. Student *t* test *P* value compares values of controls to those of indicated genotype. *$P < 0.01$; **$P < 0.005$.

Distinct Neurod1-Dependent Gene Regulation Programs in Embryonic and Adult Mouse Islet Cells

Since our studies identified novel perinatal defects associated with *Neurod1* KO and *Neurod1*$^{\Delta endo}$ mice, we wished to readdress the similarities and differences of NEUROD1 function in the embryo versus adult animals that have an impaired maturation phenotype (12,14). We generated RNA-Seq transcriptome data for adult (12 weeks old) islets from β-cell–specific *Neurod1* KO (*Neurod1*$^{fl/fl}$*; Ins:Cre*; hereafter referred to as *Neurod1*$^{\Delta\beta}$) and control (*Ins:Cre* or *Neurod1*$^{fl/fl}$) female mice (Supplementary Table 4) and compared NEUROD1-dependent gene regulation in embryonic pancreatic endocrine lineages versus adult islet β-cells (Fig. 3*A*). As expected, NEUROD1 is required in both embryonic endocrine lineages and adult β-cells for the expression of numerous genes important for β-cell function (Fig. 3*B*). Importantly, genes specifically dysregulated by loss of *Neurod1* in embryonic NEUROG3 lineages included many genes important for proliferation, which is consistent with the perinatal proliferation defects observed in these mice (Fig. 3*B*). Alternatively, β-cell–specific deletion of *Neurod1* preferentially altered the expression of genes involved in the functional maturation of β-cells, as previously described (14) (Fig. 3*B*). Interestingly, loss of *Neurod1* in embryonic endocrine cells compared with its loss in adult islet β-cells had the opposite effect on the regulation of several genes, including the β-cell immaturity marker *Npy*, proliferation gene *Prlr*, and several UPR genes. These data indicate that the gene programs and transcriptional regulatory activity of NEUROD1 are stage and context dependent.

NEUROD1 Binds Near Proliferation and β-Cell Functional Genes in Adult Murine Islets

Since there appears to be stage-specific requirements for NEUROD1 regulation of the proliferation and maturation of islet cells, we wanted to determine which genes were directly regulated by NEUROD1. To identify direct regulated targets of Neurod1, we used a previously published ChIP-Seq data set in adult murine islets that identified

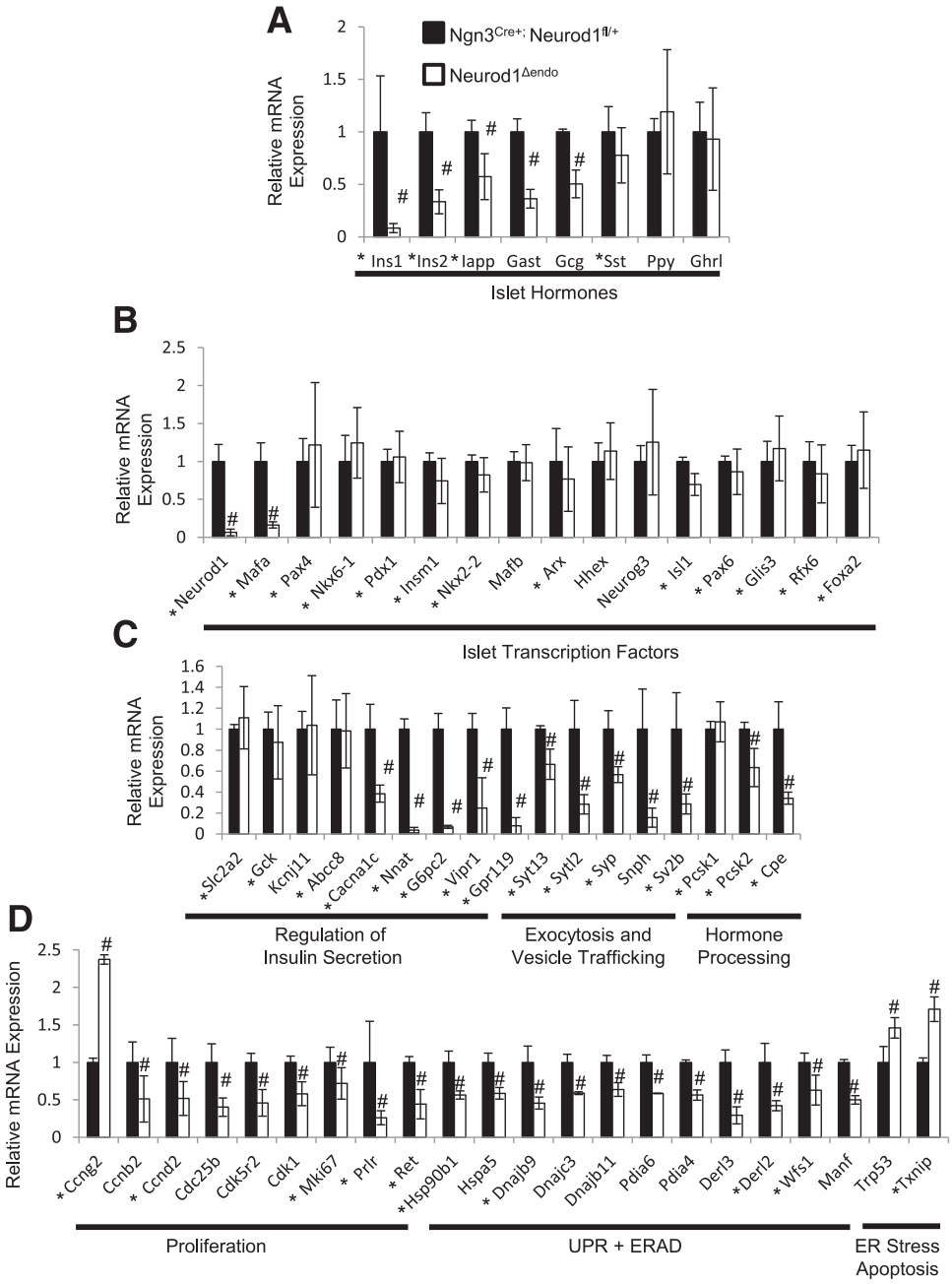
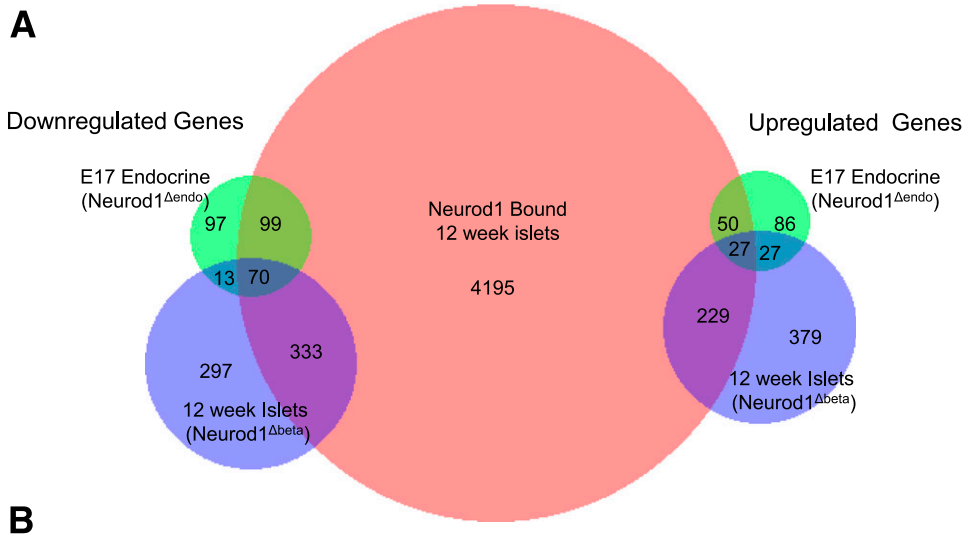


Figure 2—Murine NEUROD1 binds near and is required to regulate the transcription of genes important for the differentiation of functional β-cells, proliferation, and survival. *A–D*: RNA-Seq expression data for *Neurod1*$^{\Delta endo}$ and *Ngn3*$^{Cre+}$*; Neurod1*$^{fl/+}$ E17-sorted pancreatic endocrine cells. An asterisk indicates Neurod1-bound genes in 12-week-old mouse islets determined using a published ChIP-Seq data set (Tennant et al. [6]). #*P* value <0.01. $n = 4$ for each genotype. Error bars indicate SD.

NEUROD1 binding at islet-specific enhancers (6). We found that Neurod1 binds near many of the shared genes known to require NEUROD1 for their regulation during embryonic endocrine cell differentiation and in adult islet β-cells (Figs. 2 and 3). NEUROD1 also bound several proliferation genes that had altered expression in *Neurod1*-deficient cells specifically during embryonic endocrine differentiation but were unchanged in adult β-cells (Figs. 2*D* and 3*B*). Conversely, NEUROD1 bound and regulated a distinct set of β-cell maturation and function genes in the adult that were either unchanged or differentially regulated in the embryo, again suggesting that there are important context-dependent factors influencing NEUROD1 function. Similar to what has been reported for several other transcription factors, many genes bound by NEUROD1 in adult islets do not appear to be dependent on NEUROD1 for their regulation in adult islet β-cells. For example, NEUROD1 only appears to regulate a subset of the β-cell transcription factors that it binds to in adult islets (Figs. 2*B* and 3*A*). Hence, NEUROD1 is dispensable for the transcriptional regulation of many of its

A



B

Neurod1 dependent genes in E17 endocrine cells only	
Islet Transcription Factors	Mafa*, Myt1l*
Islet Hormones	Ins2*, Iapp*, Gcg, Gast, Gip, Sct
Secretion and Hormone Processing	Gpr119*, Pcsk2, Syt13*, Sytl2*, Syp, Sv2b*, Pak3*
β-Cell Immaturity	Npy*
ER stress + UPR	Hsp90b1*, Hspa5, Dnajc3, Dnajb9*, Dnajb11, Pdia4, Pdia6, Derl2*, Derl3, Wfs1*
Proliferation	Ccnb2, Ccnd2*, Cdc25b, Cdk5r2, Cdk1, Prlr*, Ccng2*
Neurod1 dependent genes in E17 endocrine cells and 12 week β-cells	
Islet Transcription Factors	Neurod1*, Lmx1b*
Islet Hormones	Ins1*
Secretion and Hormone Processing	Cacna1c*, Trpm5*, Vipr1*, Cpe*, G6pc2*, Nnat*, Snph, Scgn*,Sytl3*
ER stress + UPR	Jph4*, Txnip*
Proliferation	Ret*, Cdkl1*
Neurod1 dependent genes in 12 week β-cells only	
Islet Transcription Factors	Nkx2-2*, Mlxipl*, Pdx1*,
Secretion and Hormone Processing	Gipr*, Rph3al*, Cacnb1, Cacnb3*, Syt1*, Syt6, Esyt1*, Sncb*, Snca*, Sncg, Cadps2, Stx1a, Stxbp1*, Syt10, Pcsk1*, Glp1r
β-Cell Immaturity or Dysfunction	Ldha, Npy*, Aldh1a3*
ER stress + UPR	Hspa2, Hspa5, Hspa8, Dnajc3, Calr, Edem1, Insig1*, Ero1lb*
Proliferation	Cdkn1a, Prlr*

Neurod1 Δ vs Control cells: Red indicates FC < 0.67, Blue indicates F.C. > 1.5, *Neurod1 Bound

Figure 3—Stage-specific NEUROD1-dependent gene regulation in murine E17 endocrine cells and adult β-cells. *A*: Venn diagrams depicting overlap of genes bound by NEUROD1 in adult islets and either down- or upregulated ($P < 0.01$, fold change $> 1.5\times$) by NEUROD1 depletion in E17-sorted pancreatic endocrine cells vs. adult islet β-cells (beta). NEUROD1-bound genes in adult mouse islets determined using a published ChIP-seq data set (Tennant et al. [6]) *B*: Tables of Neurod1-dependent genes in E17 endocrine cells or 12-week-old adult β-cells or both. Genes down- or upregulated in NEUROD1-deficient cells are in red and blue, respectively. An asterisk indicates genes bound by NEUROD1 in adult mouse islets.

targets, indicating either compensatory mechanisms for NEUROD1-regulated gene expression or functional redundancy between Neurod1 and other islet transcription factors. The latter explanation is supported by the co-occupancy of NEUROD1 with other β-cell transcription factors at islet cell–specific enhancers (6,7).

Mutation of *NEUROD1* in HESCs Specifically Impairs β-Cell Differentiation

Aspects of murine and human pancreas development have been shown to differ in their requirements for several pancreatic transcription factors (15,16). Therefore, we wanted to determine whether NEUROD1 has conserved

essential functions in human β-cell development, which can now be modeled by the differentiation of HESCs into HESC-β cells (Supplementary Fig. 4*A*). To disrupt *NEUROD1* in HESCs, we used CRISPR/Cas9-mediated mutagenesis to target frameshift indels into the essential bHLH domain of NEUROD1 (Supplementary Fig. 4*B*). We used a previously described HESC line (MEL1 *INS*$^{GFP/wt}$) that has a GFP expression cassette knocked into a single copy of the insulin gene (25). We differentiated four mutated (*NEUROD1*$^{mut/mut}$) HESC lines (three to four experimental replicates per line) into insulin-producing cells using a protocol that recapitulates the major stages of in vivo β-cell development, including the differentiation of definitive endoderm and pancreatic progenitors (26). All four lines produced similar results, and we confirmed that targeted line *NEUROD1*$^{mut/mut}$ clone 12 had reduced NEUROD1 protein expression at the β-cell stage of differentiation (day 27) and a normal karyotype (Supplementary Fig. 4*C* and *D*). This line was used for all subsequent analyses.

During HESC-β cell differentiation, *NEUROD1* mRNA expression was induced at the pancreatic endocrine precursor stages (day 15) just prior to hormone induction (Supplementary Fig. 5*A*). At day 12 of the differentiation process, almost all *NEUROD1*$^{mut/mut}$ and control cells differentiated into cells expressing the endocrine-biased pancreatic progenitor markers PDX1$^+$ NKX6-1$^+$ (Fig. 4*A*–*C*). At the HESC-β cell stages (days 18–27) of differentiation, *NEUROD1*$^{mut/mut}$ cell populations had normal numbers of CHRGA$^+$ endocrine cells; however, there was a striking reduction in the number of cells expressing INSULIN and/or the INSULIN-GFP reporter (Figs. 4*D* and 5*A*–*F*, Supplementary Fig. 5*B* and *C*, and Supplementary Fig. 6*A* and *B*). *NEUROD1*$^{mut/mut}$ cells did have similar numbers of cells expressing the β-cell differentiation and maturation transcription factors NKX6-1 and MAFA compared with NEUROD1 wild-type controls; however, there was a noticeable reduction in their expression levels per cell, based on IF intensity (Fig. 5*E* and *F*). Loss of NEUROD1 during HESC-β cell differentiation did not appear to direct cells to an α-cell or polyhormonal cell fate: *NEUROD1*$^{mut/mut}$ cell populations had similar low numbers of GLUCAGON$^+$ C-PEPTIDE$^-$ cells compared with controls, as well as reduced numbers of GLUCAGON and C-PEPTIDE coexpressing cells (Fig. 5*A*–*D* and Supplementary Fig. 5*B* and *C*). Consistent with the reduction in C-peptide$^+$ cells, *NEUROD1*$^{mut/mut}$ cells displayed a reduction in insulin content compared with control cells, in addition to defective glucose-stimulated insulin secretion (Fig. 5*G*–*I*). Collectively, these data indicate that NEUROD1 is required downstream of NKX-6-1$^+$ PDX1$^+$ HESC-derived endocrine progenitors to produce normal numbers of HESC-β cells.

Since loss of NEUROD1 impairs the proliferation and survival of embryonic β-cells in mice, we looked for similar phenotypes in NEUROD1-deficient HESC-β cells. We measured EDU incorporation at several time points during the differentiation protocol but could not identify proliferation defects (Fig. 6*A* and *B*). Furthermore, IF staining for Ki67 and TUNEL analysis at the end of the differentiation protocol detected similarly low percentages of either proliferating or apoptotic HESC-β cells between control and *NEUROD1*$^{mut/mut}$ cells (Fig. 6*C* and *D*). Therefore, under these culture conditions, we found no requirement of NEUROD1 for the proliferation or survival of HESC-β cells.

Conserved and Distinct Gene Regulation by NEUROD1 in Mouse and HESC-β Cells

To identify the molecular defects associated with the dysfunctional *NEUROD1*$^{mut/mut}$ HESC-β cells, we performed RNA-Seq expression analysis on *NEUROD1*$^{mut/mut}$ and control FACS-sorted day 27 INSULIN-GFP$^+$ cells at the end of the differentiation protocol (Supplementary Fig. 6*A*). Consistent with the observed defect in β-cell function (Fig. 5*H* and *I*), gene ontology analysis indicated that many of the genes downregulated by *NEUROD1* ablation are involved in the regulation of insulin secretion, including *GLUCOKINASE* (*GCK*), ATP-gated K$^+$ efflux channel *ABCC8* (*SUR1*), and the calcium channel *CACNA1a* (Fig. 7*A* and *B*). To identify conserved functions for NEUROD1 between mice and humans, we compared the dysregulated genes due to loss of NEUROD1 in HESC-β cells and murine E17 endocrine cells (*Neurod1*$^{\Delta endo}$) as well as adult murine islet β-cells (*Neurod1*$^{\Delta\beta}$) (Supplementary Fig. 7). Although each population displayed distinct gene expression profiles, likely due to differences in developmental time points (embryonic vs. adult) and artifacts associated with the in vitro differentiation program, *NEUROD1*$^{mut/mut}$ cells shared a large proportion of dysregulated genes with Neurod1-deficient adult mouse islets, suggesting that NEUROD1 has a number of conserved β-cell functions. Interestingly, however, unlike in mice, mutation of *NEUROD1* in HESC-β cells caused reduced expression of many of the essential β-cell transcription factors, including MAFA, NKX6-1, PDX1, INSM1, NKX2-2, ISL1, PAX6, and RFX6 (Fig. 7*B* and Supplementary Fig. 7), suggesting that *NEUROD1* may have an expanded and/or less redundant role in regulating the essential β-cell transcription factor network in humans.

DISCUSSION

Previous studies using mouse models suggested that NEUROD1 was a survival factor for neonatal β-cells but played a predominant role in regulating β-cell maturation in adult β-cells (12,14). In this study, we used detailed phenotypic and transcriptome analysis to identify a prenatal requirement of Neurod1 in the regulation of β-cell proliferation. We also identified conserved and distinct functions of NEUROD1 in HESC-β cells. Collectively, these findings provide valuable mechanistic insight to how inactivating mutations in *NEUROD1* can cause defects in the development and function of β-cells.

In mice, NEUROD1 directly regulates a broad scope of genes important for the formation and maturation of

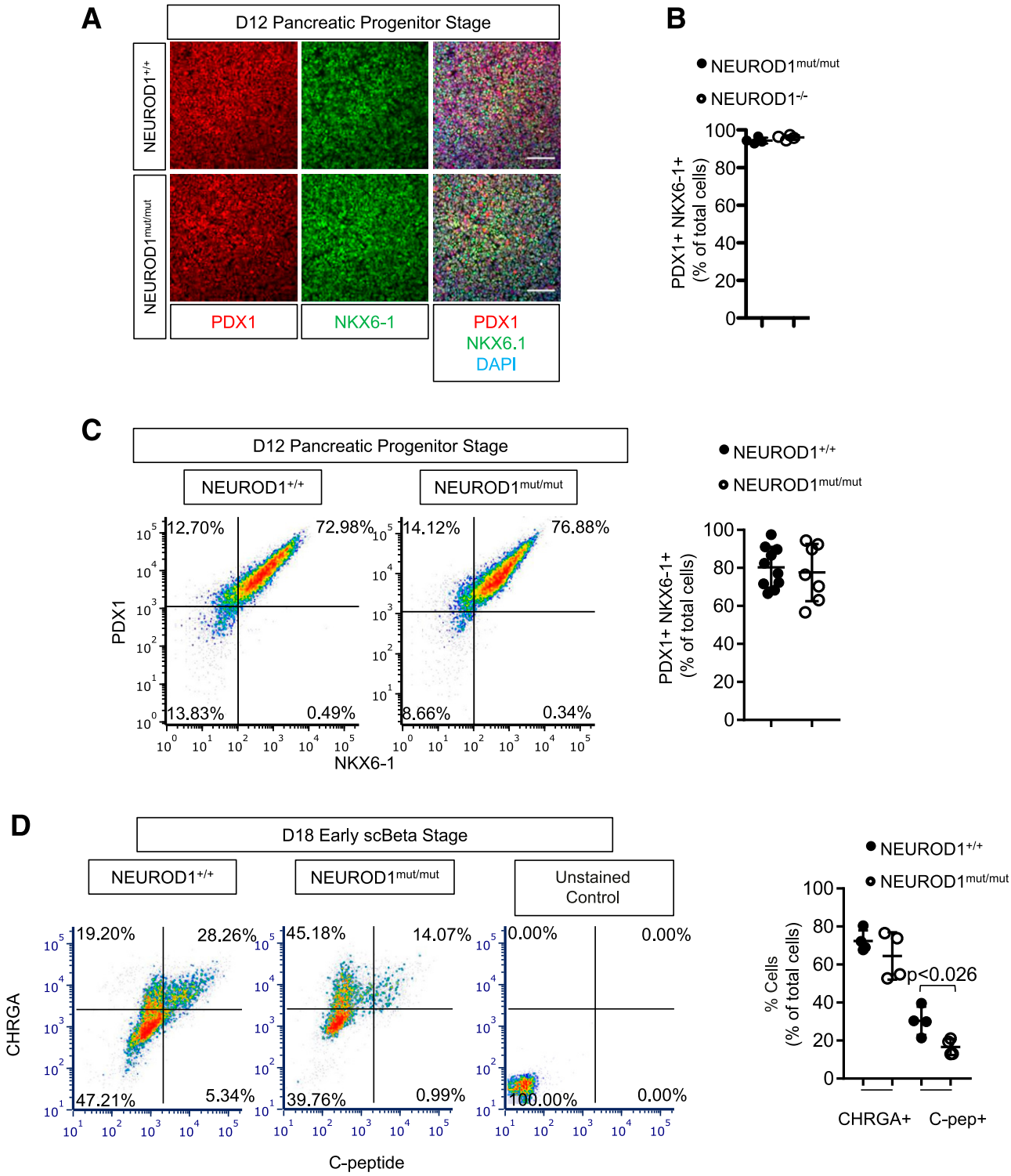


Figure 4— Impaired differentiation of *NEUROD1*$^{mut/mut}$ HESC-derived pancreatic progenitors into insulin-expressing cells. *A*: IF of pancreatic progenitor markers PDX1 and NKX6.1 at day 12 of differentiation. Scale bar = 100 μm. *B*: Quantification of PDX1$^+$, NKX6-1$^+$ cells at day 12 of differentiation. *C*: Flow cytometry expression analysis and quantification of lineage markers at day 12 of HESC-β differentiation. *D*. Flow cytometry expression analysis of lineage markers at day 18 of HESC-β differentiation. Scale bar = 50 μm. $n = 4$ for each genotype. Error bars indicate SD. Student *t* test *P* value compares values of controls to *NEUROD1*$^{mut/mut}$. scBeta, stem cell–derived β-cells. *$P < 0.01$.

β-cells. Consistent with previous reports, we found that loss of NEUROD1 in the NEUROG3 lineage prior to endocrine cell differentiation drastically reduced the number of α- and β-cells at birth (11). However, our study determined that deletion of *Neurod1* severely impairs the perinatal proliferative expansion of α- and β-cell populations while causing a relatively minor loss of β-cells by apoptosis. Therefore, we propose that the major cause of α- and β-cell

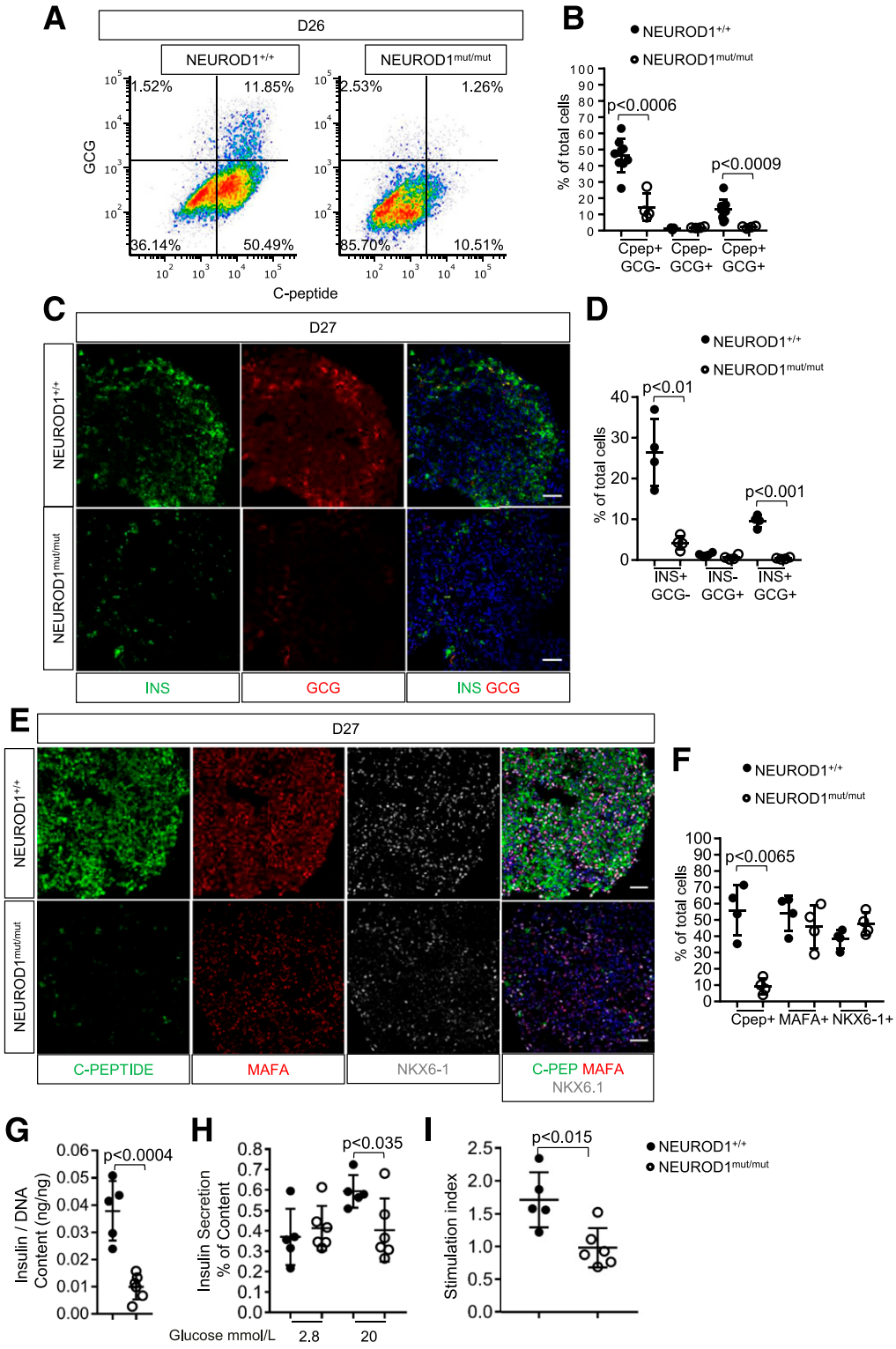


Figure 5—NEUROD1 is required for the differentiation HESC-β cells. *A*: Flow cytometry expression analysis of lineage markers for day 26 (D26) cells of HESC-β differentiation. *B*: Quantification of hormone-expressing cells determined by flow cytometry. *C*: IF expression of INSULIN and GLUCAGON by day 27 HESC–derived endocrine clusters, nuclei stained with DAPI. Scale bar = 50 μm. *D*: Quantification of hormone-expressing cells determined by IF analysis. *E*: IF expression analysis revealed reduced expression of C-peptide, MAFA, and NKX6-1 by *NEUROD1*$^{mut/mut}$ day 27 endocrine clusters compared with controls. Nuclei are stained with DAPI. Scale bar = 50 μm. *F*: Quantification of C-peptide, MAFA, and NKX-6-1–expressing cells as a percentage of total nuclei. *G*: Insulin content normalized to DNA content *H*: Secreted insulin content by day 27 endocrine clusters differentiated from HESCs in response to 2 and 20 mmol/L glucose. *I*: Fold change in insulin secretion of HESC-β cells in response to 20 mmol/L glucose relative to insulin secretion at 2 mmol/L glucose. Error bars indicate SD. Student *t* test *P* value compares values of controls to *NEUROD1*$^{mut/mut}$.

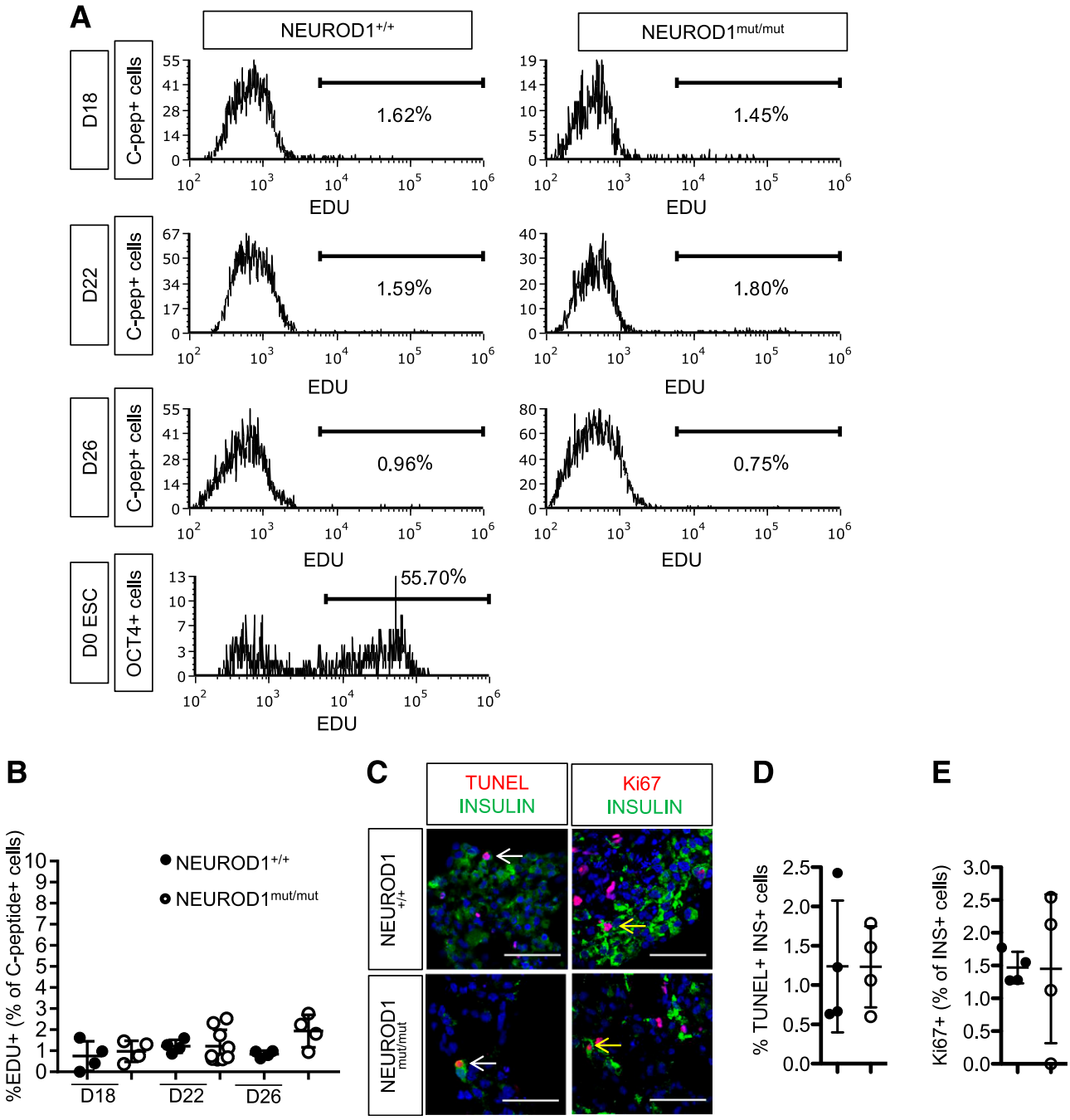


Figure 6—*NEUROD1*$^{mut/mut}$ and control HESC-β cells have similar low rates of proliferation and apoptosis. *A*: Flow cytometry histograms of EDU incorporation for C-peptide$^+$ cells at different stages of HESC-β cell differentiation as well as for OCT4$^+$ cells in ESCs. *B*: Quantification of proliferating EDU$^+$ C-peptide$^+$ cells determined by flow cytometry analysis. *C*: TUNEL and IF staining for Ki67 and insulin on sections of day 27 (D27) HESC–derived endocrine clusters. Yellow arrow points to Ki67$^+$ insulin$^+$ cells. White arrow points to TUNEL$^+$ INSULIN$^+$ cell *D*: Quantification of apoptotic TUNEL$^+$ INSULIN$^+$ cells. *E*: Quantification of INSULIN$^+$ cells expressing proliferation marker Ki67.

deficiency in *Neurod1* KO mice is primarily due to α- and β-cell proliferation defects and not from apoptosis. This novel requirement of NEUROD1 for β-cell proliferation during embryonic development may have been previously overlooked because of the limited markers and assays and/or strain background differences that are known to influence *Neurod1* mutant phenotypes (12,45).

Our data using an HESC differentiation protocol also provide support for *NEUROD1* inactivation causing permanent neonatal diabetes mellitus and defective β-cell function. Patients with *NEUROD1*-inactivating mutations have been reported to have normal pancreas size and exocrine function but reduced circulating insulin (3,4). These clinical features of NEUROD1-linked diabetes are

A

Gene Ontology category	# of genes	P value
Regulation of insulin secretion	49	0.0149
Regulation of hormone secretion	66	0.00967
Regulation of ion transport	159	4.53E-15
Calcium ion transport	68	4.60E-06
Potassium ion transport	55	4.29E-06

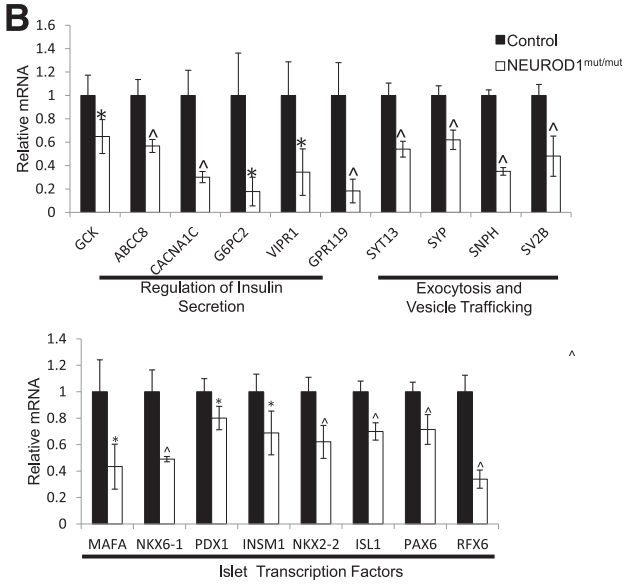


Figure 7—Reduced expression of β-cell genes in *NEUROD1*$^{mut/mut}$ INSULIN-GFP^{+} cells differentiated from HESCs. *A*: PANTHER gene ontology categories with significant enrichment of genes downregulated (adjusted *P* value = 0.05) in *NEUROD1*$^{mut/mut}$ compared with control day 27-sorted INSULIN-GFP^{+} cells. *B*: RNA-Seq data of β-cell genes downregulated in *NEUROD1*$^{mut/mut}$ relative to control D27-sorted INSULIN-GFP^{+} cells. **P* value <0.05; ^*P* value <0.01. *n* = 4 for each genotype. Error bars indicate SD.

consistent with our finding that NEUROD1 has conserved functions in mice and humans for inducing and/or maintaining insulin expression and the expression of other genes that are important for β-cell function. Consistent with *Neurod1* loss-of-function phenotypes in mice, NEUROD1 is dispensable in HESCs for pancreatic progenitor cell differentiation, but it is critical for the subsequent differentiation of HESC-β cells. However, unlike *Neurod1* KO mice, the low yield of *NEUROD1* mutant HESC-β cells in culture appeared to result not from defects in proliferation or apoptotic loss but rather from a failure of pancreatic progenitors to differentiate into insulin-expressing HESC-β cells. Furthermore, the few remaining NEUROD1-deficient HESC-β cells that did form displayed reduced expression of a β-cell transcription factor network necessary for the formation of fully functional β-cells. These data suggest there is a more robust requirement of NEUROD1 for the differentiation of fully functional insulin-expressing β-cells.

Although we were surprised by the lack of proliferation and survival defects in the HESC-derived endocrine cells, there are several possible explanations. Differences in the functional properties of human and murine NEUROD1 protein are unlikely since their amino acid sequences are 98% identical; however, differences in genetic modifiers between mice and humans could possibly influence the effects of *NEUROD1* deficiency. Genetic background, for instance, has already been shown to profoundly influence the penetrance and expressivity of diabetes caused by *Neurod1* deletion in mice (45). Similarly, varying penetrance of maturity-onset diabetes of the young 6 has been observed within pedigrees (4). Some of the observed differences in phenotypes and gene regulation could also reflect inherent differences in the HESC-β cell culture conditions, which may not accurately mimic the burst of proliferation observed in mice. We were also surprised that the *NEUROD1*-deficient HESC-β cells displayed much more severe impairment of many essential β-cell transcription factors than observed in mice. In this case, the difference in phenotypes may indicate a more critical role for NEUROD1 in regulating the β-cell regulatory network, or may suggest that the transcriptional program of HESC-β cells is more susceptible to minor transcriptional perturbations.

In summary, these studies clarify the roles for NEUROD1 in the regulation of β-cell formation and function. Whereas NEUROD1 appears to be essential for perinatal expansion of the β-cell population in mice, NEUROD1 may play a more important role in the differentiation of human β-cells. Alternatively, NEUROD1 has conserved functions in its regulation of key β-cell genes that are essential for maintaining β-cell maturation and function.

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Author Contributions. A.I.R. wrote the manuscript. A.I.R. designed and conducted the experiments with advice from D.E. and L.S. Experiments were conceived by A.I.R., D.E., and L.S. R.A.S. provided computational analysis of the RNA-Seq data with help from A.I.R., D.E., and L.S. in the interpretation of results. R.A.S. and L.S. reviewed and edited the manuscript. A.I.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Romer AI, Sussel L. Pancreatic islet cell development and regeneration. Curr Opin Endocrinol Diabetes Obes 2015;22:255–264

2. McCarthy MI, Hattersley AT. Learning from molecular genetics: novel insights arising from the definition of genes for monogenic and type 2 diabetes. Diabetes 2008;57:2889–2898

3. Rubio-Cabezas O, Minton JA, Kantor I, Williams D, Ellard S, Hattersley AT. Homozygous mutations in NEUROD1 are responsible for a novel syndrome of permanent neonatal diabetes and neurological abnormalities. Diabetes 2010;59:2326–2331

4. Malecki MT, Jhala US, Antonellis A, et al. Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. Nat Genet 1999;23:323–328
5. Naya FJ, Stellrecht CM, Tsai MJ. Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 1995;9:1009–1019
6. Tennant BR, Robertson AG, Kramer M, et al. Identification and analysis of murine pancreatic islet enhancers. Diabetologia 2013;56:542–552
7. Jia S, Ivanov A, Blasevic D, et al. Insm1 cooperates with Neurod1 and Foxa2 to maintain mature pancreatic β-cell function. EMBO J 2015;34:1417–1433
8. Moates JM, Nanda S, Cissell MA, Tsai MJ, Stein R. BETA2 activates transcription from the upstream glucokinase gene promoter in islet beta-cells and gut endocrine cells. Diabetes 2003;52:403–408
9. Kim JW, Seghers V, Cho JH, et al. Transactivation of the mouse sulfonylurea receptor I gene by BETA2/NeuroD. Mol Endocrinol 2002;16:1097–1107
10. Marsich E, Vetere A, Di Piazza M, Tell G, Paoletti S. The PAX6 gene is activated by the basic helix-loop-helix transcription factor NeuroD/BETA2. Biochem J 2003;376:707–715
11. Mastracci TL, Anderson KR, Papizan JB, Sussel L. Regulation of Neurod1 contributes to the lineage potential of Neurogenin3+ endocrine precursor cells in the pancreas. PLoS Genet 2013;9:e1003278
12. Naya FJ, Huang HP, Qiu Y, et al. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev 1997;11:2323–2334
13. Li HJ, Kapoor A, Giel-Moloney M, Rindi G, Leiter AB. Notch signaling differentially regulates the cell fate of early endocrine precursor cells and their maturing descendants in the mouse pancreas and intestine. Dev Biol 2012;371:156–169
14. Gu C, Stein GH, Pan N, et al. Pancreatic beta cells require NeuroD to achieve and maintain functional maturity. Cell Metab 2010;11:298–310
15. Xuan S, Borok MJ, Decker KJ, et al. Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. J Clin Invest 2012;122:3516–3528
16. Rubio-Cabezas O, Codner E, Flanagan SE, Gómez JL, Ellard S, Hattersley AT. Neurogenin 3 is important but not essential for pancreatic islet development in humans. Diabetologia 2014;57:2421–2424
17. Zhu Z, Li QV, Lee K, et al. Genome editing of lineage determinants in human pluripotent stem cells reveals mechanisms of pancreatic development and diabetes. Cell Stem Cell 2016;18:755–768
18. Shi ZD, Lee K, Yang D, et al. Genome editing in hPSCs reveals GATA6 haploinsufficiency and a genetic interaction with GATA4 in human pancreatic development. Cell Stem Cell 2017;20:675–688.e6
19. Miyata T, Maeda T, Lee JE. NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev 1999;13:1647–1652
20. Goebbels S, Bode U, Pieper A, Funfschilling U, Schwab MH, Nave KA. Cre/loxP-mediated inactivation of the bHLH transcription factor gene NeuroD/BETA2. Genesis 2005;42:247–252
21. Schonhoff SE, Giel-Moloney M, Leiter AB. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. Dev Biol 2004;270:443–454
22. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 2000;127:2317–2322
23. Madisen L, Zwingman TA, Sunkin SM, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 2010;13:133–140
24. Chao CS, Loomis ZL, Lee JE, Sussel L. Genetic identification of a novel NeuroD1 function in the early differentiation of islet alpha, PP and epsilon cells. Dev Biol 2007;312:523–532
25. Micallef SJ, Li X, Schiesser JV, et al. INS(GFP/w) human embryonic stem cells facilitate isolation of in vitro derived insulin-producing cells. Diabetologia 2012;55:694–706
26. Sui L, Danzl N, Campbell SR, et al. β-cell replacement in mice using human type 1 diabetes nuclear transfer embryonic stem cells. Diabetes 2018;67:26–35
27. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823–826
28. Churchill AJ, Gutiérrez GD, Singer RA, Lorberbaum DS, Fischer KA, Sussel L. Genetic evidence that Nkx2.2 acts primarily downstream of Neurog3 in pancreatic endocrine lineage development. eLife 2017;6:pii:e20010
29. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009;25:1105–1111
30. Trapnell C, Williams BA, Pertea G, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 2010;28:511–515
31. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010;11:R106
32. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: archive for functional genomics data sets–update. Nucleic Acids Res 2013;41:D991–D995
33. Zhang Y, Liu T, Meyer CA, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol 2008;9:R137
34. Zhu LJ, Gazin C, Lawson ND, et al. ChIPpeakAnno: a Bioconductor package to annotate ChIP-seq and ChIP-chip data. BMC Bioinformatics 2010;11:237
35. Suissa Y, Magenheim J, Stolovich-Rain M, et al. Gastrin: a distinct fate of neurogenin3 positive progenitor cells in the embryonic pancreas. PLoS One 2013;8:e70397
36. Brelje TC, Scharp DW, Lacy PE, et al. Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. Endocrinology 1993;132:879–887
37. Vasavada RC, Garcia-Ocaña A, Zawalich WS, et al. Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. J Biol Chem 2000;275:15399–15406
38. Mwangi S, Anitha M, Mallikarjun C, et al. Glial cell line-derived neurotrophic factor increases beta-cell mass and improves glucose tolerance. Gastroenterology 2008;134:727–737
39. Mwangi SM, Usta Y, Raja SM, et al. Glial cell line-derived neurotrophic factor enhances neurogenin3 gene expression and beta-cell proliferation in the developing mouse pancreas. Am J Physiol Gastrointest Liver Physiol 2010;299:G283–G292
40. Muñoz-Bravo JL, Hidalgo-Figueroa M, Pascual A, López-Barneo J, Leal-Cerro A, Cano DA. GDNF is required for neural colonization of the pancreas. Development 2013;140:3669–3679
41. Parsons JA, Brelje TC, Sorenson RL. Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology 1992;130:1459–1466
42. Auffret J, Freemark M, Carré N, et al. Defective prolactin signaling impairs pancreatic β-cell development during the perinatal period. Am J Physiol Endocrinol Metab 2013;305:E1309–E1318
43. Lerner AG, Upton JP, Praveen PV, et al. IRE1α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. Cell Metab 2012;16:250–264
44. Xu G, Chen J, Jing G, Shalev A. Thioredoxin-interacting protein regulates insulin transcription through microRNA-204. Nat Med 2013;19:1141–1146
45. Huang HP, Chu K, Nemoz-Gaillard E, Elberg D, Tsai MJ. Neogenesis of beta-cells in adult BETA2/NeuroD-deficient mice. Mol Endocrinol 2002;16:541–551