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RESEARCH ARTICLE

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Recovery of viable endocrine-specific cells and transcriptomes from human pancreatic islet-engrafted mice

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Abstract

Human pancreatic islets engrafted into immunodeficient mice serve as an important model for in vivo human diabetes studies. Following engraftment, islet function can be monitored in vivo by measuring circulating glucose and human insulin; however, it will be important to recover viable cells for more complex graft analyses. Moreover, RNA analyses of dissected grafts have not distinguished which hormone-specific cell types contribute to gene expression. We developed a method for recovering live cells suitable for fluorescence-activated cell sorting from human islets engrafted in mice. Although yields of recovered islet cells were relatively low, the ratios of bulk-sorted β, α, and δ cells and their respective hormone-specific RNA-Seq transcriptomes are comparable pretransplant and posttransplant, suggesting that the cellular characteristics of islet grafts posttransplant closely mirror the original donor islets. Single-cell RNA-Seq transcriptome analysis confirms the presence of appropriate β, α, and δ cell subsets. In addition, ex vivo perifusion of recovered human islet grafts demonstrated glucose-stimulated insulin secretion. Viable cells suitable for patch-clamp analysis were recovered from transplanted human embryonic stem cell-derived β cells. Together, our functional and hormone-specific transcriptome analyses document the broad applicability of this system for longitudinal examination of

Abbreviations: 7-AAD, 7-aminoactinomycin; BMI, body mass index; BSA, bovine serum albumin; DAPI, 4′6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ESAT, End Sequence Analysis Toolkit; FACS, fluorescence-activated cell sorting; FPL, FPL 64176; GCG, glucagon; GSIS, glucose-stimulated insulin secretion; HbA1c, hemoglobin A1c; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IACUC, Institutional Animal Care and Use Committee; IEQ, islet equivalent; INS, insulin; lncRNA, long non-coding RNA; MALAT1, metastasis associated lung adenocarcinoma transcript 1; NSG, NOD-*Prkdc*^{scid} *IL2rg*^{tm1wjl}; PBS, phosphate-buffered saline; PRSS1, serine protease 1 (trypsinogen 1); RRID, Research Resource Identifier; RSEM, RNA-Seq by Expectation-Maximization; SC-β, human embryonic stem cell-derived-β cells; SCRB-Seq, single-cell RNA barcoding and sequencing; scRNA-Seq, single-cell RNA sequencing; SST, somatostatin; TEA, tetraethylammonium; TXNIP, thioredoxin interacting protein; UMAP, uniform manifold approximation and projection.

Sambra D. Redick and Linda Leehy contributed equally to this work.

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human islet cells undergoing developmental/metabolic/pharmacogenetic manipulation in vivo and may facilitate the discovery of treatments for diabetes.

KEYWORDS
graft recovery, insulin, L-type voltage-gated calcium channel, RNA-Seq, β cell

1 | INTRODUCTION

Pancreatic islets are the exclusive source of insulin (INS), glucagon (GCG), and somatostatin (SST) hormones that are produced by β, α, and δ cells, respectively. The growth and differentiation of islet cells and their respective hormone secretion must be rigorously regulated to maintain glucose homeostasis. Many elegant studies report transcriptome analyses of antibody-sorted or single-cell data derived from isolated human islets obtained from pancreatic organ donors (reviewed in [1]). Although such data are invaluable, only "snapshot" information is provided from individuals with inherently variable genetics and lifestyles. Thus, the development of immunodeficient mice greatly enhanced human islet studies by permitting in vivo study of transplanted islets from individual donors under well-controlled experimental conditions.[2-9] RNA-Seq and other gene expression analyses of bulk-dissected islet graft tissue have further refined the study of human islets in vivo.[10] The recovery of sufficient live hormone-specific cells from the graft for single-cell transcriptome and functional analysis would facilitate the expansion of in vivo islet studies, yet no successful attempts have been reported.

We developed a new method to recover live single cells from long-term human islet grafts transplanted under the kidney capsule of immunodeficient mice. To validate that the posttransplant islet cells were representative of the pretransplant primary islets from the individual donor, transplants were performed using normoglycemic, experimentally unmanipulated mice. Extensive analyses of the pre and posttransplant cells confirmed that the recovered human islet cells retained their hormone-specific phenotype, gene expression, and function posttransplant. Although our focus was on pancreatic islets from individual organ donors, we also verified that our islet graft recovery method allowed efficient recovery of live cells following long-term transplantation of human embryonic stem cell (SC)-derived β cells. Taken together, these data suggest that this model system may be useful for interrogating the response of transplanted human islets or SC-β cells to various developmental, metabolic, or therapeutic regimens in vivo. In vivo assessment of the effects of such manipulations on transplanted human islets at the individual cell level, in turn, should lead to more focused clinical studies for the treatment and/or prevention of diabetes.

2 | MATERIALS AND METHODS

2.1 | Human islet procurement, SC-beta cell generation, and pretransplant analysis

Human islets were obtained from the NIH Integrated Islet Distribution Program or Prodo Laboratories Inc (Aliso Viejo, CA, USA) (Table 1). To compare the pretransplant human islet cellular composition (which can vary widely) and gene expression to that measured posttransplant, we studied an aliquot of 4000 to 7000 islet equivalents (IEQs) from each donor pretransplant. The human SC-β cells were generated as previously described.[11] The islets were stained with antibodies to INS, GCG, and SST and then separated by fluorescence-activated cell sorting (FACS) into β, α, and δ-cells, respectively, using methodology developed in our laboratory.[12] The sorted endocrine cell populations and the subpopulation of triple-negative cells were pelleted and Proteinase K digested using the Digestion Buffer from the RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA). Following protease digestion, samples were diluted in an equal volume of nuclease-free water and nucleic acids were purified using 1.9 volumes of Agencourt RNAClean XP beads (Beckman Coulter, Brea, CA, USA). Following elution with nuclease-free water, the purified nucleic acids were treated with TURBO DNase (Ambion) and purified using 1.9 volumes of Agencourt RNAClean XP beads. RNA eluted in water was quantified and analyzed for RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and stored at −80°C until further processing.

2.2 | Mice and islet and SC-β transplantation

Twelve- to 16-week-old NOD-*Prkdc*^{scid} *IL2rg*^{tm1wjl} (NSG) mice of both sexes were obtained from The Jackson

TABLE 1 Demographic characteristics of human islet donors

	Donor 15275	Donor 15306	Donor 16083	Donor 15071
RRID	SAMN08773851			SAMN08774475
Age, years	38	41	37	22
Gender	M	F	F	M
Ethnicity	White	Hispanic	Hispanic/ Hawaiian/White	African-American
Body weight, kg	86.6	70.3	72.6	110.2
Height, in	66	68	67	72
BMI, kg/m^2	30.8	23.6	25.1	33
HbA1c, %	5.5	5.6	5.5	5.4
Confirmed type 2 diabetic donor	No	No	No	No
Time in culture (hours)[a]	22	nr	nr	84
Transplanted IEQs/mouse	3800	4000	4000	4000
Sequencing analysis	Bulk RNA-Seq	Bulk RNA-Seq	Bulk RNA-Seq	Single-cell RNA-Seq

Abbreviations: BMI, body mass index; nr, not recorded; RRID, Research Resource Identifier, when available.

[a]Refers to the amount of time that the human islets were cultured following isolation until shipment to our laboratory.

Laboratory. Mice were housed in a specific pathogen-free facility. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School. Islet transplants and SC-β cell transplants were performed as previously described.[3,8,11] Briefly, mice were anesthetized and the kidney was externalized through an incision through the skin and abdominal wall; ~4000 IEQs or 5 million SC-β cells (Douglas Melton lab, Harvard University, Cambridge, MA, USA) were injected into the renal subcapsular space using a 23G winged infusion set (Terumo Medical Corporation, Somerset, NJ, USA). The kidney was reinserted into the abdomen and the incision was closed.

2.3 | In vivo glucose stimulation

To confirm human islet function in vivo, at 2 to 3 weeks post-engraftment, mice were given an intraperitoneal injection of glucose (2 g/kg body weight). Blood sample was collected from the tail vein 15 minutes post-injection into heparinized tubes; plasma was collected and stored at −80°C until analysis. Human plasma INS levels were determined using a human-specific enzyme-linked immunosorbent assay (ELISA, ALPCO, Salem, NH, USA); unengrafted NSG mouse plasma was used as a negative control.

2.4 | Islet and SC-β cell graft recovery and dissociation

At 4 to 5 weeks post-engraftment, mice were deeply anesthetized and the engrafted kidney was exposed through a midline incision. The kidney was injected with 1 mL collagenase (Sigma, St. Louis, MO, USA, C-0130, 0.125 mg/mL) in RPMI 1640/2% horse serum (Gibco, Grand Island, NY, USA), then removed and the animal was quickly euthanized according to the approved IACUC protocol. The graft was located and the kidney was bisected into two hemispheres. The capsule containing the islet graft was peeled from the kidney hemisphere and placed into a large volume of RPMI 1640 containing 50% horse serum and the sample was placed on ice. Kidney capsules with attached grafts were first washed with Hank's balanced salt solution (HBSS, Mediatech, Manassas, VA, USA), then with 10 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS, Mediatech). The capsules were placed in 1 mL of pre-warmed PBS to 37°C, and 0.5 mL of pre-warmed TrypLE Express (Gibco) was added. Capsules and attached grafts were gently passaged through a blunt 16G needle 10 times per minute for 10 minutes to release the islet cells, then 5 mL of 2% bovine serum albumin (BSA) in PBS was added to quench the enzyme. Following the addition of 5 mL PBS, capsules were removed and the dissociated islet cells were washed with PBS.

2.5 | Immunohistochemistry

To interrogate the different stages of human islet cell recovery, selected islet graft-bearing kidneys, kidney capsules with attached grafts, and post-dissociation kidney capsules were separately obtained from individual mice and fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections were stained with guinea pig anti-INS (Dako, Carpinteria, CA, USA), mouse anti-GCG (Abcam, Cambridge, MA, USA), DAPI (4′6-diamidino-2-phenylindole, Sigma), and Alexa Fluor-labeled secondary antibodies (Molecular Probes, Eugene, OR, USA). Images were

acquired with a Nikon Eclipse Ti series microscope and analyzed with Nikon Elements image analysis software.

2.6 | Islet cell staining and FACS sorting

Following PBS washes, the dissociated islet graft cell pools were subjected to live/dead staining with Zombie Violet (BioLegend, San Diego, CA, USA). After a PBS wash, cells were fixed with 4% paraformaldehyde for 5 minutes on ice. Saponin was then added to a final concentration of 0.1% and the tube was rotated end over end for 25 minutes at 4°C. Cells were washed in PBS, then stained with Zenon Alexa 488 labeled mouse anti-somatostatin (clone 7G5, Thermo Fisher Scientific, Waltham, MA, USA), Zenon Alexa 568 (Molecular Probes) labeled mouse anti-glucagon (G2654, Sigma), and rabbit anti-insulin Alexa 647 (9008, Cell Signaling Technology, Danvers, MA, USA). Stained cells were sorted using a BD Biosciences FACS Aria II (UMass Medical School Flow Core Laboratory) and collected in PBS containing 0.5% BSA and RNasin (Promega, Madison, WI, USA).

2.7 | RNA isolation and quantitation

The sorted endocrine cell populations and the population of triple-negative cells were pelleted and digested using the Ambion RecoverAll Total Nucleic Acid Isolation Digestion Buffer and Proteinase K. Following nucleic acid purification and DNase treatment, RNA was purified as described above.

2.8 | Library construction and sequencing

Libraries of bulk-sorted β and α cells (pre and posttransplant) were constructed using the SMARTer Stranded Total RNA-Seq Kit—Pico Input Mammalian (Takara Bio USA, Mountain View, CA, USA) following the manufacturer's instructions. Islet grafts from a single human donor were dissociated, fixed, and stained for islet hormones as described above, then FAC-sorted into a 384-well plate (one cell per well) for single-cell RNA-Seq using SCRB-Seq.[13] All libraries were sequenced using an Illumina (San Diego, CA, USA) NextSeq 500.

2.9 | Bioinformatics analyses

Following FASTQ file generation in BaseSpace (Illumina), sequence files from bulk cell libraries were processed as described previously,[14] except that expected counts were filtered to include only genes with > 10 counts in at least three samples prior to submission to DESeq2 for differential gene expression analysis. Genes with at least a twofold change in expression and adjusted P values of < .01 were considered differentially expressed between pretransplant and posttransplant groups. All bulk and single-cell RNA-Seq data are available in the Gene Expression Omnibus repository (DataSet Identifier GSE138748).

2.10 | Electrophysiology

Whole-cell currents from dissociated β cells were recorded at room temperature (20-24°C) with an Axon 200B patch-clamp amplifier. Cells were exposed to dithizone for 2-5 minutes to identify β cells within the field of view and then washed off before establishing the whole-cell configuration. Currents were acquired using Signal 2.15 software (Cambridge Electronic Design; CED, Cambridge, UK) and stored for later analysis. Currents were elicited by stepping from −80 mV to 0 mV for 75 msec every 5 seconds. Currents were sampled at 5 kHz, filtered at 1 kHz and digitized at 5 times the filter cut-off frequency of the 4-pole Bessel filter of the amplifier using a micro1401 interface (CED). Linear leak and capacitive currents were subtracted from all traces.

Electrodes were pulled from borosilicate glass capillary tubes (Drummond Scientific Company, Broomall, PA, USA) and each electrode was fire-polished to ~1 μm to give the pipette a resistance of 2-3 MΩ. The pipette solution consisted of (in mM): 125 Cs-aspartate, 10 HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 10 EGTA, 5 $MgCl_2$, and 4 ATP brought to pH 7.50 with CsOH. High-resistance seals were established in Ca^{2+} Tyrode's solution that consisted of (in mM): 5 $CaCl_2$, 145 NaCl, 5.4 KCl, 10 HEPES, 3 glucose, and 10 tetraethylammonium (TEA), brought to pH 7.50 with TEA. Once a seal was established and the membrane ruptured, the Tyrode's solution was exchanged for an external bath solution (in mM): 125 NMG-aspartate, 20 Ba-acetate, 10 HEPES, 3 glucose, and 10 TEA, brought to pH 7.50 with TEA. The L-type Ca^{2+} channel agonist FPL 64176 (FPL) was included in the bath solution to enhance the current amplitude and elicit a slow, long-lasting L-channel tail current.[15] A 10 mM FPL stock solution was prepared in 100% ethanol and stored at −20°C. The FPL stock was diluted daily to a final concentration of 2 μM with external solution. All reagents used for acquiring whole-cell currents were purchased from Sigma-Aldrich unless otherwise noted.

3 | RESULTS

3.1 | Human islet grafts are functional in vivo and following graft recovery

An overview of the study design is shown in Figure 1. For transplant studies, human pancreatic islets from four nondiabetic donors were used (Table 1); islets from each

donor constituted an independent study. A small aliquot of human islets was stained with dithizone to verify INS production (Figure 2A) and, for the bulk transcriptome experiments, 4000-7000 IEQs were reserved for staining and FACS to compare the pretransplant (ie, pre-engraftment) human islet cellular composition and gene expression to that measured in recovered islets posttransplant (ie, post-engraftment). Remaining islets were transplanted into the renal subcapsular space of euglycemic immunodeficient NSG mice (n = 4-8 mice for each islet donor, ~4000 IEQs per mouse).

At 2 weeks posttransplant, a representative engrafted mouse was euthanized to validate that adequate vascularization of the islet graft had occurred prior to testing for

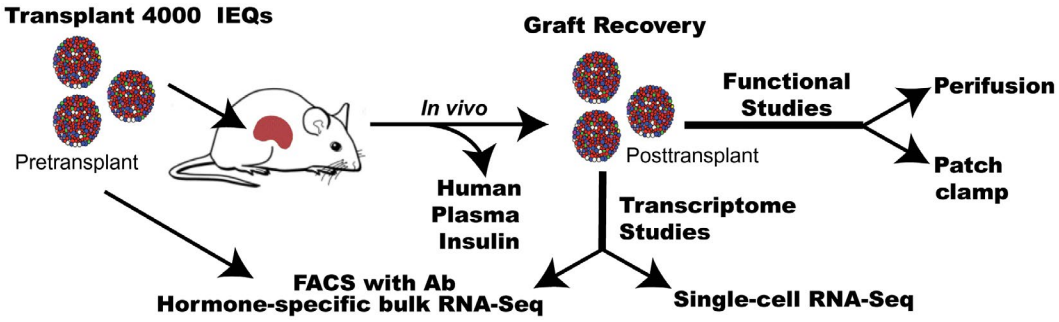

FIGURE 1 Overview. Human islet cells were transplanted into mice, recovered, and assessed as shown

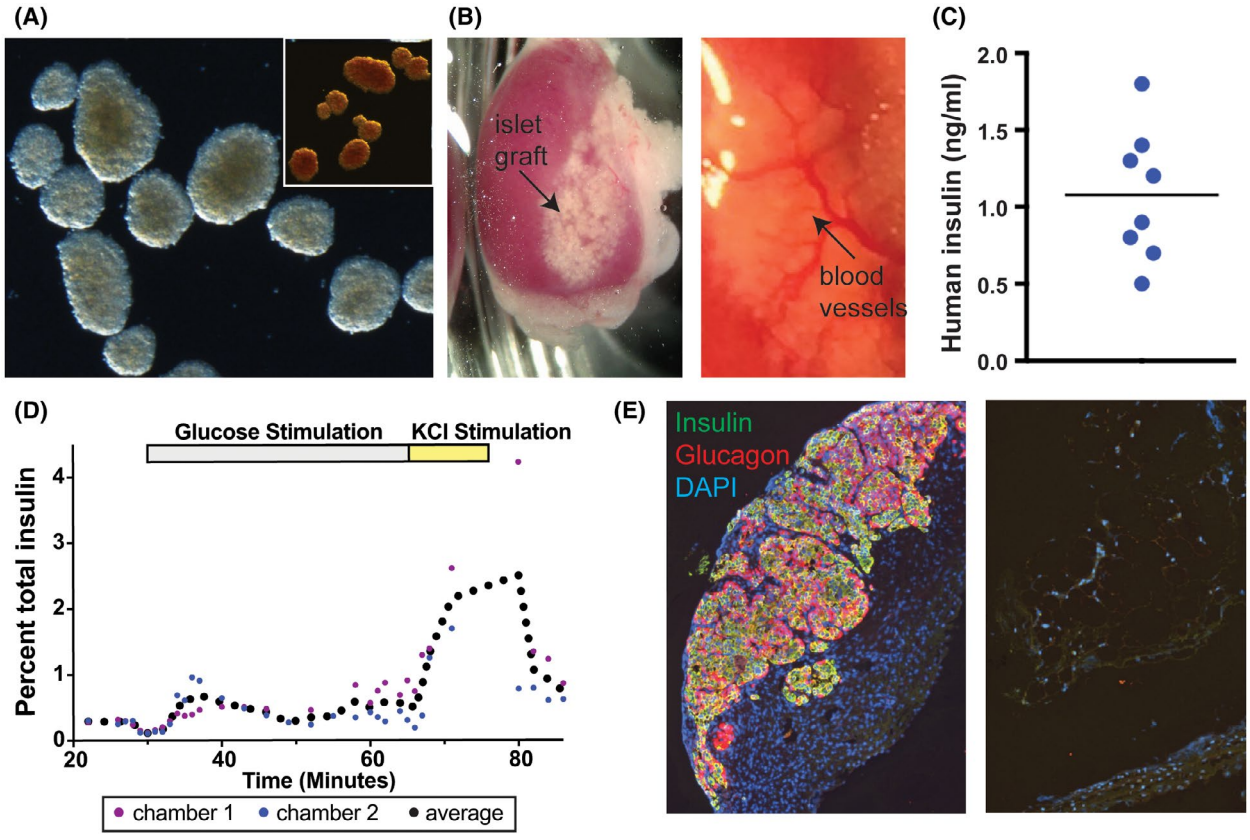

FIGURE 2 Islet graft transplant and functional assays. A, Human islets prior to transplant; stained with dithizone (inset). B, Islet graft following transplant under the kidney capsule (left); higher magnification shows vascularization of the graft (right). C, Human plasma INS recovered from mice challenged with glucose (2 g/kg body weight). D, Islet grafts (n = 2) were excised from the kidney, and GSIS was performed by perifusion. Insulin levels are shown in response to 20 mM glucose (gray bar) and KCl (yellow bar) as a percentage of total INS; the levels from each of two grafts are shown as a point with the average as a dashed line. E, INS (green) and GCG (red) staining of the kidney capsule and associated islet graft pre- (left) and post- (right) graft recovery, demonstrating successful removal of the graft from the kidney capsule

islet function (Figure 2B). At 2-3 weeks posttransplant, mice were challenged with intraperitoneal glucose (2 mg/kg body weight). Plasma collected 15 minutes post-stimulation showed robust human INS secretion (Figure 2C), confirming that the human islet grafts were functional in vivo.

At 4-5 weeks posttransplantation, the animals were anesthetized and the graft-bearing kidneys were recovered and bisected to retain the graft-containing hemisphere. To validate the function of the islet grafts posttransplant, glucose-stimulated insulin secretion (GSIS) was measured by perifusion of representative islet grafts from individual donors in independent studies. The kidney capsule was peeled away from the kidney proper and, together with its attached graft, placed in a chamber of a Biorep Technologies perifusion machine and perifused as described.[16] Briefly, the grafts were first maintained in basal (5.6 mM) glucose, then GSIS was demonstrated following treatment with 16.8 mM glucose; sufficient KCl (20 mM) to produce islet depolarization resulted in a further release of INS (Figure 2D). Together, these data indicate that the recovered posttransplant islet grafts were functional and glucose-responsive, albeit with glucose-stimulated insulin responses a bit lower than typically observed using freshly isolated human islets. The remaining graft-containing peeled kidney capsules were processed for islet cell recovery (see MATERIALS AND METHODS). Release of the capsule and attached graft by the initial collagenase treatment had no obvious effect on the engrafted islets, as INS and GCG immunostaining was robust (Figure 2E, left panel). Islets were mechanically and chemically dissociated from the kidney capsule into a single-cell suspension (see MATERIALS AND METHODS). Immunostaining of the kidney capsule following this step revealed no detectable INS and GCG staining, indicating efficient recovery of the transplanted human islets (Figure 2E, right panel).

3.2 | Ratios of β and α cells for each islet donor are similar pre and posttransplant by flow cytometry assessment

All dissociated cells from the recovered posttransplant islet grafts from a single donor were pooled, incubated with live/dead stain, fixed, and permeabilized. Immunostaining was performed with INS, GCG, and SST antibodies directly conjugated to appropriate fluorochromes. Islet cells were sorted based on immunostaining and the proportions of stained endocrine cells were compared with those obtained pretransplant (Figure 3A). We observed three major cell populations identified by their hormone expression as β (INS-positive), α (GCG-positive), and δ (SST-positive) cells, as well as a fourth population of triple-negative cells. As we and others have reported previously,[12] the relative β and α cell proportions vary considerably from donor to donor (Figure 3B). However, within each individual human donor islet prep, the percentages of β and α cells recovered from the islet grafts were essentially unchanged from their pretransplant levels, although we observed an apparent ~50% loss of SST-expressing δ cells in the posttransplant recovered cells (Figure 3B).

3.3 | The β- and α-cell transcriptomes for each islet donor are correlative pre and posttransplant

For each independent islet transplant study, the sorted cell populations from both the recovered posttransplant human islet grafts from each donor and primary islets from the same donor acquired pretransplant were processed for RNA isolation. RNA-Seq libraries from sorted β and α cells (from islets from donors 15275, 15306, and 16083, see Table 1) pre and posttransplant were constructed and sequenced to

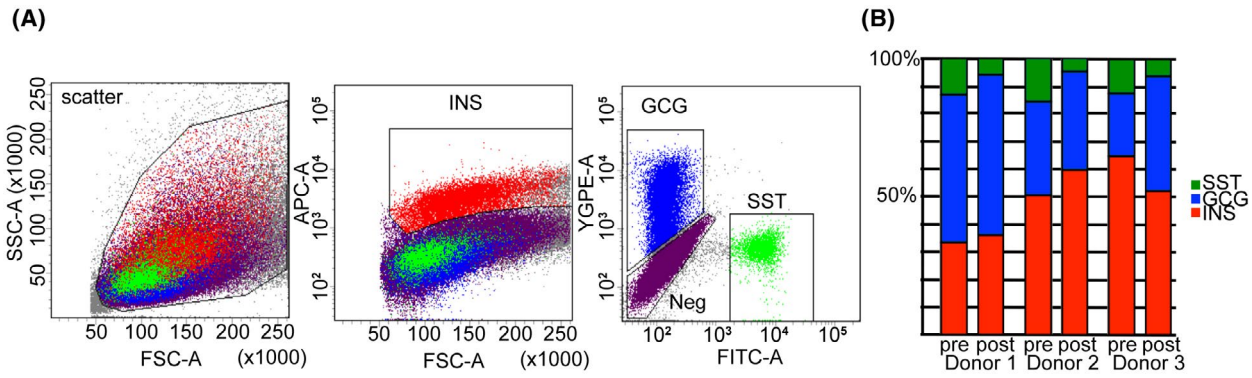

FIGURE 3 Hormone-specific islet cell recovery from human islet engrafted mice. A, Dissociated live cells from pretransplant islets and recovered human islet grafts were fixed, stained, and sorted based on their hormone expression; shown is a representative FACS sort of cells recovered from islet grafts from a single donor. B, The percentages of hormone-specific cells were similar for each individual donor pretransplant and posttransplant. Abbreviations: GCG, glucagon; INS, insulin; Neg, triple negative for INS, GCG, and SST; pre, pretransplant; post, posttransplant; SST, somatostatin

obtain an unbiased comparison of gene expression patterns between individual donor islets pre and posttransplant. Of the genes passing our filter (16 622 in β cells; 16 439 in α cells), 93.1% of β-cell transcripts and 88.7% of α-cell transcripts were expressed at similar levels, that is, < twofold difference, pre and posttransplantation. Correlations for pre and posttransplantation transcript abundance of the 200 most abundant genes (as an average of the three islet donors) are shown in Figure 4 for β and α cells. *INS* and *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) are the most abundant transcripts both pre and posttransplant in β cells (Figure 4, left); and, in the α-cell populations, *GCG* and *MALAT1* are the most abundant transcripts pre and posttransplant (Figure 4, right). *MALAT1* is a long non-coding RNA (lncRNA) highly expressed in purified human β and α cells.[17] Intriguingly, *MALAT1* resides within an active enhancer cluster containing multiple binding sites for islet transcription factors, suggesting a critical role in gene regulation of human islets.[18]

Transcripts which are notably differentially expressed between pre and posttransplant include several which are likely due to contaminating exocrine pancreas material. For example, in β cell populations, *PRSS1*, which encodes the exocrine pancreatic enzyme trypsinogen 1, is more abundant pretransplant than posttransplant (Figure 4, left), suggesting contaminating exocrine cells that are present pretransplant do not survive engraftment. In α cell populations, *PRSS1, CTRB1,* and *CTRB2* (encoding chymotrypsinogen 1b and 2b, respectively) are more abundant pretransplant than posttransplant (Figure 4, right), again suggesting that exocrine cells do not survive following engraftment. For both β and α cells, hormone transcript levels are relatively higher in the posttransplantation sample. Coupled with the decline in transcripts for protease genes of the exocrine pancreas, these data suggest that engraftment under the kidney capsule favors survival of endocrine cells and their continued secretory function.

Given that we previously reported 10 genes whose expression is distinct to highly purified human β cells and another 10 genes specific to highly purified α cells,[12] we wished to determine which of these genes might be differentially expressed pre and posttransplant. All 10 of the β cell-specific genes are expressed in both pre and posttransplant cells, and three genes (*INS*, *MAFA*, and *PDX1*) are significantly more highly expressed post-engraftment (Table 2, left). Similarly, 10 genes with distinctly high expression in α cells[12] are detected both pre and posttransplant, of which three are differentially expressed (all increased) in the posttransplant samples (Table 2, right).

In addition, we find that thioredoxin interacting protein (TXNIP) is expressed at higher levels in both α and β cells post-engraftment (β = 6.8 fold higher, P-adj = 6×10^{-31}; α = 4.7 fold higher, 3×10^{-20} P-adj). TXNIP is a thioredoxin-binding protein that regulates endoplasmic reticulum (ER) stress and is elevated by high blood glucose.[19] Since *TXNIP* upregulation induces β-cell apoptosis, we examined gene expression in the apoptosis pathway. Virtually all of the apoptosis genes with differential expression pre and posttransplant are expressed at lower levels posttransplant; while *INS* expression is also increased in the posttransplant samples, no concomitant increase in the genes of the ER stress pathway is observed (data not shown). This could indicate that the graft

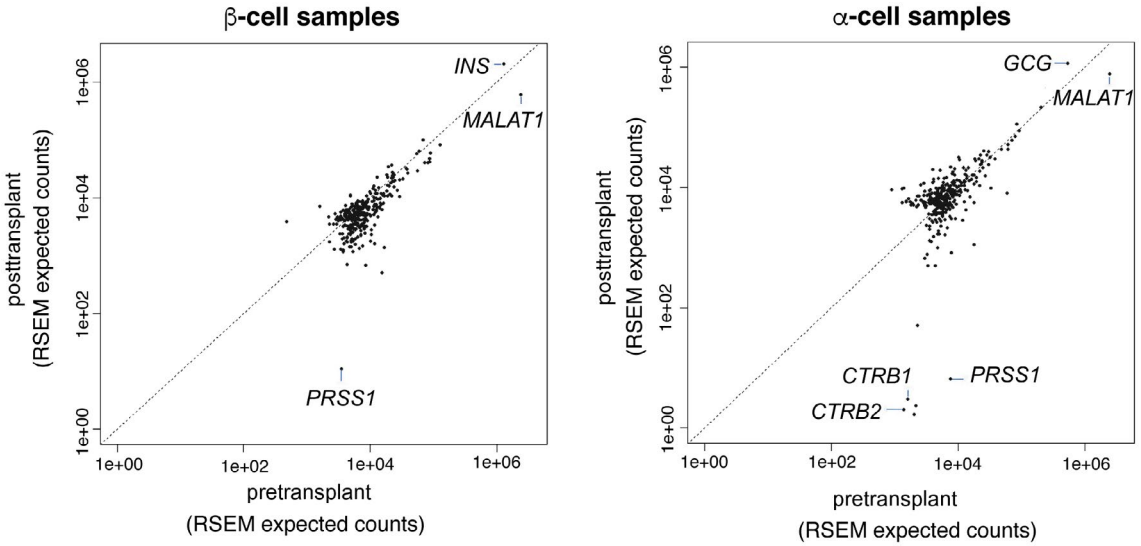

FIGURE 4 Transcript abundance pretransplant and posttransplant. The expected counts output from RNA-Seq by Expectation-Maximization (RSEM) for the 200 most abundant pretransplant transcripts (average of three human donors) are plotted on the *x*-axis and the corresponding values for posttransplant transcripts are plotted on the *y*-axis for β and α cells. Perfect correlation is indicated by the dotted line

TABLE 2 Differential expression of α- and β-associated transcripts posttransplant

β gene	Fold change	*P* adj	α gene	Fold change	*P* adj
INS	2.55	3.6×10^{-06}	*GCG*	1.78	.0007
IAPP	4.33	.2	*IRX1*	1.71	.05
PDX1	2.09	.0003	*IRX2*	1.70	.007
MAFA	2.03	.0005	*ARX*	1.57	.05
NKX6-1	1.86	.0002	*BAMBI*	1.50	.3
PCSK1	2.02	.2	***DPP4***	2.18	1.3×10^{-05}
KCNQ2	1.25	.5	***PTPRT***	2.29	2.1×10^{-06}
GLIS3	1.27	.2	*PTPRD*	1.04	.9
ACVR1B	1.42	.05	***POU6F2***	2.84	4.2×10^{-08}
CASR	1.65	.03	*KCND3*	.99	1

Note: Genes in bold have greater than 2-fold change and adjusted *P* values < .05.

environment is less stressful than standard ex vivo islet culture conditions.[20-22]

3.4 | Single-cell RNA-Seq reveals the expected islet cell populations within the recovered grafts

To further highlight the broad applicability of our islet graft recovery protocol, we performed single-cell RNA-Seq (scRNA-Seq) on human islet grafts posttransplant. In this experiment, five NSG mice were transplanted with human islets from a nondiabetic donor (Donor 15071, see Table 1). At 3 weeks posttransplant, islet grafts were dissociated, stained for islet hormones (excluding pancreatic polypeptide, made by γ cells), and the cells sorted into three 384-well plates for scRNA-Seq. Using uniform manifold approximation and projection (UMAP)[23] in tandem with our End Sequence Analysis Toolkit (ESAT)[24] and after filtering for cells with total read counts below 10% of the plate average, we find that posttransplant islet grafts segregate into five distinct groups of cells (Figure 5). Four of the major cell populations are identified by their hormone expression as β, α, δ, and γ cells; the fifth hormone-negative cell population was not further characterized. The identification of γ cells in our single-cell analysis validates the power of our graft recovery method to "capture" even minor populations of endocrine cells, as γ cells typically constitute only < 5% of the total human islet cells.[25] Of 721 cells identified as β, α, or δ by transcriptome and 710 cells identified as expressing INS, GCG, or SST by FACS, 665 cells are identified as the same endocrine cell type by both hormone staining and unbiased clustering of transcriptome data. These three human islet cell populations correspond with our bulk hormone-specific data, as well as published reports of single-cell analyses of human islets isolated from organ donors (reviewed in [1]). We did not detect subpopulations within the β cells, likely a consequence of the small

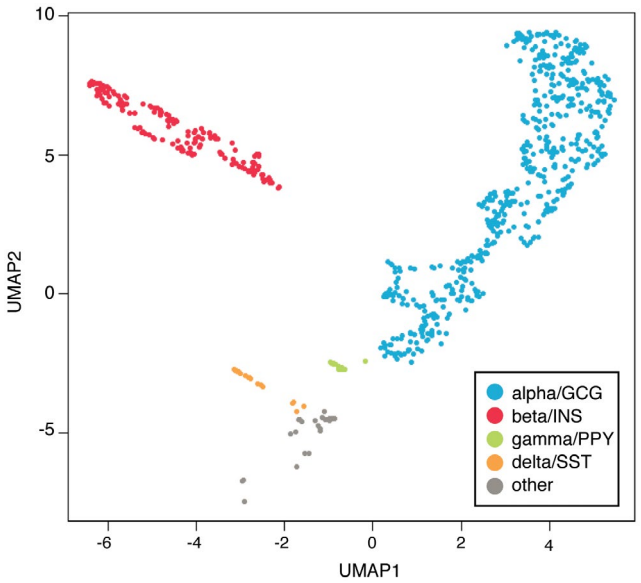

FIGURE 5 Single-cell transcriptomes from posttransplant human islets. Engrafted human islets were recovered for scRNA-Seq analysis; UMAP shows distinct subpopulations, including four hormone-specific cell subsets

number of engrafted cells recovered and the effects of fixation and staining on those cells' mRNA. In the future, sorting cells without fixation, identifying endocrine cells using autofluorescence,[26] and then capturing transcripts with SCRB-seq[13,27] will enhance our single-cell analyses. Applying such approaches to islet cells both pre- and post-engraftment will aide us in identifying islet cell subpopulations reported by others.[28,29]

Together, these data suggest that human islets maintain and/or recover much of their expression "identity" following in vivo engraftment in unmanipulated euglycemic mice. Therefore, human islets engrafted in mice are an excellent model for studying transcriptional changes in vivo following metabolic (e.g., hyperglycemia or high-fat diet) or pharmacogenetic challenges.

3.5 | Viable cells are recovered from transplanted human embryonic stem cell-derived β cells

Last, to show the feasibility of our graft recovery method for other experimental sources of β cells, an additional transplant study was performed using human embryonic SC-β cells (see MATERIALS AND METHODS) to determine the ability of our methodology to capture their in vivo development and function. At 14 days posttransplantation, grafts were recovered and the isolated SC-β cells were incubated with the live/dead stain 7-aminoactinomycin (7-AAD) (Figure 6A). For FACS, all viable (7-AAD-negative) cells were gated on the FITC channel, which detects the intrinsic autofluorescence found in β cells (Figure 6B). We recovered nearly 190 000 live autofluorescent cells, which we confirmed as INS positive by immunostaining (Figure 6C). As a test for function, whole-cell calcium currents were measured in both the pretransplant and recovered graft cells, which stained with dithizone. Patch-clamp experiments with recovered SC-β cells reveal that calcium currents underlying calcium-dependent insulin exocytosis[30-32] were robust and similar to currents observed in human primary islets in that they were sensitive to the L-type calcium channel agonist FPL 64176 (Figure 6D).

4 | DISCUSSION

In this study, we present the first report of a methodology to recover sufficient numbers of viable cells from long-term human pancreatic islet transplants to allow single-cell and/or bulk hormone-specific examination of transcriptome and functional analyses posttransplant. Whereas earlier reports of RNA analyses from bulk-dissected islet grafts were hampered by the inability to distinguish which islet cell subset contributed to gene expression, a critical advantage of our protocol is that it allows FACS-based identification of specific islet cells including α, β, δ, γ, and hormone-negative cells. Moreover, because our protocol allows recovery of viable cells, we were able to perform functional posttransplantation in vitro analyses, an essential aspect of islet graft interrogation. These data can be compared to islet graft physiology in vivo and circulating INS levels following glucose challenge. Importantly, our study also reveals the fidelity of engrafted human islets recovered from experimentally unmanipulated mice to recapitulate the hormone-specific phenotype and gene expression of the original primary donor islets, as well as the human-specific frequencies of heterotypic contacts. The slight upward trend in α-cell transcription observed posttransplant may well reflect the slightly increased purity of the sorted posttransplant cells that results from the death of

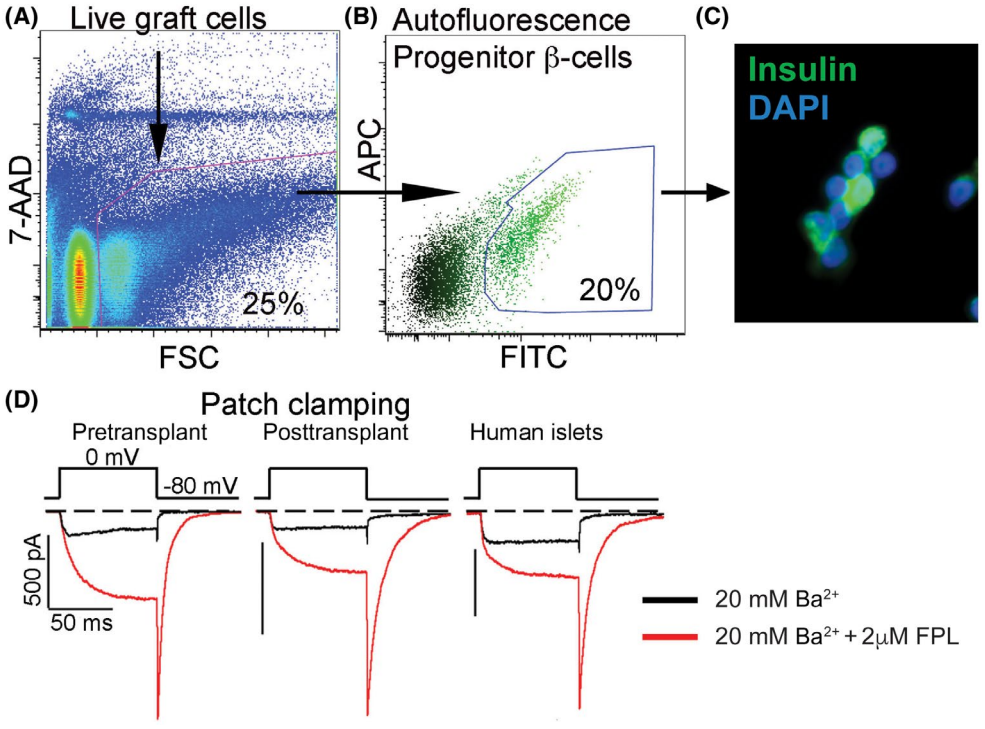

FIGURE 6 Assessment of SC-β cells recovered from grafts. A, Engrafted SC-β cells were assessed for viability with 7-AAD. B, Viable cells were measured for intrinsic autofluorescence (FITC) found in β cells. C, Live autofluorescent cells were confirmed as INS positive by immunostaining. D, Patch-clamp recordings of SC-β cells show robust calcium currents (a feature of calcium-dependent insulin exocytosis) and sensitivity to the L-type calcium channel agonist FPL 64176

contaminating acinar tissue following engraftment. Indeed, we identify a similar uptick in β-cell gene transcription post-transplant, with a significant increase in *INS* and its transcriptional activators *PDX1* (pancreatic and duodenal homeobox 1) and *MAFA* (a member of the Maf family of transcription factors).

Although *INS* and *TXNIP* are upregulated, we observe no changes in the ER stress pathways that would suggest that this minor uptick in *INS* transcription results in a sufficient increase in INS protein production to overload the ER and trigger the unfolded protein response. In addition, the vast majority of gene expression changes in the apoptotic pathway genes are decreased post-engraftment. This is key to interpreting any data from recovered human islets in which the engrafted mice have been subjected to experimental manipulation, and suggests that changes in gene expression or function that may occur in transplanted islets would be due to the in vivo effects on the experimental treatment, rather than effects from the transplant and engraftment process itself. Moreover in future experiments, analysis of increased numbers of islet cells from both pre and posttransplant conditions should reveal whether subpopulations within the five identified cell types can be detected and whether any changes within subgroups occur with transplantation and/or experimental treatment.

Accordingly, our current protocol allows for quantitative molecular analyses of specific endocrine subtypes of human islet cells during controlled experimental conditions in vivo, such as when engrafted mice are exposed to a high-fat diet, infected with viruses, or exposed to inflammatory cytokines; similarly, it can be used to interrogate the effects of potential therapeutics on pancreatic islets from diabetic donors. In addition, investigation of hormone-negative populations will enable characterization of novel cell types and their signaling molecules that may affect islet function in vivo, such as the report that islet-associated pericytes improve islet survival, proliferation, and function.[33] Finally, this methodology also permits in vivo investigation of human SC-β cell development, a necessary step in the optimization of this process and development of methodologies for personalized treatment of diabetes.[34]

In summary, studies of engrafted human islets may provide critical assessment of functional effects of in vivo manipulation of human islets that have been previously lacking. Such methodology should provide answers to fundamental questions in the field concerning dedifferentiation, compensation, and islet gene expression, and reveal how human islet cell populations adapt and compensate in vivo under stress conditions, and how they respond to treatment with potential diabetes therapies. We have shown that our islet graft recovery method, though technically challenging, yields sufficient numbers of cells suitable for single-cell and/or bulk hormone-specific RNA-Seq analysis. Importantly, we are able to recover functional β cells from grafts. Such approaches will facilitate a more complete understanding of how individual human islet cells respond during various physiologic or pathologic conditions and will lead to better strategies for studying human islets in the context of the whole system to improve overall islet function and cell survival in diabetic or at-risk individuals.

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CONFICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.D. Redick assisted in graft recovery, stained cells for hormones, purified RNA, made libraries, analyzed the bulk sequence data, and wrote the manuscript; L. Leehy developed the graft recovery protocol and collected data; A.R. Rittenhouse performed, analyzed, and interpreted the patch-clamp experiments and wrote the manuscript; D.M. Blodgett assisted in the development of the graft recovery protocol, and collected and analyzed data; S.D. Redick, A.G. Derr, A. Kucukural, and M.G. Garber were primarily responsible for the RNA-Seq analysis; L.D. Shultz provided mice. D.L. Greiner designed the study and provided mice; J.P. Wang analyzed data and wrote the manuscript; D.M. Harlan designed the study; R. Bortell designed the study, analyzed data, and wrote the manuscript; A. Jurczyk designed the study, collected and analyzed data, and wrote the manuscript. In addition, all authors reviewed and approved the manuscript.

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