Severe NEUROG3 mutation underlies pancreatic endocrine and exocrine insufficiency

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Abstract

Patients with *NEUROG3* mutations suffer from diabetes mellitus due to pancreatic endocrinopathy and chronic malabsorptive diarrhea due to enteric endocrinopathy. We have identified a severe truncation mutation in *NEUROG3* (P39PfsX38) that results in pancreatic exocrine insufficiency and significantly contributes to chronic malabsorptive diarrhea. We identified this novel phenotype by interrogating induced pluripotent stem cells from the *NEUROG3*-P39PfsX38 patient's fibroblasts and an isogenic "wild-type" control cell line generated via CRISPR-Cas9 gene editing. We discovered that *NEUROG3*-P39PfsX38 lines failed to activate pancreatic progenitor and differentiated lineage markers, suggesting that the mutation may disrupt pancreatic organogenesis and could result in endocrine and exocrine dysfunction. Isogenic corrected cell lines differentiated into all pancreatic lineages. Clinical assessments concluded that the patient has exocrine pancreatic insufficiency. Treatment with pancreatic enzyme replacement therapy improved patient outcomes, including weight gain, fat absorption, and resolution of fat-soluble vitamin deficiency. These results expose a novel role for *NEUROG3* in human pancreatic differentiation and illustrate how patient-specific stem cells can be used to interrogate disease etiology and affect patient care.

Main Text

Patients with *NEUROG3* mutations suffer from diabetes mellitus due to pancreatic endocrinopathy and chronic malabsorptive diarrhea due to enteric endocrinopathy. The role of *NEUROGENIN3* (*NEUROG3*) as a master regulator of pancreatic and intestinal endocrine differentiation has been modeled in detail using transgenic animals and human embryonic stem cells¹⁻⁸. Animal models with biallelic deletions of *Neurog3* have no pancreatic or enteric endocrine cells and die within hours after birth. Human embryonic stem cell models of *NEUROG3*-independent differentiation have concluded that very few pancreatic endocrine cells can differentiate in the absence of *NEUROG3*, and these differentiated cells are likely not functional^{1,7,9,10}. In contrast, patients with *NEUROG3* mutations develop insulin-dependent diabetes, with onset ranging from 13 days to >23 years of age¹¹. Patients also develop severe generalized malabsorptive diarrhea that fails extensive dietary challenges in the immediate postnatal period. It is thought that the clinical phenotypes are less severe than the deletion models because the hypomorphic clinical mutations retain some functionality lost in the null model systems.

We identified a 16-year-old male with a biallelic frameshift mutation in the *NEUROG3* gene (c117delC; *P39PfsX38*) that resulted in a truncated protein that is functionally null for any biochemical activity¹². At 2 weeks of age he was diagnosed with malabsorption and was temporarily managed with parenteral nutrition. At nearly 3 years of age, he was admitted with a blood glucose of >600 mg/dL and diagnosed with diabetes. He was negative for islet autoantibodies and his c-peptide levels declined over time (Supplementary Fig 1). Immunohistochemical staining of endocrine cells on the subject's duodenal biopsy was negative, confirming the null phenotype of the patient's mutation (Figure 1A). The contrast between the phenotype of null model systems and our biochemically null *P39PfsX38* subject's clinical

presentation suggests that the molecular mechanisms by which *NEUROG3* regulates human pancreatic cell differentiation are complex and require further investigation.

We created induced pluripotent stem cells (iPSCs) from the subject's fibroblasts to investigate how the *P39PfsX38* mutation affected pancreas differentiation. Native iPSC clones were referred to as *NEUROG3*^{NULL}. We replaced the mutated single exon of *NEUROG3* with a wild-type copy of the exon in the endogenous gene locus using CRISPR-Cas9 cellular engineering (Figure 1B) ¹³. Corrected clones were sequenced to verify biallelic correction of the mutation and were referred to as *NEUROG3*^{CORR} (Figure 1C). *NEUROG3*^{NULL} and *NEUROG3*^{CORR} iPSCs had normal karyotypes, expressed pluripotency markers, and had low expression of common lineage markers (Supplementary Figure 2A-D). *NEUROG3*^{NULL} and *NEUROG3*^{CORR} cells appropriately expressed endoderm, ectoderm, and mesoderm markers when subjected to lineage-specific differentiation protocols (Supplementary Figure 3).

To identify the developmental stage at which the *NEUROG3-P39PfsX38* mutation disrupted human pancreas differentiation, we assessed the cell fates of *NEUROG3*^{NULL} and *NEUROG3*^{CORR} cells at key steps of a reproducible beta cell differentiation protocol ¹⁴. Both cell lines differentiated into definitive endoderm at similar rates (Supplementary Figure 4). Next, we assessed the differentiation of mature, competent pancreatic progenitor cells by the co-expression PDX1 and NKX6.1 ^{15,16}. Immunocytochemical staining and flow cytometry established that *NEUROG3*^{NULL} cells differentiated into similar numbers of PDX1+ cells as *NEUROG3*^{CORR} cells, but significantly fewer PDX1⁺/NKX6.1⁺ competent pancreatic progenitor cells (9% vs 24% by flow cytometry, respectively, n=6, p<0.001; Figure 2A-B). Quantitative PCR confirmed that the expression of *PDX1* was similar in both cell lines, but *NEUROD1* expression, a *NEUROG3*-dependent transcription factor, was significantly decreased in *NEUROG3*^{NULL} cells (Figure 2C, p<0.05, n=4). Expression of the *NEUROG3*-independent pro-endocrine gene *MAFB* was unchanged (Figure 2C).

To understand the global impact of the *P39PfsX38* mutation on the differentiation of pancreatic progenitor cells, we assessed the RNA-transcriptome of *NEUROG3*^{NULL} and *NEUROG3*^{CORR} pancreatic progenitor cells. We normalized the expression profiles against pluripotent H1 embryonic stem cells. As expected, pluripotency genes were enriched in the H1 cells, whereas genes involved in endodermal fate commitment were similarly enriched in both *NEUROG3*^{NULL} and *NEUROG3*^{CORR} cell lines. However, the expression profiles of *NEUROG3*^{NULL} and *NEUROG3*^{CORR} cells diverged among genes associated with the pancreatic progenitor cell fate (Figure 2D).

Next, we attempted to differentiate both *NEUROG3*^{NULL} and *NEUROG3*^{CORR} cell lines into the pancreatic beta-like cell lineage (β LCs) (Figure 3A). Immunocytochemistry revealed that *NEUROG3*^{NULL} cells could not generate significant numbers of β LCs *in vitro*, while *NEUROG3*^{CORR} cells differentiated readily (Figure 3B). Rare *NEUROG3*^{NULL} endocrine cells were polyhormonal and did not coexpress c-peptide and NKX6.1, (Figure 3B) ^{15,16}. We quantified the number of c-peptide⁺ cells present in both the *NEUROG3*^{NULL} and

NEUROG3^{CORR} β LCs. Less than 1% of NEUROG3^{NULL} β LCs were c-peptide⁺, while 22.24% of NEUROG3^{CORR} β LCs were c-peptide⁺ (Figure 3C, $p \leq 0.02$, $n=5$). *INS* transcripts were very low in NEUROG3^{NULL} β LCs, but were highly expressed in NEUROG3^{CORR} β LCs (Figure 3D, $p < 0.001$, $n=3-5$). We measured the insulin secretory index, which is the ratio of insulin secreted at high glucose and low glucose, in NEUROG3^{NULL} and NEUROG3^{CORR} β LCs cultured as 3D clusters for 3 days *in vitro* using a static glucose-stimulated insulin secretion assay. We then transplanted clusters under the kidney capsule of NSG mice to facilitate maturation. After 12 weeks, we measured human insulin levels in the plasma of mice that were fasted then subjected to a glucose challenge (Figure 3E). Before transplantation, neither NEUROG3^{NULL} nor NEUROG3^{CORR} cell clusters appropriately increased insulin secretion in response to glucose (see pre-implant bars, Figure 3E). 12 weeks after transplantation, NEUROG3^{CORR} cells mounted a mature human insulin secretory response to glucose, while NEUROG3^{NULL} cell response was still immature (see post-implant bars in Figure 3E).

Because NEUROG3^{NULL} iPSCs did not differentiate efficiently into β LCs and did not express mature pancreatic progenitor cell markers, we hypothesized that these cells might have a diminished capacity to differentiate into the pancreatic exocrine lineages. To this end, we assessed exocrine differentiation. NEUROG3^{CORR} cells were able to differentiate into acinar and ductal cells, while far fewer NEUROG3^{NULL} cells were positive for the exocrine lineages (Supplementary Figure 5). This raised the possibility that the patient's mutation might compromise pancreatic progenitor cell contribution to organogenesis *in vivo* and that the subject may have undiagnosed exocrine pancreatic insufficiency (EPI). Chronic malabsorptive diarrhea is a shared clinical manifestation of enteric anendocrinosis and EPI.

EPI's formal diagnosis includes chronic malabsorptive diarrhea, low fecal elastase levels, and diminished exocrine secretory response to hormone stimulation. Diagnostic secretin-enhanced magnetic resonance cholangiopancreatography (MRCP) of the subject's pancreas indicated it was hypoplastic (Fig 4A, Supplementary video 1). Quantification of pancreatic volume from MRCP revealed a 5-fold decrease in the patient's pancreatic volume index compared to the average index of age-matched control subjects (Figure 4B). The patient's fecal elastase level was very low and exocrine pancreatic function tests (EPFT) revealed decreased exocrine pancreatic enzyme levels after secretin stimulation (Figure 4B)¹⁷⁻¹⁹. The subject was diagnosed with clinical EPI and started pancreatic enzyme replacement therapy (PERT) to treat EPI. He demonstrated weight gain, fat absorption, and resolved fat-soluble vitamin deficiency with PERT treatment.

Discussion

The assumption that *NEUROG3* is essential but not required for human pancreatic endocrine differentiation has evolved from studies that concluded that most clinically relevant *NEUROG3* mutations are hypomorphic and has been supported by clinical reports indicating that affected subjects have some residual pancreatic endocrine function for varying lengths of time^{1,9,11,20}. The novel *NEUROG3-P39PfsX38* mutation that is the subject of this study results in a truncated protein with no functional

activity; therefore these experiments served as an opportunity to interrogate the molecular and clinical consequences of a truly “null” human phenotype¹². Our study has concluded that the loss of *NEUROG3* function has consequences upstream of endocrine differentiation and is important in the pancreatic progenitor cell population during organogenesis.

Prior studies of *NEUROG3* have mostly limited its role as a master endocrine transcription factor for the pancreas and the intestine. Mouse studies have concluded that the competence of *Neurog3+* cells can change over time. It has been shown that *Neurog3+* cells can contribute to all pancreatic lineages early in development. As organogenesis progresses, *Neurog3+* cells are specified and required for endocrine lineage differentiation. The delamination and formation of *NEUROG3*-endocrine islet structures are important for exocrine branching morphogenesis late in development, but does not affect exocrine differentiation^{7,10,21}. Our data suggest that *NEUROG3* plays a role in human pancreatic progenitor competence and is critical for differentiation into both the endocrine and exocrine compartments.

While some of our results mirror reports that *NEUROG3*-deficient embryonic stem cell lines cannot make mature pancreatic endocrine cells *in vitro*^{1,3,9}, we are the first to show that transplantation of *NEUROG3*^{NULL} cells under the kidney capsule results in minimal β LC maturation. This suggests that *in vivo* maturation creates a milieu that can facilitate *NEUROG3*-independent pancreatic endocrine maturation in a manner that *in vitro* protocols cannot recapitulate. This may explain why patients with *NEUROG3* mutations are born with some functional pancreatic endocrine capacity but lose the capacity to secrete insulin over time; insufficient and immature *NEUROG3*-null beta cells may lose the capacity to produce minimal c-peptide as metabolic demands for insulin increase with body mass and age.

Taken together, we conclude that our patient-specific iPSC model revealed that *NEUROG3* is essential for human pancreatic progenitor cell competence. It is possible that EPI may result from a fundamental defect of pancreatic progenitor cell competence in patients with severe *NEUROG3* mutations. Clinically, malabsorptive diarrhea due to the underlying enteric endocrinopathy may mask the possibility that EPI significantly contributes to diarrhea. Our observation is also supported by a report documenting that other patients with *NEUROG3* mutations have hypoplastic pancreas¹¹. Thus, patients with *NEUROG3* mutations should be routinely screened for hyperglycemia and EPI and prescribed insulin and enzyme replacement therapy as appropriate.

Declarations

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Methods

Human Subjects

All studies were approved and completed in accordance with CHLA Institutional Review Board. Skin fibroblasts were collected from the patient and reprogrammed into induced pluripotent stem cells using a

vector-free approach ²². To generate a corrected control cell line, we used CRISPR-Cas9 to excise the gene by targeting guide RNAs to the single exon of *NEUROG3* and designed a HDR plasmid to facilitate homologous recombination of a wild-type copy of *NEUROG3* into the endogenous gene locus (see Figure 1B) ¹³. Clones were sequenced to verify the correction of the mutation and excision of the selection gene (see Figure 1C). Experimental results are expressed using independent differentiations from a minimum of 2 independent clones per line.

MRI analysis

Pancreata were manually outlined in each MRI plane with ImageJ software. Pancreas volume was 3D reconstructed and quantified with Arivis Vision4D v2.12.3 software.

Human Pluripotent Stem Cell Culture and Differentiation

The stem cell core at Children's Hospital Los Angeles provided the H1 embryonic stem cell line, licensed from WiCell. All human pluripotent stem cells were cultured using feeder-free conditions on plates coated in Vitronectin (StemCell Technologies) or Geltrex (Gibco) and fed with mTESR complete media (Stem Cell Technologies). Differentiation of planar hPSCs was initiated when the cells reached 90% confluency using the STEMdiff Pancreatic Progenitor (Stem Cell Technology cat#05120) to produce pancreatic progenitor cells. Cells were subjected to differentiation cocktails from Rezania et al. to progress through the pre-endocrine and immature beta-like cell stages ²³.

Karyotyping

G-band karyotyping was performed on two clones per genotype. Banding method (resolution): g-banding (500~550) number of cells counted: 20, number of cells analyzed: 20, number of cells karyotyped: 5.

RNA Isolation and qRT-PCR

RNA was extracted using Invitrogen Ambion RNA purification kit (#AM1924). cDNA was generated using Takara Primescript RT Master Mix (#RR036A). Taqman Gene Expression Master Mix (#4369016) and the AP biosystems gene expression probes and Step One real-time PCR machine. H1 pluripotent cells were used as a relative quantitative reference. GAPDH was used as the housekeeping gene. The entire probe list can be found in Supplementary Table 2.

RNA Sequencing

RNA was extracted using Invitrogen Ambion RNA purification kit (#AM1924) and samples sequence by Quick Biology Inc. FASTQ files corresponding to the pancreatic progenitor stage from NGN3NULL and NGN3CORR samples were pseudoaligned, using kallisto ²⁴, to the human transcriptome to obtain transcript per million (tpm) abundance estimates. To compare expression levels of selected genes, abundance estimates were merged with archived transcriptomic profiles (series GSE41009 on the NCBI Gene Expression Omnibus) of H1 embryonic stem cells ²⁵. A linear normalization factor was computed from the median values in the distribution of abundance estimates across all transcripts from H1 cells

and our NGN3-targeted cell lines. To select genes related to pluripotency, endoderm specification, and pancreatic differentiation, gene ontology pathways corresponding to these keywords were manually merged and curated. The resulting subset of abundance estimates was filtered to exceed an absolute log2 fold change of >0.3 of differential expression and scaled before generating heatmaps in R.

Flow Cytometry Analysis

Cells were dissociated with TrypLE and DNase, and stained with fixable viability dye 450 (BD) in dPBS with Y27632 (72304) for 12 minutes and washed 2x with PBS+0.2% BSA. Single cells were fixed with fixation/permeabilization buffer from BD (#554722) and washed 2x with Perm/Wash buffer (#554723). Cells were stained with stage-specific conjugated antibodies overnight in wash buffer and washed 2x with PBS+0.2%BSA, resuspended in PBS+0.2%BSA and analyzed on LSRII at the Saban Research Institute Flow Cytometry Core. Approximately 10,000 cells were analyzed per sample. Plots were analyzed on Flowjo version 10. Cells were gated to exclude debris, non-single cells, and non-viable cells. A list of flow antibodies and their dilutions is found in Supplementary Table 3.

Immunocytochemistry

8-well chamber slides were washed with PBS and fixed with 4% PFA in PBS for 15 minutes. Chambers were washed 3x with PBS and left in PBS + sodium azide at 4 degrees until ready to stain. Cells were permeabilized with TBS 0.4% triton for 20 minutes and washed with TBS tween for 3 minutes. Chambers were blocked with 0.2% Tween 20/3% IgG-free BSA/Tris-buffered solution for 30 mins at room temp. Primary antibodies were incubated overnight in 4C. Chambers were washed 2x with TBS tween. Secondary antibodies were applied at 1:500 for 30 minutes at room temperature. Chambers were washed with TBS tween 2x and TBS 1x. Slides were mounted with DAPI mounting media (Vector Labs) and imaged. All images were acquired at the TSRI imaging core. A list of primary antibodies and their dilutions is found in Supplementary Table 4.

Kidney Capsule Transplantation

All animal studies were approved and completed in accordance with CHLA Institutional Animal Care and Usage Committee. 8-12 week old NSG mice were anesthetized and prepped for surgery using aseptic techniques. Dermal and muscle incisions exposed the kidney. The kidney was lifted out of the body cavity using forceps and a 27G butterfly needle was used to inject beta-like cell cluster or human islets under the kidney capsule. The kidney was reinserted into the body cavity and the muscle and dermal layers sutured. Animals were given analgesic relief and monitoring post-surgery.

Hormone Content and Metabolic Analysis

Glucose Stimulated Insulin Secretion

In vitro GSIS was performed on beta cell-like clusters with two technical replicates per differentiation. N=3-4 independent differentiations per cell line. GSIS was performed as previously described.

Human Plasma Insulin Assay

12 weeks post transplantation of beta-like spheres under the kidney capsule, mice were fasted overnight. Blood glucose was measured using a standard glucometer. Mice were injected with 3mg/kg glucose. Serum was collected in silicone-coated serum gel tubes (BD Microtainers 365967) before glucose injection and 30 minutes post-injection. Serum samples were kept on ice and centrifuged for 3 minutes at 10,000 g. Serum was stored at -20°C until analysis.

Insulin Concentration

GSIS and plasma samples were analyzed for insulin content using Alpcó's Ultrasensitive Insulin Elisa kit (80-INSHUU-E01.1).

Statistical Analysis

Experiments were carried out at least three times and presented as the average values $\pm$ SD. Statistical analyses were performed with GraphPad Prism (GraphPad Software). The difference between samples was compared by the two-tailed Student's t-test and was considered significant at $p < 0.05$.

Figures

Figure 1

Generation of an induced pluripotent patient-specific NEUROG3^{NULL} stem cell line and isogenic mutation correction by CRISPR-Cas9 gene editing. (A) Immunohistochemistry of duodenal biopsy specimens from the subject and age-matched control. Chromogranin A staining (brown), a marker for endocrine cells, is absent from the subject's sample. (B) Targeting strategy for CRISPR-mediated gene editing. (C) Sanger sequencing of select clones for NEUROG3^{NULL} and NEUROG3^{CORR} cells indicated the biallelic correction of the P39Psf38 mutation.

Figure 2

NEUROG3^{NULL} cells have decreased expression of the pancreatic progenitor cell marker NKX6.1. (A) Immunocytochemistry of pancreatic progenitor cells for PDX1 (red) and NKX6.1 (green) in NEUROG3^{NULL} and NEUROG3^{CORR} cells. PDX1 levels are consistent between the cell lines, but NKX6.1 is decreased in NEUROG3^{NULL} cells. (B) Quantification of flow cytometry shows that NKX6.1+ PDX1+ competent pancreatic progenitor cells are decreased in NEUROG3^{NULL} cell line. n=6. (C) Quantitative

PCR to measure gene expression for PDX1 as a pancreatic progenitor marker, NEUROD1 as a NEUROG3-dependent pre-endocrine marker, and MAFB as a NEUROG3-independent markers. n=6-8. (D) Heatmap of select pluripotency, endoderm, and pancreatic progenitor gene expression. n=2 per cell line. Data normalized to pluripotent H1 cells. ***p<0.001.

Figure 3

NEUROG3NULL cells do not differentiate into beta-like cells in vitro. (A) Schematic of the differentiation protocol. The PancDiff kit from Stem Cell Technologies was used to differentiate pluripotent stem cells to the pancreatic progenitor cell stage. Protocols from Rezania et al. (2014) were employed to differentiate pancreatic progenitor cells into β LCs. (B) Representative immunocytochemistry images of the β LC stage for PDX1 (red) and NKX6.1 (green) in NEUROG3NULL and NEUROG3CORR cells. PDX1 levels are consistent between the cell lines, but NKX6.1 is decreased in NEUROG3NULL cells. (C) Quantification of flow cytometry experiments indicates fewer differentiated c-peptide+ cells from the NEUROG3NULL cell line. n=5. (D) Quantitative PCR to measure INS gene expression. n=3-5. (E) Glucose-stimulated insulin secretion and plasma insulin assays indicate that NEUROG3NULL cells do not have a mature secretory response to glucose challenge in vitro (pre-implantation) or in vivo (post-transplantation). n=3-5, *p<0.05, **p<0.01, ***p<0.001.

B

Clinical Characteristics				
	Subject	Reference		
Age ²³	16 y/o	15.9 y/o		
BMI ²³	21.99	21.4		
C-Peptide ²³	<0.1 ng/dL	1.6 ng/dL		
Pancreatic Volume ²³	6.5 ml	48.4ml		
Pancreatic Volume Index ²³	0.170 ml/kg	0.929 ml/kg		
Fecal Elastase ²²	29 ug/g	>200 ug/g		
Exocrine Pancreatic Function				
	Trypsin nmol/mL/min	Amylase μmol/mL/min	Lipase μmol/mL/min	Chymotrypsin μmol/mL/min
5 min (normal range values) ²¹	58.5 (188.2 - 565.3)	101.5 (105.6 - 337.0)	24.3 (276.5 - 1021.5)	8.9 (6.2 - 24.9)
10 min (normal range values) ²¹	23.1 (131.2 - 432.3)	5.9 (72.2 - 255.8)	36 (310.0 - 748.0)	1.9 (6.1 - 18.0)
15 min (normal range values) ²¹	20.3 (128.3 - 388.7)	3.2 (53.4 - 219.3)	62.2 (207.0 - 674.0)	0.9 (2.5 - 15.9)

Figure 4

Exocrine pancreatic insufficiency may be a consequence of diminished pancreatic progenitor cell competence. (A) Amylase (green) and Cytokeratin19 (red, marker for ductal cells) is decreased in NEUROG3NULL cells when compared to NEUROG3CORR cells differentiated into the exocrine lineages. (A) MRCP images of the subject and age-matched control. Control subject clinical characteristics are fall within the normal reference range stated within the literature. (B) Clinical measurements of subject and

age-matched normal controls (values referenced in the literature, 21-23). All measures of exocrine pancreatic morphology and function are abnormally low.

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