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The Future of Beta Cell Replacement

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EDITORIAL
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The Future of Beta Cells Replacement in the Era of Regenerative Medicine and Organ Bioengineering

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Keywords: islet transplantation, islets-on-chip, bioengineering, islet organoids, stem cell derived beta cells

Editorial on the Special Issue

The Future of Beta Cells Replacement in the Era of Regenerative Medicine and Organ Bioengineering

As we move into 21st century, the landscape of type 1 diabetes (T1D) management is undergoing a significant transformation. Islet transplantation, initially hailed as a breakthrough in replacing lost insulin-producing beta cells, is now confronted with challenges such as the scarcity of donor pancreata, difficulties in predicting and ensuring successful islet engraftment, graft attrition and the need for chronic immunosuppression, all of which hinder its long-term efficacy and sustainability [1, 2].

To address these impediments the scientific community has made significant strides in various domains, including stem cell technology, xenotransplantation, encapsulation techniques and immunomodulatory strategies [3–7]. This progress is further amplified by advancements in tissue engineering strategies, including the generation of on-chip technologies and biomimetic scaffolds, the development of organoids containing both therapeutic and support cells, driving the bioengineering of the endocrine pancreas [7–11]. Concomitantly, the availability of long-term clinical islet transplantation data from different regions allows mapping the activity at the worldwide level to identify regional differences, developing and validating clinical scores to correlate early graft function with long-term transplant outcomes, and the inclusion of patient perspectives and wellbeing in future clinical implementation of current research.

This Special Issue showcases the forefront of beta-cell replacement research and clinical implantation, drawing together 14 rigorously peer-reviewed articles. Each paper highlights a different, yet interrelated aspect of islet transplantation, and collectively provides an in-depth analysis of the field's current achievements and outline its future disruptive perspectives.

Bridging these comprehensive insights, the study by Lam et al. emerges as a significant highlight within this issue, introducing the BETA-2 score, as a new benchmark in predicting long-term transplant outcomes. This new approach sets a higher standard for the evaluation of transplantation procedures, by underscoring the importance of early and continued assessment of graft function, with the possibility of proactive intervention.

van de Leemkolk have provided a significant contribution with their novel technique for assessing β-cell damage in cultured islets by quantifying unmethylated insulin DNA by PCR. Their technique,

Editorial

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based on methylation-sensitive restriction enzyme digital PCR, allows for the evaluation of the islet preparation's purity and quality before transplantation, thus providing a noteworthy improvement in determining their viability and subsequent successful transplantation.

Further exploring the vital aspects of transplantation, Chetboun et al. investigate the correlation between primary graft function (PGF) and 5-year insulin independence in islet and pancreas transplant patients. Using the Beta-2 score for PGF assessment, their study shows a significant positive correlation between early PGF and long-term insulin independence in patients with T1D receiving either islet or pancreas transplantation, underscoring PGF's predictive value in long-term transplantation outcomes.

In their study, Bond et al. analyze the relationship between islet graft function and wellbeing in islet transplant recipients with T1D. This study clearly demonstrates that despite some clinical benefits, "marginal" graft function is associated with suboptimal wellbeing, thus raising the potential need for additional interventions such as re-transplantation.

Berney et al.'s study on international collaboration in islet transplantation highlights the essential role of standardized practices in a field characterized by diverse methodologies and regulatory acceptance. Their findings set the tone for global cooperation and standardization in advancing T1D treatments.

Raoux et al.'s "Islets-on-chip" study introduces a novel approach to predicting clinical islet transplant outcomes by developing a CHIP-score based on donor islets' electrical activity. This method, despite its early stage, could significantly enhance the evaluation and selection process of beta cell replacement therapies before transplantation and potentially predict their outcomes once infused.

Pignatelli et al. discuss the bioengineering of the vascularized endocrine pancreas which refers to pancreatic tissue bioengineered with a focus on the recapitulating its' endogenous vascular structure and cytoarchitecture. These building-blocks are crucial to ensure that the tissue remains functional and can integrate, once transplanted, into the circulatory system with the immediate restoration of blood flow to supply nutrients and oxygen and remove waste products. In this scenario, the authors shed light on the intricate interplay of various crucial elements necessary for successful beta cell replacement including vascularization and extracellular matrix. This report provides guidance for the future development of more effective and sustainable beta cell replacement modalities.

Wassmer et al. investigate the development of pre-vascularized islet organoids, combining therapeutic islet cells with support cells (amniotic epithelial cells and endothelial cells). Their study shows how these organoids outperform native islets *in vitro* and demonstrate improved engraftment and vascularization *in vivo*, in a murine model of T1D. This advancement, attributed to enhanced cell-cell interaction and mediated by upregulation of both pro-angiogenic and pro-β-cell survival genes, suggests a promising approach for beta replacement therapies, hypothetically enabling transplantation in more favorable extrahepatic sites.

In their review, Sackett et al. discuss the potential of genome editing for the development of immune-evasive stem cell-derived islets, a breakthrough with significant implications for advancing transplantation medicine, broadening patient inclusion and reducing procedural risks by limiting the need for chronic systemic immunosuppression.

Pellegrini et al. address a paramount concern in the field: the safety of iPSC-derived beta cells. As more of these therapies approach clinical application, their roadmap for addressing safety concerns is invaluable and can be done through four different strategies, such as somatic cell reprogramming, purification of differentiated beta cells, depletion of contaminant stem cells, and reducing the risk of tumorigenicity through suicide genes.

To translate potential surrogates for human cadaveric donors, Honarpisheh et al. study introduces an innovative approach to re-aggregate dispersed neonatal porcine islet-like cell clusters (NPICCs) as an alternative source transplantable insulin-producing cells for the management of T1D. Their research demonstrates how the re-aggregated NPICCs (REPIs) form uniform clusters with enhanced functionality and *in vivo* performance. This significant finding suggests that re-aggregated NPICCs could expand the potential donor pool for islet xenotransplantation, with improved functionality and outcomes for clinical applications.

Tol et al.'s study, surveying over 800 patients with T1D and caregivers, reveals a high willingness (97%) to receive islet delivery devices (IDDs). The study also highlights patient flexibility regarding IDD characteristics, with device functionality duration outweighing size and number of implants required, underscoring the importance of patient-centered design in future beta cell replacement strategies.

de Jongh et al. address complex ethical, legal, and psychosocial considerations in bio-artificial pancreas therapies. Their advocacy for an interdisciplinary approach that includes patient perspectives is crucial in ensuring the ethical development of these therapies.

Lastly, Piemonti et al. discuss the potential of Advanced Therapy Medicinal Products (ATMPs) in transplantation. Their emphasis on the need for enhanced funding and streamlined regulatory processes to overcome current development bottlenecks is critical for the advancement and accessibility of these therapies.

Despite heightened awareness, the multifaceted nature of diabetes continues to present a complex challenge to patients, as well as to the clinicians, basic scientists and healthcare professionals responsible for diagnosing, researching, monitoring and managing it. Beta-cell replacement is currently recognized as the only definitive therapeutic intervention that can free patients with diabetes from the need to administer insulin externally thus improving survival rates and quality of life. As witnessed by this Special Issue, regenerative medicine and organ bioengineering are undergoing a surge of dynamic advances into new horizons and the treatment of T1D.

Herein we not only showcase groundbreaking research but also illuminate the path toward a future where beta cell replacement therapies are a mainstream, safe, durable, effective, and equitable treatment option for T1D. Each contribution in this issue advances our scientific understanding on beta cell replacement and challenges us to consider the broader implications of these innovations. This compilation serves as an invaluable resource for researchers, clinicians, and patients alike, paving the way for new discoveries and applications in the field of T1D management.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Estimation of Early Graft Function Using the BETA-2 Score Following Clinical Islet Transplantation

Anna Lam[1], Richard A. Oram[2], Shareen Forbes[3], Tolu Olateju[1], Andrew J. Malcolm[1], Sharleen Imes[1], A. M. James Shapiro[1] and Peter A. Senior[1]*

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Little is known about how early islet graft function evolves in the clinical setting. The BETA-2 score is a validated index of islet function that can be calculated from a single blood sample and lends itself to frequent monitoring of graft function. In this study, we characterized early graft function by calculating weekly BETA-2 score in recipients who achieved insulin independence after single transplant (group 1, n = 8) compared to recipients who required a second transplant before achieving insulin independence (group 2, n = 7). We also determined whether graft function 1-week post-transplant was associated with insulin independence in individuals who received initial transplant between 2000–2017 (n = 125). Our results show that graft function increased rapidly reaching a plateau 4–6 weeks post-transplant. The BETA-2 score was higher in group 1 compared to group 2 as early as 1-week post-transplant (15 + 3 vs. 9 + 2, p = 0.001). In an unselected cohort, BETA-2 at 1-week post-transplant was associated with graft survival as defined by insulin independence during median follow up of 12 months (range 2–119 months) with greater survival among those with BETA-2 score >10 (p < 0.001, log-rank test). These findings suggest that primary graft function is established within 4–6 weeks post-transplant and graft function at 1-week post-transplant predicts long-term transplant outcomes.

Keywords: islet transplantation, graft survival, graft function, engraftment, BETA-2 score

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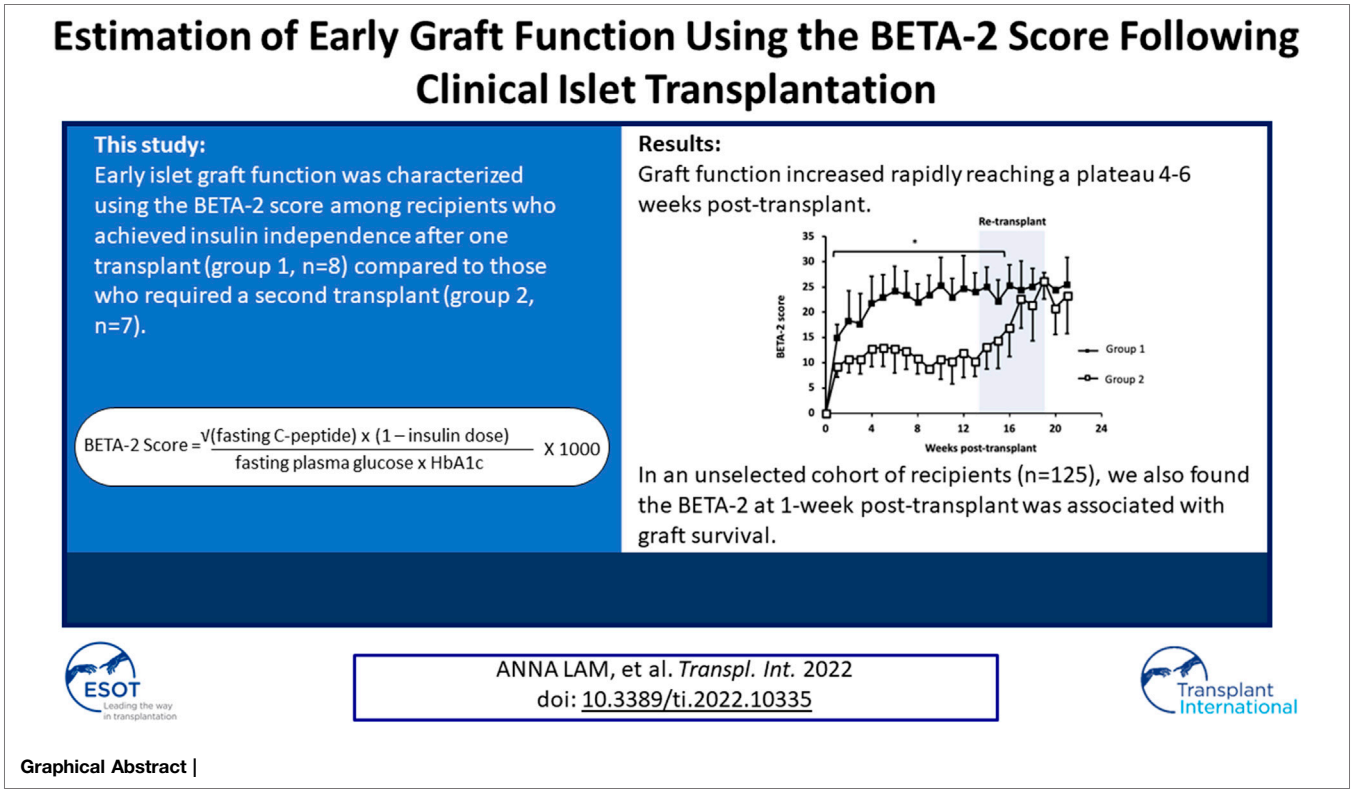
Abbreviations: BMI, body mass index; CP/G, C-peptide/glucose ratio; HbA1c, hemoglobin A1c; HOMA2-B%, homeostasis model assessment index of beta cell function; IE, islet equivalents; SD, standard deviations; SUITO, Secretory Unit of Islet Transplant Objects; TEF, transplant estimated function.



Graphical Abstract |

INTRODUCTION

Advances in clinical islet transplantation including in islet processing and immunosuppression protocols have led to improved outcomes with increased rates of insulin independence and longer-lasting graft function (1). However, most recipients will require at least two islet transplants to achieve insulin independence and will have declining graft function over time with less than 50% of recipients maintaining insulin independence at 3 years post-transplant (2).

Optimization of early islet graft function remains an important target for improving long-term islet transplant outcomes. More than 50% of transplanted islets are lost in the first few days post-transplant (3, 4) and peri-transplant interventions limiting inflammation and islet stress have been shown to promote insulin independence and long-term islet survival (5, 6). Primary graft function at one-month post-transplant has been associated with long-term islet graft function (7), however, it remains unknown how primary graft function evolves in the first weeks to months after transplant.

One of the major challenges in this area has been the inability to closely monitor islet function. Formal stimulation tests measuring insulin or C-peptide response to stimuli such as glucose or arginine provide precise information on graft function, but the metabolic stress, as well as the time and labor-intensive nature of these tests, make them impractical for frequent monitoring in the clinical setting. Taking advantage of the BETA-2 score, a validated measure of islet function that can be calculated from a single fasting blood sample (8, 9), we characterized graft function in the first-weeks post-transplant and determined whether graft function as early as 1-week post-transplant is associated with long-term transplant outcomes.

METHODS

Recipients

All subjects provided informed consent, and the analysis of data was approved by the University of Alberta Health Research Ethics Board. We performed a retrospective single-center analysis of individuals newly transplanted with allogeneic islets between 2009 and 2014. To characterize the establishment of islet graft function, BETA-2 score was calculated weekly in two selected groups representing distinct transplant outcomes: 1) subjects who achieved and maintained insulin independence for at least 12 months after a single islet infusion (group 1), and 2) subjects who only became insulin-independent (which was sustained beyond 12 months) after they received a second islet infusion after 3–6 months because they had not achieved insulin independence after their first infusion (group 2). Insulin independence was defined by no exogenous insulin use and no more than 2 self-monitored blood glucose levels >10.0 mmol/L during a 7-day period (10). A cohort of islet transplant recipients newly transplanted between 2000 and 2017 who had available lab

TABLE 1 | Baseline characteristics.

	All patients (*n* = 15)	Group 1 (*n* = 8)	Group 2 (*n* = 7)	*p*
Sex (male/female)	5/10	2/6	3/4	0.61
Age (years)	55.6 ± 9.9	56.8 + 9.4	54.3 ± 11.1	0.64
Diabetes duration (years)	34.9 ± 13.6	32.8 + 13.4	37.3 ± 14.4	0.54
Weight (kg)	68.5 ± 10.8	64.1 + 8.1	73.4 ± 11.9	0.10
BMI (kg/m^2)	25.4 ± 2.6	23.9 + 1.9	27.0 ± 2.3	0.01
HbA1c (%)	8.6 ± 1.1	9.2 + 0.9	8.0 ± 0.9	0.03
Fasting blood glucose (mmol/L)	12.3 ± 5.4	13.5 + 4.9	11.0 ± 6.2	0.41
Insulin dose (units/kg per day)	0.5 ± 0.1	0.5 + 0.1	0.5 ± 0.1	0.96
First transplant				
IEQ	525,364 ± 274,102	624,189 + 348,429	412,422 ± 75,944	0.14
IEQ/kg	7,669 ± 3,626	9,476 + 4,205	5,603 ± 846	0.03
Second transplant				
IEQ			519,886 ± 176,138	
IEQ/kg			7491 ± 2,312	
Total IEQ	767,978 ± 328,107	624,189 + 348,429	932,308 ± 224,685	0.07
Total IEQ/kg	11,164 ± 3,935	9,476 + 4,205	13,094 ± 2711	0.07

BMI, body mass index; IEQ, islet equivalents; IEQ/kg, islet equivalents per recipient body weight. Data are expressed as mean ± SD and n (%).

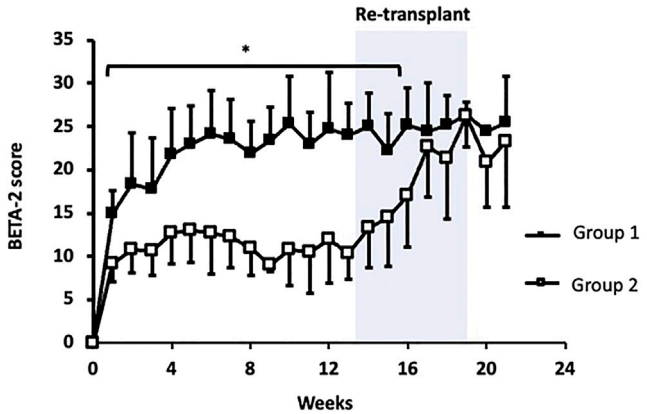


FIGURE 1 | BETA-2 score in the first 6 months post initial islet transplant. Group 1 (closed squares). Group 2 (open squares). Shaded area indicates when group 2 received their second transplant. *$p < 0.05$, group 1 vs. group 2.

results and insulin records at 1-week post-transplant were evaluated to determine whether BETA-2 score at 1-week post-transplant is associated with long-term transplant outcomes. The indications for islet transplantation, islet preparation, transplant procedure, and monitoring have been previously described (11, 12). Immunosuppression consisted of induction with alemtuzumab, thymoglobulin, daclizumab or basiliximab, and maintenance with tacrolimus and sirolimus or mycophenolate mofetil.

Clinical Assessment

All subjects were seen weekly in-clinic during the first month post-transplant and then every 3–6 months in the first year post-transplant. Subjects were asked to self-monitor blood glucose and insulin usage. No specific protocol for insulin titration was used; post-transplant insulin doses were adjusted to avoid hyper- and hypo-glycemia (i.e., target glucose 4–10 mmol/L). Insulin dose (unit/kg) was calculated based on reported insulin dose divided by body weight measured at the most recent clinical assessment. Unfortunately, data on insulin delivery method was not available for this analysis. Blood work including fasting C-peptide and fasting glucose were measured every 1–2 weeks during the first 6 months post-transplant. HbA1c (as a percentage) was measured every 1–3 months post-transplant. For fasting blood work, patients were advised not to eat or drink after midnight the night before blood work was drawn with no specific instructions regarding insulin doses.

Assays

Fasting plasma glucose concentrations were determined by the glucose oxidase method. C-peptide concentrations were measured using a commercial assay (Roche Elecsys; Roche Diagnostics, Indianapolis, IN). The lower limit of sensitivity for C-peptide in our laboratory was 0.02 nmol/L and the inter-assay coefficient of variation was 3.5%. HbA1c was measured by the Bio-Rad Variant II kit (Hercules, CA).

Calculation of BETA-2 Score

BETA-2 scores were calculated weekly post-transplant. Derivation and validation of the BETA-2 score have previously been described (10). The BETA-2 is generated based on fasting C-peptide (nmol/L), daily insulin dose (units/kg), fasting plasma glucose (mmol/L), and HbA1c (%) as follows:

$$\text{BETA-2 Score} = \frac{\sqrt{(\text{fasting C-peptide})} \times (1 - \text{insulin dose})}{\text{fasting plasma glucose} \times \text{HbA1c}} \times 1000$$

Other Indices of Islet Graft Function

Alternative simple indices of graft function were calculated at 1-week post-transplant as detailed below.

TABLE 2 | Baseline characteristics of individuals newly transplanted between 2000–2017.

	All patients	BETA-2 score at 1-week post-transplant			*p*
		<10	10–14	≥15	
n	125	61	45	19	
Sex (male/female)	55/70	29/32	16/29	10/9	0.33
Age (years)	48.3 ± 9.8	46.5 ± 10.7	49.6 ± 8.7	51.0 ± 9.0	0.12
Diabetes duration (years)	32.7 ± 10.7	31.0 ± 10.2	33.2 ± 11.1	36.7 ± 10.7	0.12
Weight (kg)	74.0 ± 12.3	73.8 ± 13.4	73.5 ± 11.3	75.5 ± 11.5	0.84
BMI (kg/m^2)	25.9 ± 3.4	25.9 ± 3.7	25.9 ± 3.1	26.2 ± 3.6	0.92
HbA1c (%)	8.3 ± 1.2	8.2 ± 1.3	8.3 ± 1.1	8.7 ± 1.3	0.25
Insulin dose (units/kg/day)	0.56 ± 0.16	0.60 ± 0.16	0.54 ± 0.16	0.50 ± 0.13	0.02
IEQ	465,565 ± 143,129	437,523 ± 147,628	483,862 ± 126,893	512,582 ± 152,524	0.08
IEQ/kg	6291 ± 1,581	5906 ± 1,482	6603 ± 1,532	6787 ± 1774	0.03

BMI, body mass index; IEQ, islet equivalents; IEQ/kg, islet equivalents per recipient body weight.
Data are expressed as mean ± SD and n (%).

C-peptide/glucose ratio (CP/G) was calculated from C-peptide (ng/ml) and fasting plasma glucose (mg/dl) levels (13).

$$\text{CP/G} = \frac{\text{fasting C-peptide}}{\text{fasting plasma glucose}} \times 100$$

The homeostasis model assessment index of beta-cell function (HOMA2-B%) was calculated from fasting C-peptide (nmol/L) and plasma glucose (mmol/L) using the HOMA calculator (www.dtu.ox.ac.uk/homacalculator).

The Secretory Unit of Islet Transplant Objects (SUITO) index was also calculated from fasting plasma glucose (mmol/L) and C-peptide (nmol/L) (14, 15).

$$\text{SUITO index} = \frac{250 \times \text{fasting C-peptide}}{\text{fasting plasma glucose} - 3.43}$$

Transplant estimated (TEF) was calculated from the daily insulin requirement (DIR; units/kg/24 h) and HbA1C (%) as previously described (16).

$$\text{TEF} = \left(\text{DIRpreTx} + \frac{\text{HbA1cpreTx}}{5.43}\right) - \left(\text{DIR} + \frac{\text{HbA1c}}{5.43}\right)$$

Statistics

Statistical analyses were performed using Stata version 14.1 (StataCorp, College Station, TX). Descriptive statistics are expressed as mean ± standard deviation (SD). Two-tailed t-test, Chi-square test, one-way ANOVA, and Tukey test were used to compare groups as appropriate. Receiver operating characteristic curves were constructed for recipients' BETA-2 score, CP/G, HOMA2-B%, SUITO index, and TEF at 1-week post-transplant based on insulin independence. The association between BETA-2 score and insulin independence was evaluated by multiple logistic regression adjusted for pre-transplant BMI, HbA1C, and insulin dose, as well as islet equivalents per recipient body weight (IEQ/kg), transplanted. Survival analysis for the duration of insulin dependence was generated using the Kaplan-Meier method and analyzed using the Mantel-Cox log-rank test. A p-value < 0.05 was considered statistically significant and all p-values were reported as two-sided. To compare the differences in survival between groups, Bonferroni-adjusted posthoc pairwise comparisons were conducted with an adjusted p-value <0.017 considered statistically significant.

RESULTS

Baseline Characteristics

The BETA-2 score was calculated on a weekly basis for the first 6 months post-transplant in 1) recipients who achieved insulin independence after a single transplant (n = 8, group 1) and 2) recipients who achieved insulin independence after having a second islet transplant 3–6 months from their first transplant (n = 7, group 2). Baseline characteristics were similar between both groups except for HbA1c which was higher in group 1 and BMI which was higher in group 2 (**Table 1**). Group 1 subjects received significantly higher islet equivalents per recipient body weight (IEQ/kg) with their first transplant compared to group 2 subjects (9476 ± 4205 IEQ/kg vs. 5603 ± 846 IEQ/kg, p = 0.03), however, there was no significant difference in total IE/kg after

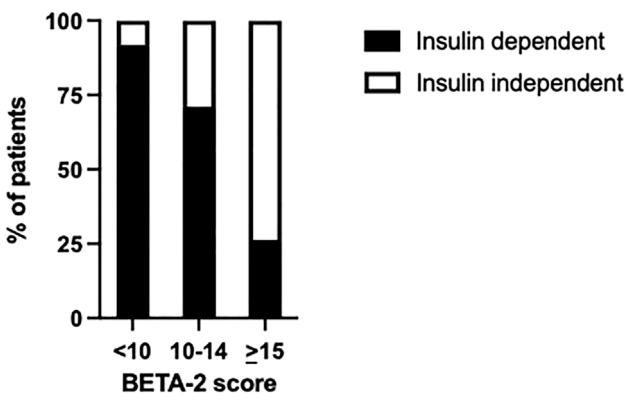


FIGURE 2 | Percentage of transplant recipients who achieved insulin independence or remained insulin-dependent according to BETA-2 score at 1-week post-transplant p < 0.001.

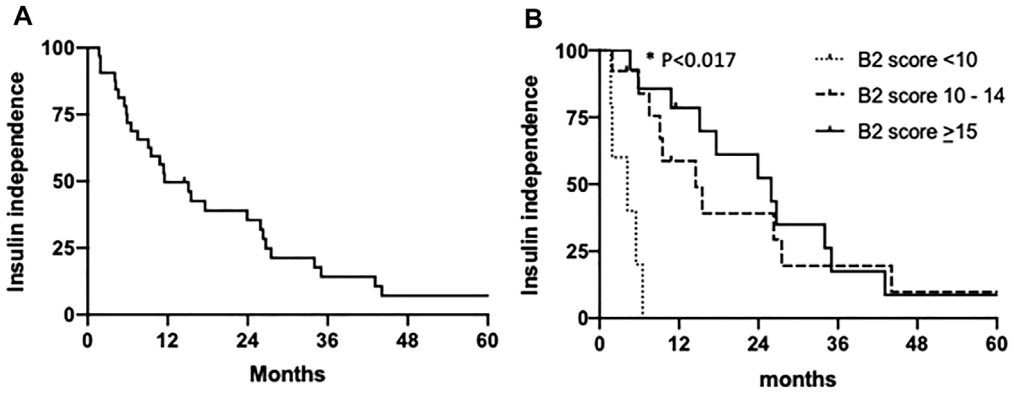


FIGURE 3 | Kaplan-Meier estimates of the proportions of patients with insulin independence **(A)** among the entire cohort and **(B)** according to 1-week post-transplant BETA-2 score <10 (n = 5), 10–14 (n = 13) or >15 (n = 14). *Durability of insulin independence was significantly lower among subjects with 1-week BETA-2 score <10 vs. 10–14 (p = 0.0002) and BETA-2 score <10 vs. > 15 (p = 0.0001) by Mantel-Cox log-rank test and Bonferroni corrected significance threshold $p < 0.017$.

recipients from group 2 received their second transplant (9476 ± 4205 IEQ/kg vs. 13,094 ± 2711 IEQ/kg, p = 0.07).

Early Graft Function

In both groups, BETA-2 score was measurable at 1-week and continued to increase before reaching a plateau 4 to 6 weeks post-transplant (**Figure 1**). BETA-2 score was significantly higher in group 1 compared to group 2 recipients as early as 1-week post-transplant (BETA-2 score 15 ± 3 vs. 9 ± 2, p = 0.001) and this difference was maintained until group 2 recipients received their second islet infusion at 4.1 ± 0.9 months (BETA-2 score 25 ± 4 vs. 17 ± 6, p = 0.07) (**Figure 1**). As expected, glycemic control as measured by HbA1c improved post-transplant in both groups (**Supplementary Figure S1**).

Early Graft Function and Transplant Outcomes

BETA-2 score at 1-week post-transplant was evaluated in an unselected cohort of recipients after their first islet transplant (n = 125) (**Table 2**). In total 26% achieved insulin independence for a median duration of 10 months (range 1.7–43 months, n = 32) while 74% remained insulin-dependent (n = 93). BETA-2 score at 1-week post-transplant was higher among those who achieved insulin independence compared to those who remained insulin-dependent (13 ± 3 vs. 9 ± 4, $p < 0.001$). BETA-2 score at 1-week also showed good discriminative ability for insulin independence (AUROC 0.83, $p < 0.001$) compared to alternative indices of graft function including the SUITO index, HOMA2-B%, CP/G and TEF (AUROC 0.55–0.77) (**Supplementary Figure S2**; **Supplementary Table S1**). Insulin independence was achieved in 8% (n = 5), 29% (n = 13), and 74% (n = 14) of recipients with BETA-2 score <10, 10–14 and ≥15, respectively ($p < 0.001$) (**Figure 2**). The odds of insulin independence increased with increasing BETA-2 score at 1 week including when adjusted for pre-transplant insulin dose, BMI, and HbA1c, as well as IE/kg transplanted (unadjusted odds ratio 1.39, 95% CI 1.21–1.59, $p < 0.001$ and adjusted odds ratio 1.44, 95% CI 1.23–1.70, $p < 0.001$). BETA-2 score at 1-week post-transplant was associated with graft survival as defined by insulin independence ($p < 0.001$, log-rank test) over a median follow-up of 12 months (range 2–119 months), with median survival of 4.2 months [IQR 1.9–5.5], 14.5 months [IQR 9.1–27.5] and 25.9 [IQR 15.1–35.0], respectively among recipients with BETA-2 score <10, 10–14 and ≥15 (BETA-2 score <10 vs. 10–14, $p < 0.002$ and vs. ≥15, $p < 0.001$) (**Figure 3**).

CONCLUSION

This study describes the evolution of islet graft function in the early period post-islet transplant using the BETA-2 score. This validated clinical score assessed weekly shows that graft function is established rapidly and increases over the first 4–6 weeks post-transplant before stabilizing. Furthermore, early engraftment estimated by the BETA-2 score as early as 1-week post-transplant is key to predicting longer-term transplant outcomes.

Vantyghem et al have shown that primary graft function as measured by the original BETA score at 1-month post-transplant is associated with prolonged graft survival (7). More recently, Witkowski et al demonstrated that the BETA-2 score on day 75 post-transplant is an early predictor of graft decline (15). In keeping with these studies, we found that it takes approximately 4–6 weeks before primary islet graft function is established and supports the association of graft function in the first 1–2 months with islet transplant outcomes.

Interestingly, our results suggest that it is possible to assess how well a graft will function even before primary graft function is fully established. We compared transplant recipients who achieved insulin independence for at least 1 year after a single transplant to those who remained insulin-dependent and found that the BETA-2 score was significantly higher at 1-week post-transplant among those who achieved insulin independence. We confirmed this in an unselected cohort of islet transplant recipients where a significantly higher BETA-2 score at 1-week was observed among those who achieved insulin independence

post-transplant. In clinical practice, this may translate into earlier identification of recipients who are unlikely to achieve insulin independence and allow for earlier intervention including repeat transplantation in recipients who are already immunosuppressed/lymphodepleted. An early endpoint such as the BETA-2 score 1-week post-transplant could serve as an intermediate outcome and allow for shorter and more efficient clinical trial testing strategies designed to improve islet engraftment.

Ourselves and others have shown previously that BETA-2 scores >13 and >15 reliably predict insulin independence (8, 9) and a BETA-2 score >17.4 on day 75 post-islet transplant has been found to be associated with durable (5 years) insulin independence (15). This is similar to our current findings: that islet transplant recipients who achieved and maintained insulin independence for at least 1 year after a single infusion had an average BETA-2 score of 15 at 1-week post-transplant, and in our unselected cohort, recipients who achieved insulin independence (minimum duration 1 month) had average BETA-2 score of 13. In both analyses, for recipients who were unable to come off insulin, the average BETA-2 score at 1-week was 9. We also found that BETA-2 score at 1-week post-transplant was associated with long-term graft survival with a longer duration of insulin independence among recipients with BETA-2 scores of 10–14 and ≥15 compared to those with BETA-2 scores <10. Taken together, it appears that a BETA-2 score cut-off of >13 at 1-week post-transplant may be useful in identifying recipients who are likely to achieve insulin independence with higher scores being associated with a longer duration of insulin independence.

A potential limitation of the current analysis is the small number of subjects being compared in groups 1 (insulin-independent for > 1 year after a single transplant) and group 2 (recipients who did not become insulin-dependent until after a second transplant 3–6 months after the first infusion which was maintained at 12 months). This was necessary to be sure that the effect of each transplant could be assessed independently by selecting groups of recipients with distinct transplant outcomes, i.e., those with optimal vs. sub-optimal graft function. Thus, patients receiving a second transplant before 3 months were not included in case they might have been able to achieve insulin independence with the first transplant. Neither were recipients of second transplants who did not remain insulin independent at 12 months since the decline in graft function might be due to other factors such as rejection, rather than engraftment estimated by BETA-2. Most recipients at our center are re-listed for a second transplant at 4 weeks and priority is given to second infusions while recipients are still lymphodepleted. Furthermore, we confirmed that early graft function (1-week post-transplant) is associated with long-term transplant outcomes in an unselected cohort of transplant recipients with BETA-2 scores consistent with previous studies showing an association between BETA-2 scores and transplant outcomes (8, 9, 15).

A limitation of using the BETA-2 score soon after islet transplant is the inclusion of 1) HbA1c which is not expected to change in the short term and 2) insulin dose which may vary depending on several factors including diet, activity, and care provider discretion. However, in our study the BETA-2 score at 1-week post-transplant had better discrimination for insulin independence compared to other simple indices of islet function (SUITO index, HOMA2-B%, TEF and CP/G) suggesting that there is merit in including these additional variables even in short term assessment of graft function. Practical considerations for calculating the BETA-2 score peri-transplant may be to measure HbA1c less frequently (i.e., bi-weekly to monthly) than fasting C-peptide and glucose and to use standardized protocols regarding insulin dose adjustments.

Our study was not designed to explore how recipient and/or donor factors relate to graft function. However, we found that higher islet equivalents were associated with insulin independence and higher 1-week BETA-2 score in keeping with previous studies demonstrating single islet transplant success in recipients who had received higher transplanted islet mass(16, 17). Lower pre-transplant BMI and insulin requirements were also associated with higher BETA-2 scores at 1-week post-transplant suggesting that transplant success appears to depend not only on the number and function of transplanted islets but also on the metabolic demand placed on them. Importantly, however, we found that the association between insulin independence and BETA-2 score at 1-week post-transplant remained relatively unchanged when adjusted for pre-transplant BMI, insulin dose, and HbA1c, as well as transplanted IE/kg.

We characterized islet function in the early period post-transplant and show that primary graft function is established over the first 4–6 weeks post-transplant and that graft function as early as 1-week post-transplant is associated with long-term graft survival. Importantly, we demonstrated that frequent and close monitoring of islet graft function soon after transplantation is possible in the clinical setting and that this may be useful in routine clinical care as well as in the development and evaluation of interventions targeted at improving islet transplant outcomes.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: This study was a retrospectives analysis of single center data of individuals newly transplanted with allogenic islets between 2009–2014. The data is not publicly available. Requests to access these datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Alberta Health Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL and PS drafted the manuscript and analyzed and interpreted the data. AL and SI researched the data. All authors contributed to revision of the article and approved the final version of the article.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10335/full#supplementary-material

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Quantification of Unmethylated Insulin DNA Using Methylation Sensitive Restriction Enzyme Digital Polymerase Chain Reaction

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Assessment of specific β-cell death can be used to determine the quality and viability of pancreatic islets prior to transplantation and hence predict the suitability of the pancreas for isolation. Recently, several groups have demonstrated that unmethylated insulin (*INS*)-DNA is correlated to β-cell death in type 1 diabetes patients and during clinical islet isolation and subsequent transplantation. Here, we present a step-by-step protocol of our novel developed method for quantification of the relative amount of unmethylated *INS*-DNA using methylation sensitive restriction enzyme digital polymerase chain reaction This method provides a novel and sensitive way to quantify the relative amount of β-cell derived unmethylated *INS*-DNA in cellular lysate. We therefore suggest that this technique can be of value to reliably determine the purity of an islet preparation and may also serve as a measure of the quality of islets prior to transplantation measuring unmethylated *INS*-DNA as a reflection of the relative amount of lysed β-cells.

Keywords: transplantation, biomarker, unmethylated insulin DNA, methylation sensitive restriction enzyme, digital PCR, β-cell, islets

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INTRODUCTION

β-cell replacement therapy has been established as a therapy for patients with complex Type 1 Diabetes (T1D) not amenable to optimal conventional diabetes management (1). One example of β-cell replacement therapy is the transplantation of deceased donor derived pancreatic islets that has proven its long-term efficacy during the past 20 years (2, 3). In order to aim for optimal post-transplant outcomes, the use of high-quality pancreatic islets is essential. Reliable assays are needed to assess the quality and viability of islets prior to transplantation. Soluble β-cell specific biomarkers may serve as a relevant diagnostic target to determine the quality and viability of islets at an early

Abbreviations: ddPCR, Digital Droplet polymerase Chain Reaction; DNA, DeoxyriboNucleic Acid; DTZ, Dithizone Staining; IEQ, Islet Equivalent; INS, Insulin; MSRE, Methylation Sensitive Restriction Enzyme; PCR polymerase Chain Reaction; T1D, Type 1 Diabetes; TTC5, Tetratricopeptide Repeat Domain 5.

stage as they can be used to assess the amount of β-cell loss during islet isolation and subsequent transplantation.

Recently, several groups have reported unmethylated Insulin (*INS*)-DNA as a specific β-cell death marker during the early development of T1D. During the progression of the disease, autoimmune destruction of β-cells occurs and unmethylated *INS*-DNA is released in the bloodstream that can be identified (4-11). As the concentration of this marker is extremely low, digital polymerase chain reaction (PCR) is often used to detect the amount of β-cell death in a quantitative manner. Recent studies using digital PCR to analyze unmethylated *INS*-DNA were based on a sodium-bisulfite conversion method that chemically converts unmethylated cytosine into uracil (6, 8-10, 12). However, this method comprises an insurmountable problem as regards heterogeneity since it depends on the completeness of the chemical conversion. Overshooting or incomplete bisulfite conversion can lead to reduced sensitivity and may hamper quantitative and qualitative interpretation (13).

To avoid bisulfite conversion whilst still allowing the possibility to specifically quantify the methylation fraction of a specific allele, we recently published a methylation sensitive restriction enzyme (MSRE) digital PCR assay (14). MSREs are used to differentiate between methylated and unmethylated alleles and in combination with digital PCR it provides the opportunity to determine specific allele quantification.

Based on this methodology we now describe here the step-by-step approach how to quantify the unmethylated *INS*-DNA fraction using a MSRE and digital PCR assay. In this proof-of-concept study, we aim to demonstrate that this novel assay can be used as a helpful method to determine the purity of an islet preparation by measuring the amount of β-cells specific genomic DNA in an islet suspension. The subsequent step to then test this particular assay as a clinically quality marker of islets prior to transplantation by measuring the relative amount of lysed β-cells was beyond the scope of this proof-of-concept study. .

METHOD

Sample Collection and DNA Isolation

Human insulinoma EndoC-βH1 cells (Univercell-Biosolutions (15), Toulouse, France) and human monocytic THP-1 cells (Invivogen, Toulouse, France) were used as a positive and negative control, respectively. Isolated human pancreatic islets with different purities were obtained from seven individual pancreases (Leiden University Medical Center, Netherlands). Human donor pancreases were used that were declined for clinical purposes according to national criteria. Written informed consent for research of pancreatic tissue from donors was present, according to local guidelines of the medical ethical committee (Leiden University Medical Center, Netherlands) and of the Dutch Transplantation Foundation as the competent authority for organ donation in Netherlands. Regarding the culture of the EndoC-βH1 and THP-1 cells and isolation and maintenance of human islets, please find further details in the Supplemental document.

1) Stored pellets of $2.5 \times 10\hat{6}$ EndoC-βH1 cells, $2.5 \times 10\hat{6}$ THP-1 cells and 10 µL tissue of different purities from human islets were resuspended with phosphate buffer up to a final volume of 200 µL.

From these samples genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.

2) DNA concentrations were measured using NanoDrop TM 1000 Spectrophotometer (Thermo Fisher Scientific, Landsmeer, Netherlands).

Treatment With Methylation Sensitive Restriction Enzyme

The restriction enzyme, HpaII (Thermo Fisher Scientific), was used according to manufacturer's instructions. The restriction enzyme was added for the *INS target DNA* (**Figure 1A**) as it cleaves the unmethylated *INS*-DNA and leaves the methylated *INS*-DNA intact. Each sample was either left untreated or treated with HpaII.

1) Take two separate units of 100 ng genomic DNA from each sample and add each of these units to a separate PCR tube (8-strip PCR tubes). Mark the first strip as "with MSRE" and the second strip as "without MSRE". Include at least one sample in each strip containing only nuclease-free H_2O (negative control).
2) Add 2 units/reaction of HpaII, 1.0 µL CutSmart Buffer (Bioké, Leiden, Netherlands), and nuclease-free H_2O up to a total volume of 10 µL to the strip marked as "with MSRE".
3) Add 1.0 µL CutSmart Buffer (Bioké, Leiden, Netherlands), and nuclease-free H_2O up to a total volume of 10 µL to the strip marked as "without MSRE".
4) Incubate both strips at 37°C for 1 hour.

Duplex Analysis Using Digital PCR

Primers and FAM-labelled hydrolysis probes (both Sigma-Aldrich) were designed to be 1) gene specific, 2) to contain an MSRE specific CpG site and 3) to possess optimal melting temperature (±55°C) based on the region identified previously (**Supplementary Figure S1**) (11, 16). Probes directed to the *INS target DNA* were labelled with FAM (**Supplementary Table S1**). The probe directed to the reference TTC5 (tetratricopeptide repeat domain 5) gene was labelled with HEX (BioRad, Veenendaal, Netherlands).

1) To prepare the PCR mastermix, add 11 µL per reaction of Droplet PCR Supermix™ (No dUTP) (BioRad) (e.g., 110 µL per 10 samples), 0.5 µL per reaction 36uM forward *INS* primer (e.g., 5 µL per 10 samples), 0.5 µL per reaction 36uM *INS* reverse primer (e.g., 5 µL per 10 samples), 0.5 µL per reaction 10uM *INS* FAM probe (e.g., 5 µL per 10 samples), 1 µL per reaction 20x TTC5 HEX assay (e.g., 10 µL per 10 samples) and 6.5 µL per reaction nuclease-free H_2O (e.g., 65 µL per 10 samples).

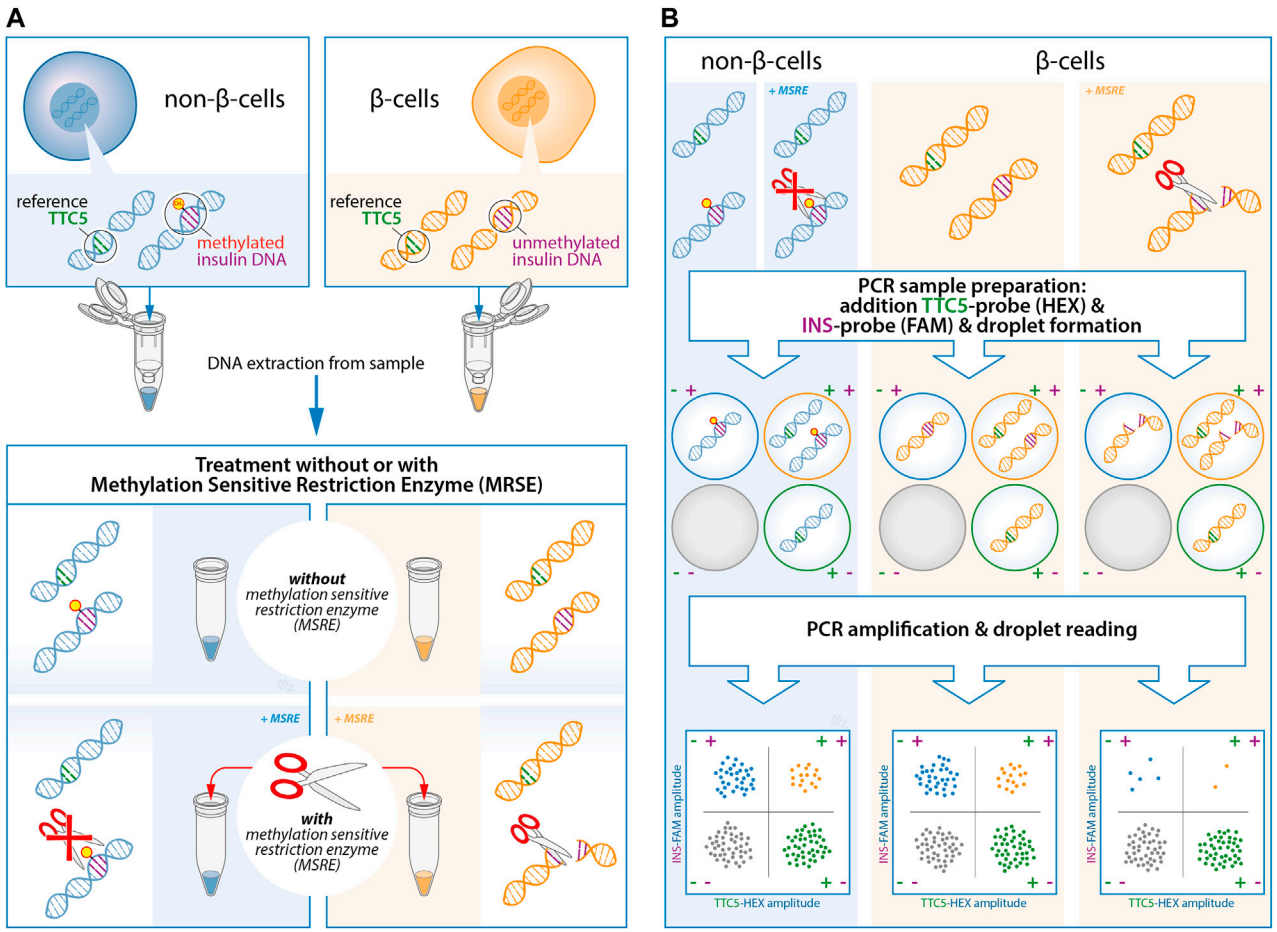

FIGURE 1 | Experimental design of the quantification of unmethylated insulin (*INS*)-DNA using methylation sensitive restriction enzyme (MSRE) digital droplet polymerase chain reaction (ddPCR). **(A)** DNA is isolated from the samples and subsequently split in two and treated with or without the MSRE. The MSRE cleaves the unmethylated *INS*-DNA and leaves the methylated *INS*-DNA intact. **(B)** The DNA sample is partitioned into thousands of droplets followed by PCR amplification with FAM-labelled hydrolysis probes directed to the *INS target DNA* and probes directed to a reference gene (HEX-labelled). Droplet reading takes place after amplification. Droplets that are positive or negative for the *INS target DNA* and/or reference gene are counted to calculate the fraction of unmethylated *INS*-DNA in the sample. Abbreviations: ddPCR, Digital Droplet polymerase Chain Reaction; INS, Insulin; MSRE, Methylation Sensitive Restriction Enzyme; PCR, polymerase Chain Reaction.

2) In order to set up a PCR reaction in a 96-well plate, first, add 20 µL mastermix to each well. Add 2 µL of cleaved unmethylated *INS*-DNA (from the "with MSRE" PCR-strip) or uncleaved unmethylated *INS*-DNA (from the "without MSRE" PCR-strip) to each appropriate well. Mix wells by pipetting up-and-down several times.

All eight wells in a column must contain cleaved unmethylated *INS*-DNA (from the "with MSRE" PCR-strip) or uncleaved unmethylated *INS*-DNA (from the "without MSRE" PCR-strip).

3) Seal the 96-well PCR plate with foil and centrifuge shortly to remove liquid from the sides of the wells.
4) Digital PCR is performed using the digital droplet PCR (ddPCR) method described below (**Figure 1B**).
   4.1) Use the Automated Droplet Generator (BioRad) to generate droplets according to manufacturer's instructions.
   4.2) In order to prevent evaporation of the newly formed droplets, the droplets should be collected in a second 96-well PCR plate placed into a properly frozen cooling block.
   4.3) When finished, remove the 96-well PCR plate including the newly formed droplets and use a Plate Sealer (BioRad) in order to cover the 96-well PCR plate with a heat-sealed foil.

NOTE: Careful handling is strongly advised as the newly formed droplets are fragile in this stage.

5) Perform a PCR reaction in a T100 Thermal Cycler (BioRad) using the following protocol:
   - 10 min of activation at 95°C
   - 30s at 94°C denaturation and 60s at 60°C for 40 cycles
   - 10 min inactivation at 98°C
   - Cooling at 12°C until droplet reading

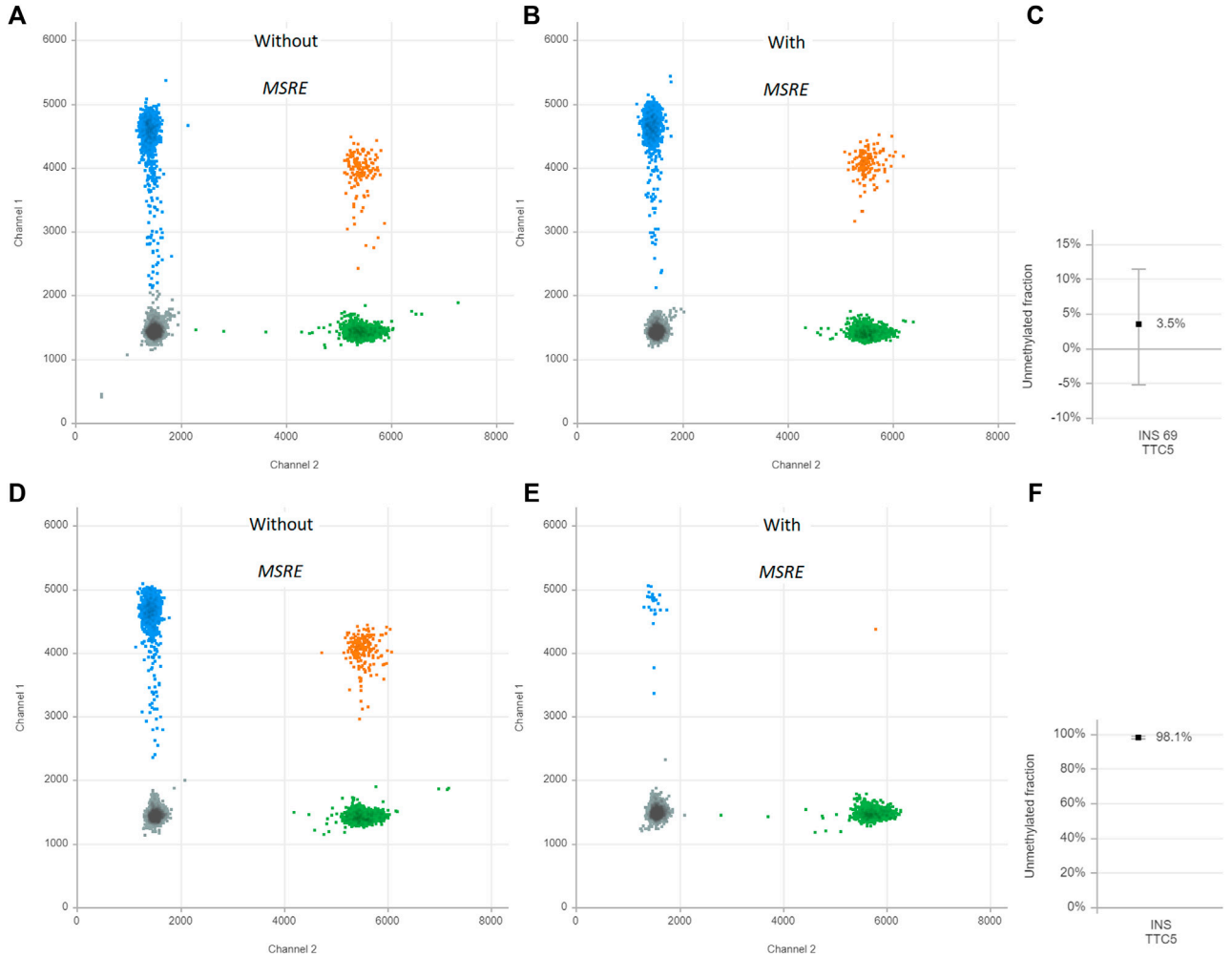

FIGURE 2 | Unmethylated *INS*-DNA fraction in EndoC-βH-1 cells **(A–C)** and THP-1 cells **(D–F)** as positive and negative control samples, respectively. The two-dimensional plots from the digital droplet polymerase chain reaction (ddPCR) demonstrate four different clusters each representing different DNA containing droplets: Gray cluster: FAM- HEX-, containing no *INS target DNA* and no reference copies. Green cluster: FAM- HEX+, containing no *INS target DNA* but contains reference copies. Blue cluster: FAM+ HEX-, containing *INS target DNA* but no reference copies. Orange cluster: FAM+ HEX+ containing both *INS target DNA* and reference copies. The control samples are both split in two and either treated with or without methylation sensitive restriction enzyme (MSRE). The unmethylated *INS*-DNA fraction is calculated: $1 - \frac{\frac{[INS]}{[TTC5]}_{with\ MSRE}}{\frac{[INS]}{[TTC5]}_{without\ MSRE}} * 100\%$. Abbreviations: ddPCR, Digital Droplet polymerase Chain Reaction; INS, Insulin; MSRE, Methylation Sensitive Restriction Enzyme.

6) Analyze the DNA content of the droplets using the QuantaSoft™ software with the QX200 Droplet Reader (BioRad) according to manufacturer's protocol.
7) Calculate for each sample the unmethylated *INS*-DNA fraction as follows:
   - Unmethylation fraction = $1 - \frac{\frac{[INS]}{[TTC5]}_{with\ MSRE}}{\frac{[INS]}{[TTC5]}_{without\ MSRE}} * 100\%$

RESULTS

With attention to previous studies (11, 16) on target areas of DNA methylation in the human *INS* gene, we designed a methylation sensitive restriction enzyme (MSRE) duplex digital PCR assay to determine the relative amount of unmethylated *INS*-DNA fraction in our DNA samples of interest.

First, the assay was validated in cell line models. DNA was isolated from EndoC-βH1 cells, a cell line that was derived from human β-cells(17). The MSRE duplex digital PCR assay was performed. This results in two-dimensional plots that demonstrate four different clusters each of them representing different DNA containing droplets (**Figure 2**). The green cluster contains no *INS target DNA* but only TTC5 copies; the blue cluster contains only *INS target DNA* but no TTC5 copies; the orange cluster contains both *INS target DNA* and TTC5 copies; the gray cluster includes the empty droplets. Without treatment of the MSRE (**Figure 2A**), the *INS target DNA* reflects the quantification of both unmethylated and

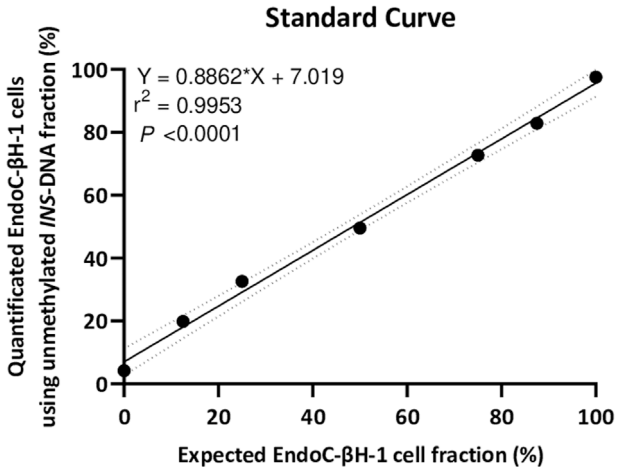

FIGURE 3 | A seven point standard curve demonstrates the relation between input percentage of EndoC-βH-1 cells DNA (diluted in a background of THP-1 cells DNA) that could be expected and EndoC-βH-1 cells DNA quantified as unmethylated *INS*-DNA was measured using digital PCR. (Y = 0.8862 *X + 7.019, r^2 = 0.9953, $p < 0.0001$). Abbreviations: PCR; polymerase Chain Reaction.

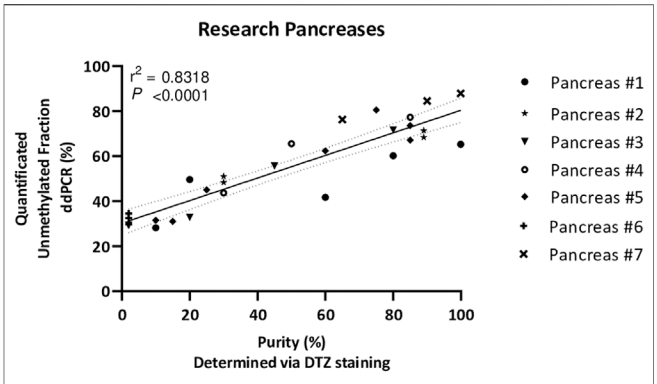

FIGURE 4 | Unmethylated *INS*-DNA fraction quantified by digital PCR in different purities of islets, determined via DTZ staining, after isolation from seven donor research pancreases. (r^2 = 0.8318, $p < 0.0001$). Abbreviations: DTZ; Dithizone Staining, PCR; polymerase Chain Reaction.

methylated *INS target DNA*. After treatment with the MSRE HpaII (**Figure 2B**), the unmethylated *INS target DNA* is digested, resulting in less blue and orange droplets. For both, with and without treatment of MSRE, a stable independent reference, TTC5, was used to correct for input differences as it is not digested by the MSRE. When using both ratios from *INS target DNA* and reference TTC5 in the samples with and without treatment with MSRE, an unmethylated *INS*-DNA fraction of 98.1% (95% CI 97.3–98.8) was determined (**Figure 2C**). With regards to DNA isolated from THP-1 cells, both ratios from *INS target DNA* and reference TTC5, when treated with (**Figure 2E**) or without (**Figure 2D**) the MSRE HpaII, were calculated and this resulted in an unmethylated *INS*-DNA fraction of 3.5% (95% CI -5.2–11.5) (**Figure 2F**).

As isolated DNA from EndoC-βH1 cells was essentially unmethylated for the *INS target DNA* whilst isolated DNA from THP-1 cells was mainly methylated for the *INS target DNA*, a 7-points standard curve was generated to technically validate the quantitative experimental setup. Isolated DNA from EndoC-βH1 cells diluted in the background of isolated DNA from THP-1 cells resulted in a strong linear correlation (r^2 = 0.9953, Y = 0.8862*X + 7.019, $p < 0.0001$) (**Figure 3**).

Next, the unmethylated *INS*-DNA fraction was determined in 24 human islets preparations which were isolated from seven different human donor pancreases obtained for research. For each sample, islet purity was determined, varying from <5 to 99%, via dithizone staining which is currently used by most centers to estimate the fraction of pancreatic islets in an isolated islet preparation (18, 19). In the case of a sample with <5% purity, the sample was categorized as islet depleted tissue (i.e., pancreatic tissue left over from islet isolation). After using this MSRE duplex digital PCR assay on DNA isolated from all the different purities of the islets, the unmethylated *INS*-DNA fraction was quantified (**Figure 4**). When comparing the purity of the pancreatic islets a significant linear correlation was observed (R squared = 0.8318, $p < 0.0001$). In the samples containing islet depleted tissue an unmethylated *INS*-DNA fraction of 29.4%–34.5% was observed.

DISCUSSION

Previous studies have demonstrated that the human *INS* gene is controlled epigenetically by methylation as it is unmethylated in β-cells and methylated in most other cell types (4, 20-22). When cells are dying or lysed - either *in vivo* or for experimentation purposes - their genomic DNA is released into the milieu. This makes unmethylated-*INS* DNA a highly interesting marker to detect the death of β-cells. Several research groups have developed assays to measure the circulating fraction of unmethylated *INS*-DNA in humans, often aiming to be used in the context of early detection of β-cell death in type 1 Diabetes. In 2020 Speake et al. (23) assessed the performance of three different methodologies (5, 9 11) to quantify circulating levels of unmethylated *INS*-DNA in patients undergoing total pancreatectomy and subsequent islet auto-transplantation. This was considered a reliable model as damage or cell death of β-cells is known to occur during transplantation. Not only did the group measure a different CpG site or sites in the human *INS* gene in these three assays, they also applied different sample collection methods and measurement techniques (e.g., next generation sequencing or digital PCR). We agree with Speake's group that to further develop these assays, optimization of the three different techniques might be beneficial. A similarity between all three assays was that DNA was treated with sodium bisulfite. This technique, which was first described by Frommer et al. (24), is still regarded as the gold standard to analyze DNA methylation. To prevent partial conversion and subsequent misinterpretation, the chemical conversion is performed at high concentrations. As a result, however, fragmentation and degradation of DNA will occur that may lead to an incorrect quantitative interpretation

(13, 25). In addition, with regard to the bisulfite conversion kits used in these studies focusing on unmethylated-*INS* DNA, it remains a relatively time consuming technique e.g., as approximately 12–16 h are needed for the incubation period.

To circumvent or even avoid these limitations, we report in this protocol a proof-of-concept study where we have combined the MSRE with digital PCR techniques to measure unmethylated-*INS* DNA. As an MSRE can differentiate between methylated and unmethylated alleles, MSRE treatment for only 1 hour results in digestion of unmethylated DNA, with the methylated DNA remaining intact. This allows for the rapid calculation of the fraction of unmethylated alleles in our target of interest (*INS target DNA*). When using two different cell lines, a strong correlation was observed (**Figure 3**) demonstrating a high sensitivity and specificity of this assay.

Next, we extended the use of this assay to measure the unmethylated *INS*-DNA fraction in different purities of islets obtained after pancreas isolation (**Figure 4**). Interestingly, the purity of the samples was not directly proportional to the quantified unmethylated *INS*-DNA fraction as was found in the standard curve obtained from the 2 cell lines (**Figure 3**). When using the MSRE duplex digital PCR in islet depleted tissue (i.e. containing <5% islets) an unmethylated *INS*-DNA fraction of 29.4–34.5% was observed. Of note is that this observed fraction is likely not a limitation of the assay itself but an indication that the biological variability in methylation of the human *INS* gene promotor in non β-cells may play an important role. Our result is in line with the study by Kuroda et al. (22) who investigated nine CpG sequences in the human *INS* gene promotor and compared the methylation pattern in this region in the 'islet cell fraction' and in the 'non-islet cell fraction'. In their study they demonstrated that the human *INS* gene promotor was mainly unmethylated in the islet cell fraction and predominantly methylated in the non-islet cell fraction (i.e., 13 of 15 clones (86%) in the non-islet cell fraction exhibited at least one unmethylated CpG out of the nine CpG sequences investigated).

With regard to the samples including high purity of islets, the quantified unmethylated *INS*-DNA fraction did not reach 100% which could be explained as the ratio of β-cells versus non-β-cells (e.g., alpha and delta) in human islets is generally assumed to be 50–70% (26). This is in line with the ±70% unmethylated *INS*-DNA fraction we have found (**Figure 4**).

A limitation of this proof-of-concept study is that our protocol was performed in cell lines and in different purities of human islet preparations obtained after isolation. Further validation experiments of this assay during islet isolation, islet culture and subsequent islet transplantation are necessary. During these next steps of the process an unknown amount of β-cell destruction occurs. To be able to specifically quantify the amount of β-cell loss using this promising assay could be helpful to differentiate between low or high quality and viability of islet preparations (12, 27). In clinical islet transplantation the accurate determination of the number of (viable) β-cells in a pancreatic islet preparation is essential. Not only assessment of the islet depleted tissue fraction, but more important the total number of isolated islets in the preparation is key for a successful transplant (28). In islet transplantation, the islet yield has previously been determined using various methods such as size-dependent islet counting by visualizing islets under a microscope and subsequent measurement of their volume (19), calculating both islet purity and graft volume or specific β-cell counting (28-31). To date, in most centers the estimation of the fraction of pancreatic islets in an isolated islet preparation is based on a method that uses dithizone staining (DTZ) (18, 19). Dithizone is a zinc chelating agent that, when added to an islet prep, results in a rapidly and reversibly red staining of islets which can therefore be distinguished from exocrine tissue. Importantly, this method cannot be used to determine the total number of β-cells in an isolated islet preparation. In addition, in case of β-cell degranulation, the red staining will not take place. Therefore, due to the human error that is intrinsic to this subjective method, an over- or under-estimation of the islet equivalent (IEQ) may easily occur. As such, determination of IEQ by eye or by digital image analysis has proven difficult within and between different centers (32).

Based on these notions, we suggest in this preliminary study that our newly developed MSRE duplex digital PCR assay using unmethylated *INS*-DNA may be a fast and easy method to specifically quantify β-cells. As shown previously, the combination of MSRE with digital PCR provides both specificity and sensitivity by quantitative assessment of target alleles (14). By measuring the concentration of the targeted unmethylated *INS*-DNA and therefore the number of lysed β-cells, this combined technique may be a promising tool to determine the fraction of β-cells immediately after islet isolation, during culture and immediately prior to islet transplantation. Pending further validation trials, the MSRE duplex digital PCR assay using unmethylated INS-DNA may therefore help decision making on islet quality (through the measurement of β-cell death) and islet quantity in islet transplantation centers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Leiden University Medical Center, Leiden, Netherlands. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FL, RN, PV and ME participated in conception and research design. The acquisition of data was performed by FL and MV. FL, RN, MV, Ed, VH, IA, RP, PV and ME participated in data analysis and interpretation. FL and ME drafted the manuscript. FL, RN, MV, Ed, VH, IA, RP, PV and ME participated in critical revision and final approval of the manuscript. PV and ME participated in study supervision. FL and ME are the guarantors of this work and, as such, had full access to all of

the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10167/full#supplementary-material

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ORIGINAL RESEARCH
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Primary Graft Function and 5Year Insulin Independence After Pancreas and Islet Transplantation for Type 1 Diabetes: A Retrospective Parallel Cohort Study

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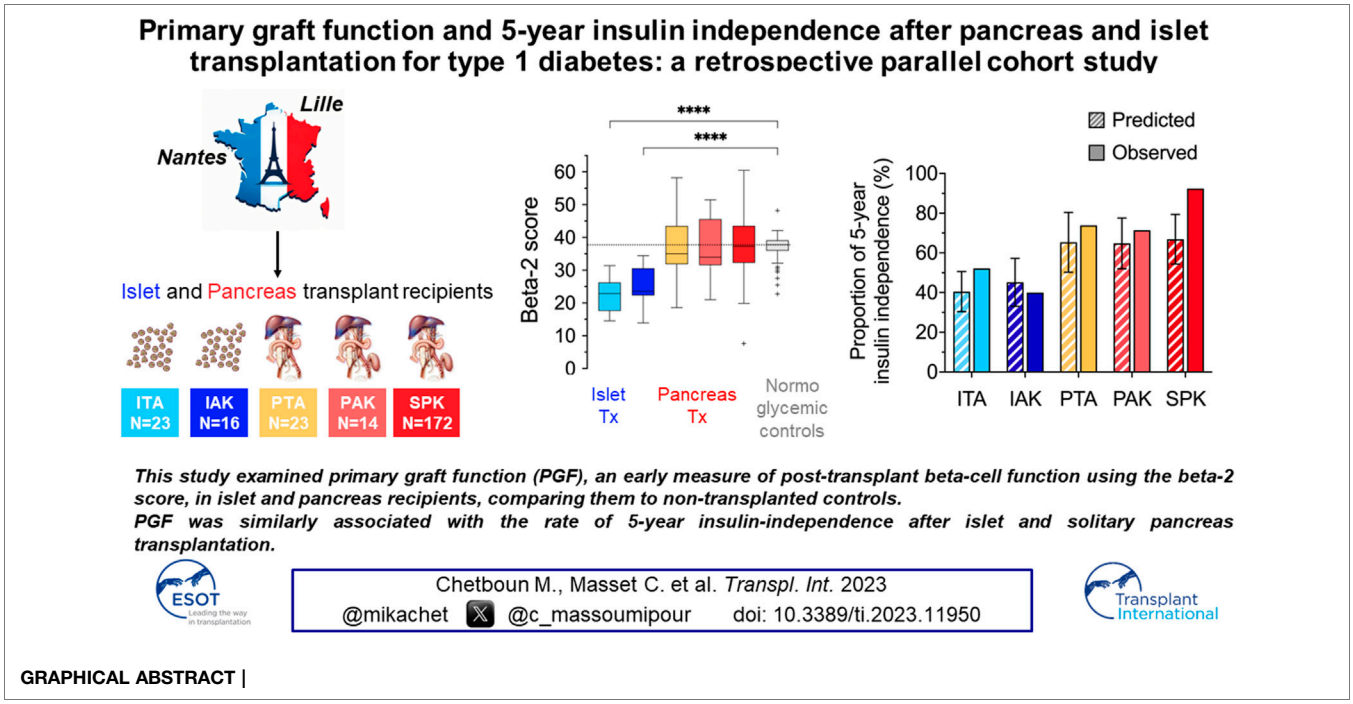
In islet transplantation (ITx), primary graft function (PGF) or beta cell function measured early after last infusion is closely associated with long term clinical outcomes. We investigated the association between PGF and 5 year insulin independence rate in ITx and pancreas transplantation (PTx) recipients. This retrospective multicenter study included type 1 diabetes patients who underwent ITx in Lille and PTx in Nantes from 2000 to 2022. PGF was assessed using the validated Beta2-score and compared to normoglycemic control subjects. Subsequently, the 5 year insulin independence rates, as predicted by a validated PGF-based model, were compared to the actual rates observed in ITx and PTx patients. The study enrolled 39 ITx (23 ITA, 16 IAK), 209 PTx recipients (23 PTA, 14 PAK, 172 SPK), and 56 normoglycemic controls. Mean[SD] PGF was lower after ITx (ITA 22.3[5.2], IAK 24.8[6.4], than after PTx (PTA 38.9[15.3], PAK 36.8[9.0], SPK 38.7[10.5]), and lower than mean beta-cell function measured in normoglycemic control: 36.6[4.3]. The insulin independence rates observed at 5 years after PTA and PAK aligned with PGF predictions, and was higher after SPK. Our results indicate a similar relation between PGF and 5 year insulin independence in ITx and solitary PTx, shedding new light on long-term transplantation outcomes.

Keywords: insulin independence, prediction, islet transplant, pancreas allograft, primary graft function

Abbreviations: PGF, Primary Graft Function; T1D, Type 1 Diabetes; PTx, Pancreas transplantation; ITx, Islet transplantation; ITA, Islet Transplantation Alone; IAK, Islet After Kidney transplantation; PTA, Pancreas Transplantation Alone; PAK, Pancreas After Kidney; SPK, Simultaneous Pancreas-Kidney.



GRAPHICAL ABSTRACT |

INTRODUCTION

Type 1 diabetes is caused by the autoimmune destruction of pancreatic beta-cells, leading to a complete deficiency of insulin secretion [1]. While exogenous insulin therapy remains the standard treatment, allogeneic transplantation of either whole pancreas organs or isolated pancreatic islets have emerged as validated therapeutic approaches in patients with severe forms of type 1 diabetes (T1D). The choice between pancreas (PTx) or islet transplantation (ITx) depends on various factors, including recipient characteristics, risk of immunosuppressive regimen and associated comorbidities [2–6].

In PTx, the vascularized transplanted organ rapidly restores endogenous insulin production, resulting in a substantial improvement in glycemic control, sustained insulin independence over years and the potential for regression of diabetic degenerative complications, including nephropathy lesions [7]. In patients with end stage renal failure, simultaneous pancreas-kidney transplant (SPK) was also linked to enhanced patient survival [8]. On the other hand, the transplantation of a vascularized pancreas requires a major surgical procedure which carries specific risks, such as bleeding, infection, and vascular thrombosis. Stringent patient selection is therefore crucial to minimize risks and ensure successful outcomes [9–11].

ITx entails only a minimally invasive procedure consisting of the infusion of few milliliters of isolated pancreatic islets into the portal vein, typically using a radiological or mini-surgical approach [3, 12–14], resulting in limited risks.

Although partial islet graft function is sufficient to suppress severe hypoglycemia [15], multiple islet infusions are often required to achieve sustained insulin independence [16–18].

Overall, PTx results in better long-term metabolic results than ITx [19–22], with the best long term outcome being reported after SPK. The reasons underlying these discrepancies are not fully elucidated. Assessing and predicting long-term graft function is an important objective for optimizing patient outcomes.

In the field of ITx, long-term graft survival has been related to the early estimate of transplanted beta-cell function, also named primary graft function (PGF) [16, 23]. A recent global study analyzing 1210 islet recipients from the international Collaborative Islet Transplant Registry [17], confirmed this tight relation between primary graft function, estimated 1 month after last islet infusion with the Beta2-score, a validated index of beta-cell function [24], and the overall 5 year success of ITx. Importantly, this association was independent of graft characteristics such as the number of islet infusions, the total transplanted islet mass, and also of the immunosuppressive regimen. These findings designate primary graft function as a robust early endpoint, which can be used to predict long-term outcomes in ITx [17]. In contrast, the evaluation of primary graft function (within first weeks after surgery) and its relation with long-term success (i.e., insulin independence) has not been explored in PTx recipients.

The primary objective of the present study was therefore to analyze and compare the potential association of primary graft function estimated soon after transplantation, and the 5 year rate of insulin independence in patients receiving an ITx and for the first time in patients receiving PTx.

PATIENTS AND METHODS

Study Design

This retrospective multicentre cohort study was designed to estimate primary graft function in patients who received beta-cell replacement with either PTx or ITx, and to analyze its relation with the rate of 5 year insulin independence. In addition, we also compared primary graft function in transplanted patients with beta-cell function estimated in non-transplanted normoglycemic individuals.

Study Population

Pancreas and Islet Transplantation Recipients

We enrolled participants from two single-center cohorts of ITx and PTx in whom all variables required to calculate primary graft function were available within weeks after transplantation, and a follow-up of at least 5 years.

PTx was performed at Nantes University Hospital between 2000 and 2022. Recipients aged from 18 to 65 years old were included if they received pancreas transplantation alone (PTA), pancreas after kidney (PAK) or simultaneous pancreas and kidney transplantation (SPK), had a functional pancreas graft, and available variables to calculate the Beta-2 score (HbA1c mostly available after the third post-operative month), and a follow-up of at least 5 years. Procurement of pancreases, for both PTx and ITx, was obtained from ABO-compatible/MHC-unmatched brain-dead deceased donors with a negative T-cell cross-match. Whole organ pancreas was transplanted following procurement (i.e., less than 12 h) using a duodeno-enteric anastomosis, either with or without Roux-en-Y. Portal or systemic venous diversion was performed. Kidney transplantation was performed according to standard surgical procedure [25]. The induction immunosuppressive strategy consisted of a T-cell depleting agent (anti-thymocyte globulin for 5 days) and TNF-alpha inhibitor Etanercept (since 2017), tacrolimus and antitiproliferative agent mycophenolate mofetil or mycophenolic acid, all at standard and recommended doses. Steroids were administered for only 7–10 days.

ITx was performed at Lille University Hospital between 2003 and 2017, as previously described [14]. Briefly, recipients were patients with C-peptide negative type 1 diabetes, aged from 18 to 65 years old who received an islet transplantation alone (ITA) or after kidney transplantation (IAK) in the context of three prospective trials (ClinicalTrials.gov Identifier: NCT00446264/NCT01123187 [16] and NCT01148680 [26]) and a follow-up of at least 5 years. Islets were isolated within 12 h following pancreas procurement and cultured for up to 72 h prior to transplantation [27]. ITx consisted of two to three sequential intraportal islet infusions within 3 months, with the aim of reaching adequate metabolic control (i.e., HbA1c ≤ 6.5% without severe hypoglycemia) without exogenous insulin. No re-transplantation was performed during the follow-up even when the patient had lost his islet graft. Access to portal vein was obtained under general anesthesia by percutaneous transhepatic catheterization of a peripheral portal branch under ultrasound guidance or by a surgical mini-invasive laparotomy with vascular approach of a proximal mesenteric vein. Heparin (35 units/kg of recipient body weight) was added to the final human islet preparation, gently infused by gravity with portal pressure monitoring as previously described [16, 23]. Participants received Interleukin-2 receptor antagonist (Daclizumab^{TM}) induction with sirolimus and tacrolimus maintenance (trials NCT00446264 and NCT01123187) [16]. Participants from trial NCT01148680 [26] received induction with TNF-alpha inhibitor (Etanercept^{TM}), T-cell depleting agent (anti-thymocyte globulin) for first infusion or with Interleukin-2 receptor antagonist for second or third infusions followed by maintenance therapy using tacrolimus and antiproliferative agents (mycophenolate mofetil).

Controlled Non-transplanted Population

In addition, we also analyzed data from normoglycemic adult individuals enrolled in two prospective cohorts (OBEDIAB, ClinicalTrials.gov Identifier: NCT00688974; and ABOS, ClinicalTrials.gov Identifier: NCT01129297), at Lille University Hospital between 2004 and 2022 for surgery. Participants with a body mass index comprised between 18 and 40 kg/m2 and normal glucose control (fasting plasma glucose <5.6 mM/L, 2 h plasma glucose <7.8 mM:L, HbA1c<5.7%), in whom the four variables required to calculate the Beta-2 score were available at the baseline visit, were included in the present study.

Data Collection

Recipient, donor and transplantation characteristics were collected in the ITx and PTx cohort prior to transplantation. Including recipient age, sex, body mass index (BMI), pre-transplant glycemic status, immunosuppressive regimens, graft characteristics. The total islet mass transplanted was expressed in islet-equivalent (i.e., one islet-equivalent corresponds to the tissue volume of one spherical islet with a diameter of 150 µm [28]). Allogeneic immunization prior to transplantation was evaluated by complement-dependent lymphocytotoxicity assay prior to 2007 and by the LABScreen Mixed Luminex flow bead assay (One Lambda^{TM}) after 2007 and preformed donor-specific antibodies (DSA) were defined as positive if minimum mean fluorescent intensity (MFI) was equal to or greater than 500 in ITx and 1000 in PTx recipients.

Study protocols were approved by the Institutional Review Board and were previously published [16, 23, 25, 26, 29]. PTx data were extracted from the French Nantes DIVAT cohort approved by the French CNIL (n°914184). The quality of the DIVAT data bank is validated by an annual cross-center audit and has been reviewed by the appropriate ethics committee in accordance with the ethical standards laid down in the Declaration of Helsinki 2000 as well as the Declaration of Istanbul 2008. The database was locked on July 1, 2023. The implementation of the database refers to the standard operating procedures established in accordance with the European Data Protection Directive (95/46/EC) and, upon its entry into force, Regulation (EU) 2016/679, also referred as the General Data Protection Regulation (GDPR), with the French CNIL concerning the processing of personal data in clinical studies. Data were de-identified before analysis in order to respect confidentiality. A signed informed consent was obtained from all ITx, PTx and OBEDIAB/ABOS patients.

Exposure of Interest

The study exposure of interest was primary graft function, an early estimation of the functional beta-cell mass after transplantation. In ITx, primary graft function was assessed as previously described, 1 month after the last islet infusion (2–6 months after first islet infusion) [16, 17]. In PTx, since HbA1c level was rarely measured before the end of the third month after surgery, primary graft function was assessed at this time period. In all cases, primary graft function was estimated with the Beta-2 score, a continuously validated variable (in which 0 represents no beta-cell function) calculated using a fasting blood sample based on values of fasting C-peptide (nmol/L), fasting blood glucose (mmol/L), HbA1c (%), and daily exogenous insulin needs per kg of body weight (IU/kg per day) [24]. In the OBEDIAB/ABOS cohort, beta-cell function was similarly estimated with the Beta-2 score using the fasting values of C-peptide, blood glucose, and HbA1c measured during a 75 g oral glucose tolerance test prior to surgery and allowed to classify the glucose tolerance disorder of each patient according to the criteria of the American Diabetes Association. In this population only normoglycemic controls were included in the present study.

Outcome

The success of transplantation was defined as insulin independence, i.e., no exogenous insulin needs for a minimum of 14 consecutive days, assessed 5 years after transplantation.

Statistical Analysis

Quantitative variables were expressed as means ± standard deviation in cases of normal distribution or medians (interquartile range, IQR) otherwise. Categorical variables were expressed as numbers (percentage). Normality of distributions was assessed using histograms and the Shapiro-Wilk test.

Pre-transplant recipient and transplantation characteristics were described for three different subgroups: ITA/IAK, PTA/PAK, and SPK. Beta-2 score and its determinants, fasting serum C-peptide, and HbA1c, were described for different subgroups in ITx, PTx recipients, and in OBEDIAB/ABOS individuals and continuous variables were compared using the One-way Welch ANOVA test. Note that only patients with a functional pancreatic graft at 3 months were analyzed in this study (per-protocol analysis, excluding patients with primary graft failure), whereas all islet-transplanted patients had a functioning graft at 1 month and were included in the analysis (intention-to-treat analysis).

For each subgroup of recipients (ITA, IAK, PTA, PAK, and SPK), we calculated the mean observed 5-year rate of insulin independence. For this analysis, only patients transplanted between 2000 and 2018 were analyzed. We estimated the mean predicted 5 year rate of insulin independence using an online calculator based on PGF [30]. As previously outlined [30], this calculator solely depending on the value of the primary graft function was constructed and validated using a cohort of islet recipients and predicts diverse outcomes validated in ITx [17].

All statistical analyses were performed using SAS Studio Statistics (version 3.81) and Prism GraphPad (Version 10.0.1) software.

RESULTS

Characteristics of the Study Population

Among 476 patients who benefited from PTx in Nantes (377 SPK/43 PAK/56 PTA), 209 recipients did not meet the inclusion criterion of the study, mainly because the lack of available HbA1c and/or C-peptide at 3 months after transplantation ($n = 207$), or because of early graft loss ($n = 60$). The individuals excluded for missing values showed no clinically relevant differences when compared to the included recipients (**Supplementary Table S1**). Baseline and transplantation characteristics of the study participants are described in **Table 1**. Of these recipients, 172 (82%) underwent SPK, 23 (11%) received PTA, and 14 (7%) received PAK transplantation.

All 39 patients who underwent ITx in Lille during the study period (16 IAK/23 ITA) were enrolled. The baseline recipient's and transplantation characteristics are provided in **Table 1**. Among them, 28 (72%) received three islet cell infusions, while 11 (28%) received two infusions. A total of 106 infusions of human islets were carried out, with recipients experiencing a median overall transplantation duration of 2.7 months (IQR 1.6–4.1). There were no further infusions conducted throughout the follow-up period. The median total islet mass transplanted was 13.6 thousand islet-equivalents per kg of body weight (IQR 11.7–15.9).

A total of 56 non-transplanted normoglycemic individuals were included in this study. Of these, 43 (77%) were women, their median age was 41 (IQR 34–48) years, and their median BMI was 37.6 (IQR 27.0–38.9) kg/m^2

Primary Graft Function

Mean[SD] primary graft function estimated with the Beta-2 score in ITA, IAK, PTA, PAK, and SPK recipients was 22.3[5.2], 24.8 [6.4], 38.9[15.3], 36.8[9.0], and 38.7[10.5], respectively. Mean beta-cell function estimated with the Beta-2 score in normoglycemic controls was 36.6[4.3]. As displayed in **Figure 1A**, the mean values of primary graft function in ITA and IAK recipients were significantly lower than the mean beta-cell function measured in normoglycemic controls ($p < 0.0001$). Conversely, the mean value of primary graft function in PTx recipients with a surviving graft and the mean beta-cell function measured at the time of enrolment in normoglycemic controls were similar.

The mean fasting C-peptide levels were significantly higher in the controls compared to ITA ($p < 0.0001$) and IAK ($p = 0.001$), but they were significantly lower compared to SPK ($p < 0.0001$) and similar to those of PTA ($p = 0.643$) and PAK ($p = 0.310$) (**Figure 1B**).

Of note, the overall HbA1c values of normoglycemic controls did not significantly differ from those in IAK, PTA, PAK and SPK recipients but were significantly lower compared to ITA ($p = 0.191$) (**Figure 1C**).

Five-Year Insulin Independence

Among the 39 islet-transplanted recipients, two never achieved insulin independence, and 22 patients (56.4%) were not insulin independent at 5 years. At the last follow-up, 32 ITx recipients out of 39 had a functional graft (serum C-peptide ≥0.3 ng/mL).

TABLE 1 | Recipient, graft and transplantation characteristics in the Islet transplantation cohort.

	ITA/IAK *n* = 39	PTA/PAK *n* = 37	SPK *n* = 172
Pre-transplantation recipient's characteristics			
Female gender, n (%)	20 (51%)	18 (49%)	63 (37%)
Age (years), mean (SD)	45 (8)	42 (±9)	40 (±7)
Body mass index (kg/m^2)	24 (±3)	25 (±4)	23 (±3)
HbA1c (%)	8.2 (±1.0)	9.4 (±2.6)	8.3 (±1.5)
Preformed donor specific antibody	1 (3%)	2 (6%)	14 (10%)
Transplantation characteristics			
Islet transplantation			
Number of islet infusions	2.7 (±0.5)		
Time between first and last infusion, months	2.7 (1.6–4.1)		
Total islet mass transplanted, 10^3 IEQ/kg of recipient weight	13.6 (11.7–15.9)		
Total tissue volume (mL)	12.9 (9.7–14.9)		
Islet purity[a] (%)	47 (44–54)		
Islet viability[a] (%)	93 (91–96)		
Pancreas transplantation			
Female donor		12 (32%)	59 (34%)
Donor age (years)		32 (±14)	33 (±11)
Donor body mass index (kg/m^2)		23 (±3)	23 (±3)
Cold ischemia time (min)		603 (±161)	658 (±177)
Immunosuppression			
T-cell depleting agent induction	11 (28%)	35 (95%)	161 (95%)
Calcineurin inhibitor	39 (100%)	37 (100%)	167 (98%)
m-TOR inhibitor	28 (72%)	37 (100%)	3 (2%)
Corticosteroid therapy	0 (0%)	32 (86%)	153 (90%)

Recipient, and transplantation characteristics are reported as n (%), mean (SD), or median (IQR) as appropriate.

[a]*The overall islet purity and viability were the weighted median (IQR) of the two or three islet infusions transplanted by the volume of each preparation. The total tissue volume was the sum of the volume of each infused preparation in the recipient.*

ITA, islet transplant alone; IAK, islet after kidney; PTA, pancreas transplant alone; PAK, pancreas after kidney; SPK, simultaneous pancreas kidney; m-TOR inhibitor, mammalian target of rapamycin inhibitor.

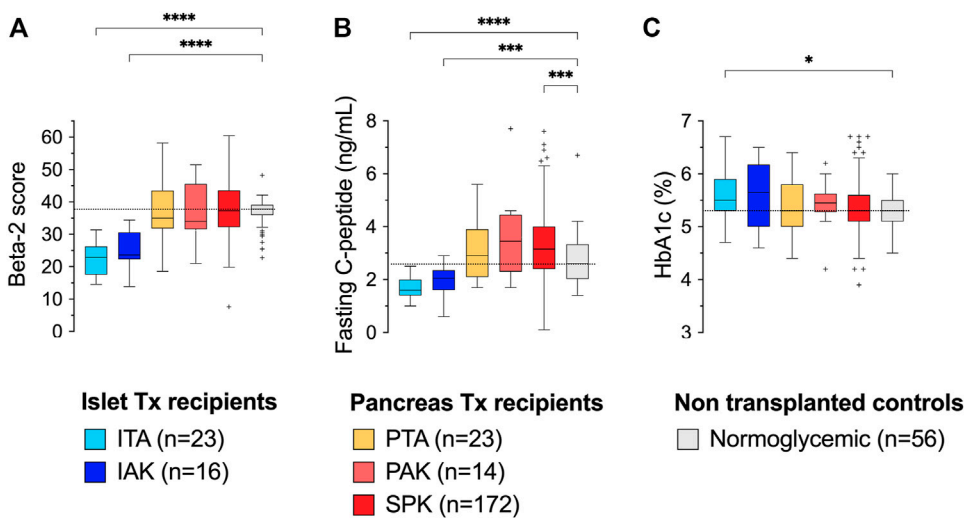


FIGURE 1 | Beta-2 score **(A)**, fasting serum C-peptide **(B)** and HbA1c **(C)** values in islet recipients, pancreas recipients and non transplanted control individual The distribution is represented in the form of a box plot using the Tukey method, where the line in the middle of the box is drawn at the median, the box limits represent the 25th and 75th percentiles, and the whisker limits are represented from the value of the 25th percentile minus 1.5 times the interquartile range (IQR) to the value of the 75th percentile plus 1.5 times the IQR. Outliers are represented individually. *p* values ≤0.001 are summarized with an asterisk. Groups were compared with Welch ANOVA tests. Symbol meaning: $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****) ITA, Islet Transplantation Alone; IAK, Islet After Kidney transplantation; PTA, Pancreas Alone; PAK, Pancreas After Kidney; SPK, Simultaneous Pancreas-Kidney.

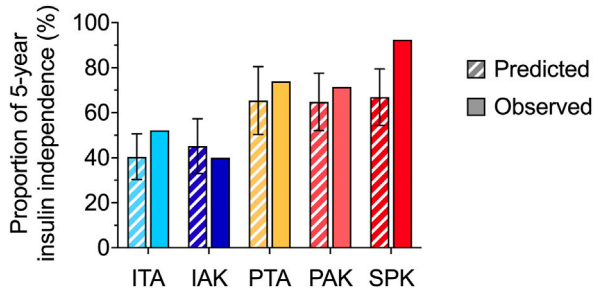


FIGURE 2 | Predicted and observed proportion of 5 years insulin independence among islet and pancreas recipient with initial graft function The mean (95% confidence interval) of predicted 5 year rate of insulin independence (hatched bar) and the observed 5 year rate of insulin independence (solid bar) are reported for the various recipient subgroups. IA, Islet Transplantation Alone; IAK, Islet After Kidney transplantation; PA, Pancreas Alone; PAK, Pancreas After Kidney; SPK, Simultaneous Pancreas-Kidney.

Among the 209 recipients who received PTx and had a functional pancreas graft at 3 months, 23 patients (11.0%) had lost insulin independence during the 5 years follow-up. Of note, 12.5% of the overall cohort of PTx recipients experienced a graft loss before 3 months and were therefore excluded from the present analysis (60 out of 476 pancreas recipients).

Relation Between Primary Graft Function and 5 Year Outcome

We used the PGF-based calculator available online [30] to estimate the mean (95% CI) proportion of patients in each subgroup with 5 year insulin independence, as illustrated in **Figure 2**, the proportion of insulin-independent patients observed at 5 years remained within the prediction confidence interval determined by the calculator in islet and solitary pancreas recipients but not in SPK. Indeed, in this subgroup of patients, the observed rate of 5 year insulin-independence was significantly higher than the rate predicted with the PGF-based calculator.

DISCUSSION

In the current study, we analyzed the early post-transplant beta-cell function, referred to as primary graft function, in islet transplantation and pancreas transplantation, and examined its relationship with the 5 year rate of insulin independence across all transplantation modalities.

Our study demonstrated that primary graft function values were comparable between ITA and IAK, as well as between PTA, PAK, and SPK. However, mean value of primary graft function was significantly lower in ITx recipients compared to PTx recipients. Primary graft function values in ITx recipients were also significantly lower than the beta-cell function observed in normoglycemic controls. In contrast, pancreas transplant recipients exhibited primary graft function values similar to beta-cell function values in normoglycemic controls.

Notably, serum C-peptide levels in normoglycemic controls were higher than in the ITA and IAK groups. However, these levels were similar to those in solitary pancreas recipients but lower than in SPK recipients. Every islet and pancreas recipient exhibited marked improvements in HbA1c levels, aligning with the American Diabetes Association's recommended targets, compared to their pre-transplant values. Additionally, mean HbA1c values were not significantly different between the various types of transplantation, except for ITA, where recipients exhibited significantly higher values.

Secondly, our findings indicated that for PTA and PAK recipients, the calculator's predictions of 5 year insulin independence rates, which were based solely on primary graft function, were relatively precise. In contrast, the calculator tended to underestimate the outcomes in SPK recipients.

These results are in line with a recent study demonstrating the independent linear association between primary graft function and various 5 year outcomes of ITx [17], including graft function, insulin independence, adequate glucose control, and overall transplantation success assessed with the Igls 2.0 criteria [31]. To our knowledge, the present study is the first to extend these results in the context of PTx. These findings indicate that the difference in long-term outcomes of PTx and ITx are likely attributable to the superior initial function of islets that survive the transplantation of a vascularized pancreas, in contrast to isolated islets infused in the portal vein. Of note, all subgroups transplanted with a vascularized pancreas had similar primary graft function. The 5 year insulin independence rate observed in patients who simultaneously received a kidney from the same donor (SPK) was, however, superior than in those who received a solitary pancreas (PTA/PAK). This difference between SPK and solitary pancreas transplant was also reported in the International Pancreas Transplant Registry and related to the reduction of immunologic graft loss [32]. In a study on SPK recipients, synchronous pancreas and kidney rejection occurred in 73%, kidney-only rejection occurred in 23% and pancreas-only rejection occurred in only 3% of biopsies [33]. Taken together, these results suggest a positive impact of monitoring kidney function for early detection and treatment of the overall allogenic immune response. Diagnosing immune rejection remains therefore challenging in solitary pancreas or islet transplantation [34].

Several limitations need to be considered when interpreting our study. First, the retrospective design of the study and the limited sample size for certain groups could have introduced selection bias. A prospective study in a larger cohort of patients could yield more robust and generalizable results. Additionally, data were collected from two parallel single center cohorts, which could introduce variations in patient selection and follow-up protocols. Multicenter studies with standardized protocols could help mitigate this potential bias and strengthen the study's findings.

Second are the method and timing used to estimate primary graft function. Several composite indexes have been proposed to estimate beta-cell function [35]. We chose here to use the Beta-2 score, a simple and continuous score validated in ITx [24]. The

use of more sophisticated tests to estimate primary graft function, such as dynamic tests of insulin secretory reserve, could have refined the prediction of long-term outcomes [36]. As previously described, primary graft function was assessed 1 month after the last islet infusion [17], which corresponds to the necessary time for full revascularization of islets transplanted in the liver [37]. In practice, this also resulted in a mean duration of 4.3 [2.8] months after the first islet infusion. The optimal timing for assessing PGF after the transplantation of a vascularized pancreas is unknown. Here, we used 3 months for this was the earliest data available in the study's participants.

Of note, PTx recipients who experienced graft failure before that date (60 cases) had to be excluded from this retrospective study, since 5 year follow-up data were not available, resulting in a twelve percent overestimation of the reported 5 year rate of insulin independence after PTx. This exclusion of early pancreatic graft failures may be debatable. However, since our main objective was to evaluate the predictive value of early beta-2 score in functional pancreas transplant recipients for long-term graft function, we assume this exclusion did not introduce bias into our study's analysis and conclusions. It is also important to note that half of the eligible pancreas transplant recipients were excluded from the analysis due to missing data. Nonetheless, as the included and excluded groups were comparable (**Supplementary Materiel**), we assume these exclusions did not introduce bias into our analysis.

Finally, it should be noted that organ allocation rules differ for islet and pancreas recipients in France and many countries. This practically favors the use of organs from donors with lower BMI and younger age in pancreas Tx. This potential selection bias, may have contributed to higher primary graft function observed in pancreas Tx recipients.

In summary, this study showed that the beta cell function restored in patients with Type 1 diabetes following islet Tx, even after multiple infusions, remains generally inferior to the levels observed in recipients of pancreas Tx and to those measured in control individuals. Our results also suggest that this difference in PGF likely explains the difference in 5 year rate of insulin independence generally observed between islet and pancreas Tx. Overall, this study suggests for the first time, a potential use of primary graft function as an early predictor of long-term outcome of PTx, principally PTA and PAK. Optimal primary graft function indicates better graft function and a higher likelihood of maintaining long-term insulin independence. However, to better understand this predictive role, further research is needed in the context of PTx. Prospective, larger scale, long-term studies remain warranted to distinguish the respective role of primary graft function and confounding factors, such as recipient allogeneic and autoimmune reactions, and the effects of immunosuppressive treatments.

In conclusion, the present study supports the value of primary graft function in the management of type 1 diabetes patients undergoing beta-cell replacement with various modalities, such as PTx, ITx, or other insulin-secreting cell transplantation.

DATA AVAILABILITY STATEMENT

Datasets presented in this article are available upon reasonable request to corresponding authors.

ETHICS STATEMENT

The studies involving humans were approved by European Data Protection Directive (95/46/EC). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC, CM, DC, and FP contributed substantially to the conception and design of the study, the acquisition of data, or the analysis and interpretation. MC and CM conducted the data analysis. MC, CM, DC, and FP drafted the article. MC, CM, DC, and FP reviewed/edited the manuscript. MC, CM, MM, FD, VG, VR, TH, CB, LS, CK, GB, JB, GK, IC, AH, MG, CG, JD, KL, AJ, MH, RC, JK-C, M-CV, DC, and FP contributed to the interpretation of data and critical revision of the article. FP and DC are 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. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11950/full#supplementary-material

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ORIGINAL RESEARCH
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Validation of Igls Criteria for Islet Transplant Functional Status Using Person-Reported Outcome Measures in a Cross-Sectional Study

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Associations between islet graft function and well-being in islet transplant recipients requiring exogenous insulin remain unclear. This cross-sectional analysis compared person-reported outcome measures in 15 adults with type 1 diabetes whose islet transplants were classified according to Igls criteria as "Good" (n = 5), "Marginal" (n = 4) and "Failed" (n = 6) graft function. At a mean of 6.2 years post-first islet transplant, 90% reduction in severe hypoglycaemia was maintained in all groups, with HbA1c (mean ± SD mmol/mol) 49 ± 4 in recipients with "Good" function; 56 ± 5 ("Marginal"); and 69 ± 25 ("Failed"). Self-reported impaired awareness of hypoglycaemia persisted in all groups but those with "Good" function were more likely to experience symptoms during hypoglycaemia. "Marginal" function was associated with greater fear of hypoglycaemia (HFS-II score: "Marginal": 113 [95, 119]; "Failed": 63 [42, 93] (p = 0.082); "Good": 33 [29, 61]) and severe anxiety (GAD7: "Marginal"): 21 [17, 21]; "Failed": 6 [6, 6] "Good": 6 [3, 11]; (p = 0.079)), diabetes distress and low mood. Despite clear evidence of ongoing clinical benefit, Igls criteria 'Marginal' function is associated with sub-optimal well-being, including greater fear of hypoglycaemia and severe anxiety. This study provides person-reported validation that "Good" and "Marginal" graft function are differentiated by general and diabetes-specific subjective well-being, suggesting those with "Marginal" function may benefit from further intervention, including re-transplantation.

Keywords: hypoglycaemia, Igls, PROMs, islet, transplant

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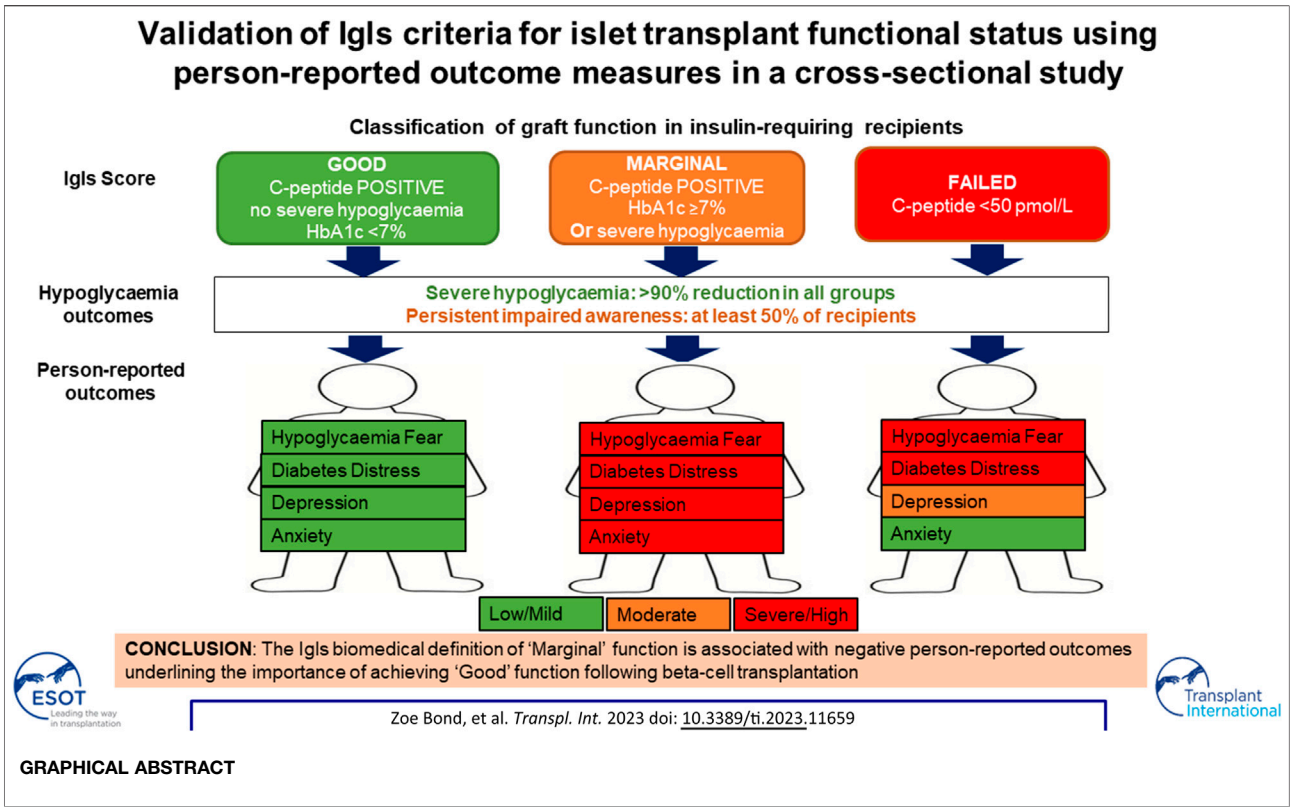
INTRODUCTION

Following seminal success in Edmonton [1], intraportal transplantation of deceased donor isolated pancreatic islets has become established as standard-of-care for selected individuals with type 1 diabetes in healthcare systems around the world [2]. A National Health Service (NHS) funded integrated programme for islet transplant was commissioned in the United Kingdom in 2008 to provide equitable access to adults with C-peptide negative type



GRAPHICAL ABSTRACT

1 diabetes complicated by life-threatening hypoglycaemia despite optimal conventional medical management.

The goal of the NHS programme was to prevent further severe hypoglycaemia without the expectation of insulin independence. At the outset, the National Institute for Health and Care Excellence identified core audit criteria as confirmation of graft function through C-peptide positivity; reduction in numbers of severe hypoglycaemic events; attainment of HbA1c less than 53 mmol/mol (7%); and reduction in exogenous insulin dose [3]. In 2017, the International Pancreas & Islet Transplant Association (IPITA) and the European Pancreas & Islet Transplant Association (EPITA) proposed the Igls criteria using these outcome measures to define islet graft status. "Optimal function" necessitated insulin independence and a consensus was reached around definitions of "Good" and "Marginal" graft function [4, 5].

In the absence of insulin independence, associations between level of islet graft function and overall health status/well-being remain unclear. Validation of the Igls classification using patient-reported outcome measures (PROMs) was advocated in the original consensus statement but has not previously been undertaken [4]. We aimed to examine associations between Igls criteria and person-reported hypoglycaemia awareness; behaviours and fears around low and high glucose levels; diabetes distress; and anxiety/depressive symptoms in a cross-sectional study of previous islet transplant recipients, with an ongoing requirement for self-administered insulin therapy, at a single UK centre.

METHODS

Study Design

The study was conducted between April and June 2022 following ethical approval (REC number 07/Q0904/11) to recruit participants who had received one or more percutaneous, transhepatic, intra-portal deceased donor pancreatic islet infusions at the Newcastle upon Tyne Hospitals NHS Foundation Trust within the NHS islet transplant programme. Inclusion criteria included ≥2 episodes of severe hypoglycaemia requiring assistance in treatment [6] over the 2 years before first islet transplant, with pre-transplant meal tolerance test stimulated C-peptide of <50 pmol/L and current requirement for exogenous insulin. In this cross-sectional study primarily designed to interrogate the Igls criteria proposed "boundary" between those with "good" and "marginal" islet graft function, we agreed *a priori* to exclude recipients with insulin independent "optimal" function.

A questionnaire pack was compiled for participant completion to assess hypoglycaemic episodes and impaired awareness; attitudes and behaviours towards hyper- and hypoglycaemia; diabetes-associated distress and problems; and anxiety/depression (**Supplementary Table S1**). Instruments which have previously been established as acceptable to, and validated in, adults with established type 1 diabetes were selected through a consensus reached by a consultant diabetologist, a diabetes clinical research fellow with experience in qualitative data collection, a health psychologist

and a clinical psychologist. Acceptability, understandability, utility and face validity were confirmed in previous islet transplant recipients before finalisation. In keeping with published scoring systems, it was agreed that missing items would be replaced by the mean score of the non-missing items where less than 20% of items were missing in the Attitudes to Awareness of Hypoglycaemia (A2A) [7], Problem Areas in Diabetes (PAID) [8] (PAID), Hospital Anxiety and Depression Scale (HADS) [9], and 9-item Patient Health Questionnaire (PHQ-9) [10]; or where less than 25% of items were missing in Hypoglycaemia Fear-Survey-II (HFS-II) [11] and Hyperglycaemia Avoidance Scale (HAS) [12]. The Gold score [13], 7-item Generalised Anxiety Disorder Scale (GAD-7) [14] and Type 1 Diabetes Distress Score (T1DDS) [15] scortes were included when completed without missing items. The Hypoglycaemia Awareness Questionnaire (HypoA-Q) [16] was completed in long-form with novel analysis of "Symptom Frequency" and "Symptom Level" subscales. Participants were asked to comment on the utility and acceptability of each questionnaire in addition to any preference for particular measures.

In parallel with questionnaire completion, demographics, transplant history and biomedical data enabling Igls graft status classification according to published criteria [5] were obtained from participants' most recent follow-up visit recorded on electronic healthcare records. C-peptide samples were analysed in a central reference laboratory by Siemens Immulite 2000 assay (Erlangen, Germany).

Statistical analysis was completed using SPSS statistics software version 28.0. Data normality was determined by Shapiro-Wilks test with age, diabetes duration and other parametric data presented as mean ± standard deviation and non-parametric data as median [quartile 1, quartile 3]. Categorical data are shown as number (%). Means were compared using one-way ANOVA with post-hoc testing using Tukey's test. Medians were compared using Kruskal-Wallis test and categorical variables using Fisher's exact test. $p < 0.05$ was considered statistically significant.

RESULTS

Twenty-one islet transplant recipients fulfilling inclusion criteria were approached for potential participation and 15 returned questionnaires following written informed consent (**Figure 1**). In participants with ongoing graft function, biomedical data were retrospectively collected from a follow-up visit within 6 months of questionnaire completion, with the exception of a single participant providing questionnaire data during pregnancy in whom pre-conception biomedical data were used. In participants with graft failure, biomedical data including C-peptide <50 pmol/L were collected from a single visit which may have preceded questionnaire completion by >6 months (but with confirmed clinical stability between biomedical and patient-reported data collection).

All participants had type 1 diabetes (absolute C-peptide negativity confirmed by pre-transplant meal tolerance test)

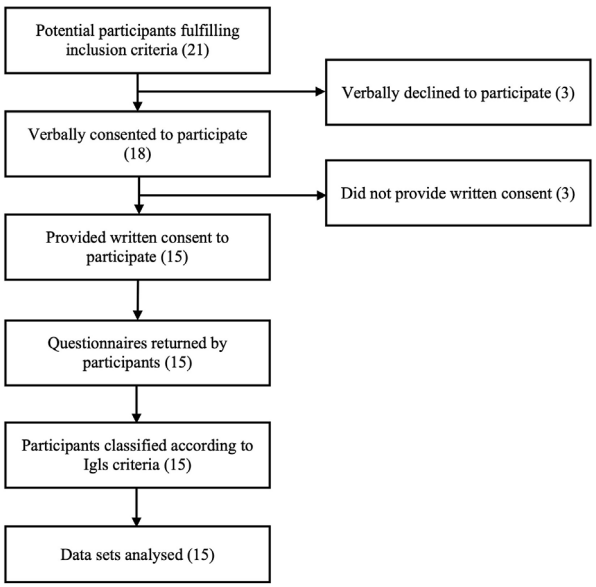


FIGURE 1 | CONSORT diagram.

complicated by recurrent severe hypoglycaemia requiring assistance with treatment, with 52 ± 98 events over the 12 months prior to transplantation. Twelve (80%) were female with age 60 ± 10 years and diabetes duration 45 ± 11 years. Twelve (80%) had received islet transplants alone and three (20%) islet after kidney transplants. Questionnaires were completed at a mean of 6.2 years following first islet transplant.

Ten (67%) participants were using multiple dose insulin therapy with the remaining five (33%) on continuous subcutaneous insulin infusion pumps. Eight (53%) participants were using continuous glucose monitoring and the remaining seven (47%) flash glucose monitoring. Pre-transplant total daily insulin dose in the cohort as a whole was 0.47 ± 0.19 units/kg.

At the time of cross-sectional assessment, five (33%) participants' transplants were classified as having "Good" function, four (27%) had "Marginal" function and six (40%) had "Failed." "Good" function required absence of severe hypoglycaemia and HbA1c <7.0% (53 mmol/mol) [6]. C-peptide increase compared to baseline was defined as stimulated C-peptide >50 pmol/L, as all had mixed meal tolerance test 90 min values below this cut-off at baseline. Those in the 'Good' function group had reductions in total daily insulin dose of >50% with the exception of one participant with current insulin dose of 0.15 units/kg where pre-transplant insulin dose was not available and another with very low pre-transplant insulin dose (0.34 units/kg) reduced by only 44% to 0.19 units/kg at post-transplant assessment. "Marginal" function was defined as HbA1c ≥7.0 (53 mmol/mol)), severe hypoglycaemia with less than baseline frequency, insulin requirement ≥50% of baseline and C-peptide level greater than baseline [4]. Those who had stimulated C-peptide <50 pmol/L were defined as having a graft that had "Failed."

Age, duration of diabetes and number of transplants were comparable in all groups (**Table 1**). Time since first transplant

TABLE 1 | Cross-sectional metabolic status of transplant recipients classified, by Igls graft status.

	Islet graft function			*p*-value
	Good	**Marginal**	**Failed**	
Number	5 (33%)	4 (27%)	6 (40%)	—
Age (years)	62.0 ± 16.6	61.3 ± 5.3	57.0 ± 4.1	0.696
Duration of diabetes (years)	50.0 ± 16.8	38.8 ± 7.8	44.7 ± 4.5	0.389
Female	4 (80%)	3 (75%)	5 (83%)	1.000
Islet transplant alone	4 (80%)	4 (100%)	4 (67%)	0.736
Number of islet transplants	2 (2.2)	2 (1.2)	2 (2.2)	0.857
Insulin independence achieved	3 (60%)	1 (25%)	1 (17%)	0.397
Time since first transplant (months)	59.0 ± 35.3	59.8 ± 44.7	97.7 ± 30.3	0.173
CSII	0 (0%)	2 (50%)	3 (50%)	0.201
CGM	2 (40%)	3 (75%)	3 (50%)	0.674
Severe hypoglycaemia:				
Frequency per year	0.0 ± 0.0	1.5 ± 1.0	1.7 ± 2.3	0.093
Participants per year	0 (0%)	3 (75%)	3 (50%)	0.069
HbA1c (mmol/mol)	49 ± 4	56 ± 5	69 ± 25	0.389
Daily insulin dose (units/kg)	0.15 ± 0.03	0.15 ± 0.09	0.53 ± 0.15	<0.001
Percentage reduction in daily insulin dose (compared with pre-transplant)	54.1%	49.0%	3.7%	0.033[+]
C-peptide (pmol/L)	658 ± 372	218 ± 59	0.0 ± 0.0	0.002
Concomitant glucose (mmol/L)	6.8 [6.8, 8.2]	8.65 [6.4, 11.3]	7.3 [5.2, 9.4]	0.639
C-peptide: glucose ratio (nmol/L:mmol/L)	0.055 [0.045, 0.072]	0.028 [0.025, 0.029]	0.001 [0.000, 0.001]	0.002

Data are n (%); mean ± SD or median (Q1,3). Means were compared by one way ANOVA and medians by Kruskal-Wallis test. Categorical data were compared by Fisher's exact test. CSII, continuous subcutaneous insulin infusion; CGM, continuous glucose monitoring; HbA1c, glycated haemoglobin.

TABLE 2 | Hypoglycaemic awareness, by Igls criteria.

	Islet graft function			*p*-value
	Good	**Marginal**	**Failed**	
Awareness of hypoglycaemia: Gold Score	6 [6, 6]	4 [1, 7]	6 [3.5, 7]	0.849
Impaired awareness of hypoglycaemia: Gold Score ≥4	4 (80%)	2 (50%)	4 (67%)	0.800
Hypoglycaemia Awareness: HypoA-Q				
Impaired Awareness (/20)	10 ± 4	10 ± 5	9 ± 4 (*n* = 5)	0.862
Symptom Frequency (/30)	10.8 ± 4.2	23.7 ± 7.8 (*n* = 3)	19.4 ± 2.7 (*n* = 5)	0.008
Symptom Level (/18)	16 [13, 17]	18 [17, 18]	13 [12, 17] (*n* = 5)	0.152

Data are n (%); mean ± SD or median (Q1,3). Means were compared by one way ANOVA and medians by Kruskal-Wallis test. Categorical data were compared by Fisher's exact test. When data incomplete, number with available data denoted by n number in parentheses. IAH, Impaired Awareness of Hypoglycaemia.

tended to be longest in those with graft failure. Reduction in severe hypoglycaemia event rate of >90% was sustained following islet transplantation even in those with graft failure. Nevertheless, only the group with "Good" function had no individuals experiencing severe hypoglycaemia at the time of assessment (**Table 1**). There was a trend towards incrementally higher HbA1c with worsening graft status.

Insulin dose was significantly lower in both groups with graft function, compared to the graft failure group, with comparable dose (~50% of baseline requirements) in individuals with Igls "Marginal" and those with "Good" function. A period of insulin independence was achieved in 60% of those with current "Good" function but in ≤25% within the other two groups.

Random C-peptide was significantly lower in the "Marginal" compared with the 'Good' function group with C-peptide/glucose ratio falling incrementally with worsening graft function.

All participants with ongoing graft function were on a comparable immunosuppression regimen (tacrolimus with/without mycophenolate mofetil). Three (50%) of those classified as 'failed' were no longer taking immunosuppression. Two remained on immunosuppression for a functional renal transplant and one remained on low dose tacrolimus alone immunosuppression on the active waiting list for islet retransplantation.

Although only 15 individuals (71% of those who fulfilled inclusion criteria) consented to participate and completed questionnaires, they appeared representative of the overall cohort. The remaining 6 exogenous insulin-requiring islet transplant recipients in this single site cross-sectional study included a comparable distribution of those with "good," "marginal" and "failed" function. All had received islet transplants alone. Age and duration of diabetes were comparable to the study participants and the majority were female.

Self-Reported Hypoglycaemia Awareness and Experience

Across all three insulin-requiring Igls groups, most recipients self-reported unresolved impairment in hypoglycaemia

TABLE 3 | Worries, behaviours and attitudes to hyper- and hypoglycaemia, by Igls graft status.

	Islet graft function			*p*-value
	Good	**Marginal**	**Failed**	
Fear of hypoglycaemia: HFS-II				
Behaviour	30 [28, 33]	51 [43, 52]	28 [24, 36] (*n* = 4)	0.303
Worry	4 [3, 28]	62 [49, 69]	57 [17, 64] (*n* = 5)	0.067
Total	33 [29, 61]	113 [95, 119]	63 [42, 93] (*n* = 4)	0.082
Hyperglycaemia avoidance: HAS				
Immediate Action	10 [8, 11]	9 [7, 10]	10 [4, 12]	0.944
Worry	14 [14, 27]	22 [22, 28]	32 [22, 33] (*n* = 5)	0.307
Low Blood Glucose Preference	4 [3, 5]	4 [2, 6]	7 [5, 13]	0.207
Avoid Extremes	4 [1, 5]	7 [4, 12]	6 [4, 9] (*n* = 5)	0.372
Attitudes to Awareness: A2A				
Asymptomatic hypoglycaemia normalised	0 [0, 1]	1 [1, 1]	0 [0, 4] (*n* = 5)	0.755
Hypoglycaemia concerns minimised	1 [1, 2]	1 [1, 2]	0 [0, 1] (*n* = 5)	0.246
Hyperglycaemia avoidance prioritised	5 [3, 5]	3 [3, 4]	4 [3, 6] (*n* = 5)	0.899

Data are median (Q1,Q3). Medians were compared by Kruskal-Wallis test. When data incomplete, number with available data denoted by n number in parentheses. HFS, Hypoglycaemia Fear Survey; HAS, Hyperglycaemia Avoidance Scale; A2A, Attitudes to Hypoglycaemia.

TABLE 4 | Diabetes distress and general anxiety/depressive symptoms, by Igls graft status.

	Islet graft function			p-value
	Good	**Marginal**	**Failed**	
Diabetes distress: PAID	23 ± 26	44 ± 29 Severe distress	41 ± 17 (*n* = 5) Severe distress	0.211
Diabetes distress: T1DDS				
Powerlessness	2.1 ± 0.9 Moderate	3.6 ± 1.7 High	2.8 ± 1.2 (*n* = 5) Moderate	0.272
Management distress	1.0 [1.0, 1.3] Little/none	1.5 [1.2, 2.5] Moderate	1.5 [1.5, 1.5] (*n* = 5) Little/none	0.136
Hypoglycaemia distress	2.2 ± 1.3 Moderate	4.0 ± 1.5 High	3.0 ± 1.4 (*n* = 5) High	0.188
Negative social perceptions	1.6 ± 1.7 Little/none	2.8 ± 2.4 Moderate	2.4 ± 1.5 (*n* = 5) Moderate	0.649
Eating distress	1.3 [1.0, 1.3] Little/none	1.3 [1.0, 2.8] Moderate	2.0 [2.0, 2.3] (*n* = 5) Moderate	0.173
Physician distress	1.0 [1.0, 1.0] Little/none	2.5 [1.8, 3.1] Moderate	1.0 [1.0, 1.0] (*n* = 5) Little/none	0.032
Friend/family distress	1.5 [1.0, 1.5] Little/none	2.8 [2.1, 3.1] Moderate	1.0 [1.0, 2.3] (*n* = 5) Little/none	0.361
Anxiety symptoms:				
HADS - A	8 ± 4 Mild	13 ± 6 (*n* = 3) Moderate	9 ± 2 Mild	0.216
GAD-7	6 [3, 11] Mild	21 [17, 21] (*n* = 3) Severe	6 [6,6] (*n* = 5) Mild	0.079
Depressive symptoms:				
HADS - D	6 [4, 8] Normal	11 [8.5, 11] (*n* = 3) Moderate	7 [6, 8] Normal	0.548
PHQ-9	7 [5, 8] Mild	27 [19, 27] (*n* = 3) Severe	10 [7, 11] (*n* = 5) Moderate	0.077

Data are mean ± SD or median (Q1,3). Means were compared by one way ANOVA, and medians by Kruskal-Wallis test. When data incomplete, number with available data denoted by n number in parentheses. PAID, Problem Areas in Diabetes; T1DDS, Type 1 Diabetes Distress Scale; HADS - A, Hospital Anxiety and Depression scale–Anxiety subscale; HADS-D, Hospital Anxiety and Depression scale–Depression subscale; PHQ-9, Patient Health Questionnaire-9.

awareness without significant differences in Gold or HypoA-Q "Impaired Awareness" scores in those with "Good," "Marginal" or "Failed" graft function (**Table 2**).

In addition to HypoA-Q hypoglycaemia awareness scoring, novel analysis of HypoA-Q "Symptom Frequency" (**Supplementary Table S2**) and "Symptom Level" subscales (**Supplementary Table S3**) was undertaken. The Symptom Frequency question was scored in two parts. In the first, participants indicated whether in the past month they had experienced blood glucose readings in the following ranges: 3.5–3.9 mmol/L (1 point); 3.0–3.4 mmol/L (2 points); 2.5–2.9 mmol/L (3 points); <2.5 mmol/L (4 points). In Part 2, participants were asked how often symptoms are experienced if they encounter glucose levels within each of these ranges: never (5 points); rarely (4 points); sometimes (3 points); often (2 points); always (1 point). Higher scores indicate more experience of more profound biochemical hypoglycaemia with less frequent symptoms.

Analysis of the HypoA-Q "Symptom Frequency" subscale showed 100% with "Marginal" graft function and 80% with "Failed" function reported experiencing glucose <2.5 mmol/L within the last month, whereas none of those with "Good" graft function reported levels in this range. When hypoglycaemia was experienced, participants with "Good" graft function experienced symptoms more often than those with "Marginal" ($p = 0.01$) or "Failed" ($p = 0.039$) function (**Table 2**).

The HypoA-Q "Symptom Level" subscale (question 6) asks the participant "how low does your blood glucose usually need to be

TABLE 5 | Participant feedback on questionnaires.

Questionnaires	Feedback
Overall pack	"Questionnaire was fine" "Overall they were ok, not time consuming" "A lot were repetitive, parts I do not understand due to terminology" "Time consuming" "I thought it covered everything relevant" "Absolutely fine and went through lots of helpful information via the questions asked"
Were the questions within the questionnaires relevant to you? If so, which	"Very relevant" "Yes" "Most of the questions" "Yes very relevant, well most of them"
Do you feel that the pack addresses all aspects of living with diabetes and/or following islet transplantation?	"How I felt after transplant and how it improved my quality of life" "Yes" "I think yes the questions covered everything" "Could consider a part about side effects of transplant/transplantation medications and how that affects your diabetes" "I think it is extremely difficult to remove a questionnaire relating specifically to circumstances transplant history and feelings, everyone is different"
Gold Score HypoA-Q	"Excellent" "Hard to think back 6 months" "I liked the way the signs of a hypo were so accurate" "I liked that it was thorough" "Very good it makes one think about how they might manage hypo-awareness"
HFS-II	"That is first a question for individuals however I think every diabetic is scared or worried about hypos"
A2A	"Should add do people understand what is going on with you a lot of the time people think you are drunk or having a fit" "Made me realise I act on my sensor telling me that sugar is low, not very aware and not concerned as I do not go below 3" "This is interesting for me because I have always managed my diabetic control in a way that I run a low blood sugar. I am aware of the problems associated with this way of doing things but I do not want to have high BG's."
HAS vs. A2A	"Attitudes to awareness of hypos was more about how you feel" "I could not answer some of the questions" "Covered feelings I have when my blood sugars are high and actions I have taken" "Personally speaking 'high blood glucose' is a real worry for all the outlined listed issues"
PAID	"Worries a lot about not having long to live. I think I am going to die before I am 66. I also think I am going to be ill, very ill and needing to have dialysis, so doing the questionnaire was both good and bad. I am ok right now."
T1-DDS	"A bit complicated and hard to think back over the last month" "All very thorough and included different aspects of life"
PAID vs. T1-DDS	"More options to explain how things affect you and make you feel in the T1-DDS" "I am aware of the problems associated with diabetes"
HADS	"It is good that you recognised anxiety and depression as part of diabetic life because a lot of the time it is ignored" "I do suffer from anxiety and I think that sometimes my diabetes plays a part in this feeling, the constant worry and concern about being well, having hypos etc. is always going to be part of this"
HADS vs. GAD-7/PHQ-9	"Both are big problem areas with diabetes" "Not really have a preference" "Extremely similar"

before you feel" one or more symptoms, clustered into: autonomic, neuroglycopaenic and non-specific. Higher scores are allocated to lower glucose thresholds with a maximum score of 18 (**Supplementary Table S3**). All groups scored highly on HypoA-Q "Symptom Level" subscale (**Table 2**), consistent with unresolved impairment of awareness evidenced by absence of symptoms

regardless of how low glucose falls or, at least, requirement for glucose levels below those required for normal cognitive functioning before any symptoms are experienced.

Fears, Attitudes and Behaviours Around Hypo- and Hyperglycaemia

Worry about hypoglycaemia appeared low only in those with "Good" graft function (**Table 3**), although differences between groups did not reach statistical significance. Worry related to high glucose levels (measured by the Hyperglycaemia Avoidance Scale; HAS) appeared to increase incrementally with worsening graft function category, although again differences were not significant.

Drive to take immediate action to reduce high blood glucose (measured by the HAS) and prioritisation of hyperglycaemia avoidance (measured by the Attitudes to Awareness questionnaire; A2A) were scored highly in all groups suggesting underlying behavioural preferences which are not influenced by islet graft function. Low blood glucose preference (HAS) and asymptomatic hypoglycaemia normalised/hypoglycaemia concerns minimised (A2A) were scored relatively low by participants in all three Igls groups, consistent with the approach within the UK islet transplant programme of only listing individuals who recognise concerns regarding dangerous hypoglycaemia risk as their primary motivator for proceeding with transplantation despite the need for life-long immunosuppression.

Diabetes Distress and General Anxiety/ Depressive Symptoms

Median PAID scores of those in both the "Marginal" and "Failed" graft function groups indicated they were experiencing elevated diabetes distress (PAID score >40), contrasting with those with "Good" graft function who reported lower median PAID scores (**Table 4**). Assessed using the Type 1 Diabetes Distress Scale (T1DDS), diabetes distress scores were highest in the group with "Marginal" graft function who reported "moderate/high distress" in all domains, incrementally lower in the group with "Failed" function, but were within the little/no distress range for 5 of 7 domains in those with "Good" islet graft function (**Table 4**). Highest scores in those with "Marginal" function reached statistical significance for "physician distress."

Similarly, self-reported generalised anxiety and depressive symptom scores were highest, and in the "severe range" in those with "Marginal" graft function (**Table 4**), and lowest among those with "Good" function.

Participant Experience of PROMs

Feedback on individual questionnaires and overall usefulness/ burden of questionnaire completion was provided by 11 particiopants and was largely positive (**Table 5**). All were perceived as valuable with no consistent strong preference for one questionnaire over another. The importance of reviewing questionnaire responses with those completing them, acknowledging issues arising and acting on these where appropriate was emphasised, with one participant stating: "I would like it to be compared to other questionnaire answers and if anything was significantly different for this to be addressed. Hopefully information gathered will help to work on giving diabetics a better way of managing their every day lives."

DISCUSSION

In this study, we set out to determine whether "Good" and "Marginal" islet graft function (defined by the EPITA/IPITA consensus) were able to differentiate person-reported experience and outcomes. Fifteen adults with (pre-transplant) C-peptide negative type 1 diabetes were studied cross-sectionally at a mean of 6 years following their first islet transplant at a single centre. All required at least low dose insulin replacement but were continuing to benefit from significantly reduced severe hypoglycaemia. Despite maintained biomedical benefit, person-reported measures of health status revealed significant concerns in those with "Marginal" graft function including persistent fear regarding hypoglycaemia, diabetes distress and overall anxiety symptoms in contrast to those with "Good" function. This provides validation of the Igls criteria in meaningfully defining overall clinical outcomes through a simple biomedical scoring system. In addition, the current study provides evidence of unmet needs in those with "Marginal" function justifying further intervention, including re-transplantation.

Recurrent life-threatening severe hypoglycaemia remains the primary indication for deceased donor islet transplant, both within the NHS-adopted integrated UK programme and more widely. We and others have previously reported that significant biochemical hypoglycaemia (glucose <3 mmol/L) can be successfully avoided even in those with relatively low levels of restored C-peptide secretion following islet transplant [17], in keeping with the ongoing reduction in severe hypoglycaemia in all groups in the current study. Nevertheless, 75% with "Marginal" and 50% with "Failed" graft function had experienced at least one episode of severe hypoglycaemia over the preceding year. Following careful assessment of person-centred outcomes in a cohort of islet transplant recipients in Edmonton, it has been proposed that meal tolerance test stimulated C-peptide of >680 pmol/L is required for freedom from serious, clinically important hypoglycaemia [18]. Although MTTs were not undertaken in this cross-sectional study, it is clear from random C-peptide values that only those classified as Igls "Good" had sustained this level of graft function.

Classification of graft function as "Marginal" in the presence of any severe hypoglycaemic events has been widely accepted. Exclusion of all with HbA1c >7% from the "Good" function category has been more contentious, even though HbA1c <7% was agreed by NICE as a key performance indicator for reimbursed islet transplantation and formed part of the primary outcome measure for the US Phase 3 trials towards islet transplant licensing [3, 19]. The current analysis supports this cut-off as a meaningful marker for graft impairment sufficient to negatively impact recipient confidence and well-being. Forty-two percent (3 of 7) of those with HbA1c >7% versus 25% (2 of 8) of those with HbA1c <7% had experienced severe hypoglycaemia over the

preceding 12 months. In our continuous glucose monitoring analysis of an earlier cohort [17], HbA1c post-islet transplant was shown to be intrinsically related to graft function, regardless of exogenous insulin dose adjustment and individual therapeutic goals, with mean HbA1c 6.9% in those with stimulated C-peptide in the range 500–1,000 pmol/L, mirroring data in the current cross-sectional study. In a recent analysis of 677 islet transplant alone recipients within the Clinical Islet Transplant Registry, C-peptide was highly associated with concurrent metabolic status, with mixed meal tolerance test-stimulated C-peptide-to-glucose ratio (CPGR) having optimal predictive value [20]. Optimal CPGR cut-points for predicting absence of severe hypoglycaemia and HbA1c <7% were 0.044 nmol/mmol (fasting) and 0.071 nmol/mmol (stimulated). This is in keeping with the mean random CPGR of 0.055 nmol/mmol in the Igls "Good" group in the current study.

The evidence that optimal primary graft function predicts long term graft function is now incontrovertible [21]. In the current study, 60% of those with sustained "Good" function at a mean of 6.2 years post-first islet transplant had attained a period of insulin independence, whereas only 25% of those with "Marginal" function had experienced this.

Previous studies have confirmed the potential to restore counter-regulatory response and improve symptomatic response to hypoglycaemia following optimal islet engraftment [22]. In the current study, however, at least 50% of participants continued to self-report impaired awareness of hypoglycaemia regardless of level of graft function. Although no differences between groups were manifest using validated IAH scores, a novel analysis of HypoA-Q "Symptom Frequency" and "Symptom Level" subscales (**Supplementary Table S2**) showed that only participants with "Good" function were not experiencing glucose levels <2.5 mmol/L and that, when this was experienced, it was associated with more frequent symptoms when glucose was 2.5–3.9 mmol/L). Although two scores were used, the validated Clarke survey was not employed and may be a more sensitive instrument for differentiating degrees of impaired awareness [23, 24]. We did not include the Clarke survey given the inclusion of questions within the score around hypoglycaemia severity as well as those specifically assessing awareness. Continuous glucose monitoring metrics were also not included. These may have revealed biochemical hypoglycaemia exposure sustaining IAH even in those with "good" function, whereas absolute hypoglycaemia avoidance would be envisaged in insulin independent recipients with "optimal" function.

The factors associated with incomplete recovery of awareness even following successful biochemical hypoglycaemia avoidance remain unclear [25]. We hypothesise that cognitive and physical frailty in those being considered for islet transplantation may be contributory factors given mean age 60 years with diabetes duration 45 years in the current cohort. This study demonstrates the value of using the long-form of HypoA-Q, incorporating two additional questions to more fully characterise symptomatic response to hypoglycaemia, enabling discrimination between groups with differing C-peptide levels.

There have been relatively few previous studies in islet transplant recipients evaluating wider person-reported impact using validated questionnaires, and PROMs have not previously been utilised to validate Igls criteria [26–29]. Reduced fear of hypoglycaemia has been reported in a number of cohorts following successful islet transplant [28, 29]. The current analysis suggests that this requires "Good" graft function, with highest overall HFS score in recipients with "Marginal" function.

Attitudes and behaviours related to high glucose levels have not previously been reported in islet transplantation and were undertaken using the validated Hyperglycaemia Avoidance Scale (HAS) [12] and Attitudes to Awareness questionnaire [15]. High scores for "hyperglycaemia avoidance prioritised" and "immediate action for hyperglycaemia" were reported irrespective of islet transplant function reflecting underlying cognitions contributing to increased hypoglycaemia tendency in this cohort. These values are comparable to those at baseline in the HypoCOMPaSS multicentre trial of UK participants with long-standing type 1 diabetes and impaired awareness of hypoglycaemia [30], suggesting that islet transplantation does not impact these long-established attitudinal beliefs. Worry regarding hyperglycaemia appears to be attenuated in islet transplant recipients with "Good" graft function mirroring reduced fear of hypoglycaemia.

Islet transplant recipients in all groups continued to report a strong preference for low versus high blood glucose levels but neither "minimisation of concerns regarding hypoglycaemia" nor "normalisation of asymptomatic low blood glucose levels" were endorsed strongly in this cohort. This likely reflects the screening process within our programme, which requires each individual to be clear that dangerous hypoglycaemia is their over-riding concern, justifying progression to beta-cell replacement therapy despite the associated risks.

Worry about and avoidance of circumstances that might lead to high glucose (constituting the HAS Avoid Extremes subscale) was scored particularly highly in islet transplant recipients, compared with those in the HypoCOMPaSS trial [30], potentially evidencing a strong desire to minimise glucotoxic stress to the transplanted cells.

Reduced diabetes distress following successful islet transplantation has been reported previously [29]. In this study, T1DDS scores were highest in the "Marginal" graft function group, suggesting that having biomedical evidence of persistent graft function with a parallel increase in HbA1c and risk of severe hypoglycaemia may be particularly distressing, even compared to those who have lost function all together. Scores for "physician distress" were significantly higher in those with "Marginal" function. This sub-scale includes the "feeling that I do not get help I really need from my diabetes doctor about managing diabetes," This loss of confidence may be at least partially driven by healthcare professional assurances that the graft is still functioning, without fully acknowledging recipients' recognition that glucose unpredictability and hypoglycaemia risk has recurred associated with diabetes distress. Global anxiety and depressive symptoms (GAD-7 and PHQ-9) scores were in the severe range for those with "Marginal" function.

Significant anxiety and low mood were, however, reported by all groups. This may be attributable to their high-risk status and requirement for prolonged "shielding" during the COVID-19 pandemic, but nevertheless confirms that successful islet transplantation (certainly in the absence of predictable,

sustained insulin independence) does not completely allay ongoing concerns, given the need for ongoing close monitoring and the potential risks of concomitant immunosuppression.

It is possible that high anxiety and depression scores in those with "marginal function" were contributed to by continued immunosuppression despite marginal perceived benefits. We do not, however, advocate immunosuppression withdrawal in this C-peptide positive group where biomedically meaningful graft function is maintained and given the associated risk of alloantibody sensitisation [31]. In contrast those with failed function were only continuing to take immunosuppression to maintain a functional renal graft or in preparation for further islet transplantation.

As currently configured, Igls "Good" status is dependent on meeting criteria for all four included parameters (C-peptide; severe hypoglycaemia; HbA1c; and insulin dose). We strongly endorse the need for graft function in all islet transplant recipients to be primarily evidenced by robust demonstration of higher C-peptide compared to pre-transplantation. We believe that the current analysis provides strong supportive evidence for classifying all with HbA1c >7% as "Marginal." While understanding that lower HbA1c may be attainable by higher dose exogenous insulin, we conclude that there is now sufficient evidence for an intrinsic impact of C-peptide on composite hypoglycaemia/HbA1c outcomes [17, 18]. Moreover, exogenous insulin needs may be influenced by concomitant oral glucose-lowering agents. We thus suggest that the absolute requirement for >50% reduction in insulin dose post-islet transplant is removed from the criteria justifying designation of islet transplant function as "Good" as proposed at the International Pancreas and Islet Trasnplant Consortium 2021 Igls criteria symposium and further supported by the current PROM analysis.

Limitations of this study include relatively small numbers of participants within a single centre and the cross-sectional design without inclusion of pre-transplant PROMs. Further studies will be important to confirm the current findings in larger prospective cohorts including insulin independent recipients with "optimal" function.

A key strength is the comprehensive assessment of PROMS, and the robust process that we undertook to select appropriate validated measures and to confirm face-validity and utility for implementation in people undergoing islet transplantation. Despite the effort required in completion, participants fed-back positively regarding the value of collecting and reviewing PROM data in parallel with biomedical outcomes. An unmet need to assess anxiety and depression was clearly identified.

For holistic assessment following islet transplantation, we recommend a "minimum dataset" PROM pack quantifying hypoglycaemia awareness, hypoglycaemia fear, diabetes distress, anxiety and depression. Suggested specific measures are Gold score, HFS-II, PAID and HADS.

In addition to providing person-reported outcome validation of cut-offs selected by the healthcare professional community for Igls islet transplant function criteria, this study has confirmed the importance of collecting, reporting and responding to more holistic assessment of wider factors necessary for overall well-being and truly optimal outcomes, using a standardised PROM questionnaire pack in parallel with interval biomedical data collection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Sunderland Research Ethics Committee, NHS Health Research Authority (REC number 07/Q0904/11). The participants provided written informed consent to participate in this study. This ethical approval allowed recruitment of participants who had received one or more percutaneous, transhepatic, intra-portal deceased donor pancreatic islet infusions at the Newcastle upon Tyne Hospitals NHS Foundation Trust within the NHS islet transplant programme.

AUTHOR CONTRIBUTIONS

JAS, AB, RS, and JB contributed to the study design. Selection of PROMS was undertaken by JAS, AB, JB, and JSp. ZB contributed to data collection, data analysis, interpreted data and wrote the first draft of the manuscript. SM contributed to study design, data collection and data interpretation. JAS and JSp contributed to data interpretation. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

JSp has served on advisory boards for Janssen, Medtronic, Omnipod, Roche Diabetes Care, and Sanofi Diabetes; received

unrestricted educational grants and in-kind support from Abbott Diabetes Care, AstraZeneca, Medtronic, Roche Diabetes Care, and Sanofi Diabetes; received sponsorship to attend educational meetings from Medtronic, Roche Diabetes Care and Sanofi Diabetes, and consultancy income or speaker fees from Abbott Diabetes Care, AstraZeneca, Insulet, Medtronic, Novo Nordisk, Roche Diabetes Care and Sanofi Diabetes. In all cases, JSp's research group (ACBRD) has been the beneficiary. JAMS has served on an Advisory Board for Mogrify.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11659/full#supplementary-material

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ORIGINAL RESEARCH
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A Worldwide Survey of Activities and Practices in Clinical Islet of Langerhans Transplantation

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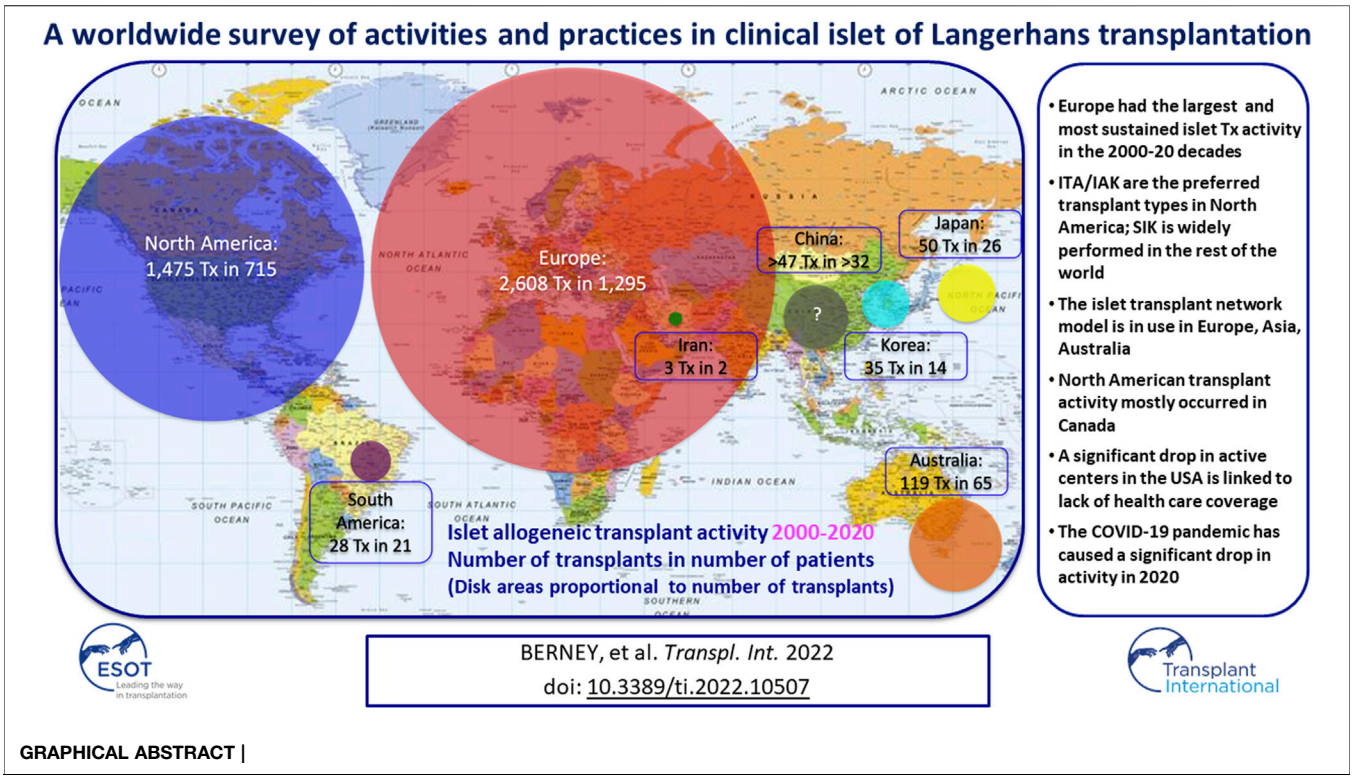
A global online survey was administered to 69 islet transplantation programs, covering 84 centers and 5 networks. The survey addressed questions on program organization and activity in the 2000–2020 period, including impact on activity of national health care coverage policies. We obtained full data from 55 institutions or networks worldwide and basic activity data from 6 centers. Additional data were obtained from alternative sources. A total of 94 institutions and 5 networks was identified as having performed islet allotransplantation. 4,365 islet allotransplants (2,608 in Europe, 1,475 in North America, 135 in Asia, 119 in Oceania, 28 in South America) were reported in 2,170 patients in the survey period. From 15 centers active at the start of the study period, the number of simultaneously active islet centers peaked at 54, to progressively decrease to 26 having performed islet allotransplants in 2020. Notably, only 16 centers/networks have done >100 islet allotransplants in the survey period. Types of transplants performed differed notably between North America and the rest of the world, in particular with respect to the near-absence of simultaneous islet-kidney transplantation. Absence of heath care coverage has significantly hampered transplant activity in the past years and the COVID-19 pandemic in 2020.

Keywords: islet transplantation, type 1 diabetes mellitus, activity, indications, health care coverage, ITA, IAK, SIK



GRAPHICAL ABSTRACT |

INTRODUCTION

It has almost become commonplace to state that islet transplantation has become an established beta-cell replacement therapy since the seminal publication of the Edmonton protocol (1). The significant improvement of outcomes reported has led to a multiplication of islet transplant centers and transplant procedures. In comparison to the 237 procedures performed during the 1990–1999 decade and reported to the now defunct International Islet Transplant Registry (ITR) (2), 2,150 islet allotransplants have been reported to the Collaborative Islet Transplant Registry in the 1999–2015 period alone (3). The CITR is funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and previously received support in part from the Juvenile Diabetes Research Foundation (JDRF). Therefore, collection, analysis, and communication of comprehensive and current data on human-to-human islet transplants is limited to those performed in transplant sites in North America, Europe and Australia, with NIDDK and JDRF sponsoring (3). As a result, the CITR data are skewed toward North American activity, with 1,146 procedures (53%) reported in Canada and the US alone, and the true number of islet transplant procedures performed worldwide is unknown.

The outcomes reported by the University of Alberta in 2000 have not only rekindled the interest in the procedure and boosted activity, but also led to a radical change in indication. Prior to the publication of the Edmonton protocol, simultaneous islet-kidney transplantation (SIK) was the most common indication (55%), followed by islet-after-kidney (IAK; 37%), islet transplant alone (ITA) being very rarely performed (4%) (2), and it is not exaggerated to say that, at least in North America, the Edmonton protocol has led to a true paradigm shift, with problematic hypoglycemia becoming the major indication for an islet transplant (4). However, CITR reports have provided hints that this change of practice may not have been as abrupt outside North America (3).

High-quality prospective clinical trials have been conducted in the past 2 decades and have demonstrated the value of islet transplantation in controlling complicated type 1 diabetes (5–8). Despite these achievements, islet transplantation still doesn't benefit from third party health care coverage, most conspicuously, in the United States (9). Although this has not yet been studied, it is likely that reimbursement and activity should be correlated.

Finally, and more recently, the COVID pandemic has severely, albeit not in a uniform fashion, affected organ donation and transplantation activities globally (10). The impact on islet transplantation activity has not been studied.

The lack of actual activity data has prompted the authors to conduct an international survey with the aim to better characterize not only activity volumes, but also practices, including program organization, types of transplants performed, trends in activity and factors influencing those trends.

METHODS

Survey Construction and Administration

In preparation for the American Diabetes Association's 81st Scientific Sessions held virtually in 2021, the lead author (TB) of this study was tasked to give a lecture entitled "Successful implementation of clinical islet transplantation across the world: What can the US learn?". A survey investigating worldwide islet transplant activity was designed to prepare for the lecture.

Survey was constructed and study data were collected and managed using the Microsoft Forms electronic data capture tool, hosted at the University of Geneva Hospitals. All centers, or at least one center per islet transplantation network, identified to have performed clinical islet allogeneic transplantation in the 2000–2020 period (11) received the questionnaire. Fifteen US centers who had terminated their allogeneic islet transplant activities (each of which had performed <10 transplants) could not be invited for lack of a contact. Questions were formulated to obtain information only on center practices and activity and included no request for outcome data. The survey included a combination of open and scroll-down menu questions. Questions were written to include "other" for all sections in order to allow for full description of alternative practices. The questionnaire is presented as **Supplementary Appendix S1**.

Incomplete or Missing Data

Some datasets were completed, as per survey respondent instructions, with data obtained from the NHS-BT (UK National Health Service-Blood and Transplant) or ANZIPTR (Australia and New Zealand Islet and Pancreas Transplant Registry) activity reports available online (12, 13).

A minimal dataset (number and types of transplants performed on a yearly basis) was obtained from the CITR from North American institutions that had not been invited (N = 15) or had not responded to the survey (N = 5). For centers for which allotransplant numbers were obtained from the CITR, the number of transplanted patients was estimated, based on a theoretical ratio of 2 islet infusions for 1 recipient, as reported by North American centers to the CITR (infusion/recipient ratio: 1.95) (3). For these centers, activity was considered as "apparently terminated" if they had not performed/reported an islet transplant in >5 years.

A similar minimal dataset was obtained for 7 institutions in other continents who had terminated their islet transplant activities: from the Organizacion Nacional de Trasplantes of Spain (N = 3), from a survey administered in 2013 by the lead author of the present study (TB) and presented at the 14th World Congress of the International Pancreas and Islet Transplant Association (N = 3) and from a personal contact (N = 1) (14).

For 4 non-responding centers, all located in China, no current data could be retrieved. Data on patients transplanted in 2 centers in China were added to the activity calculation as obtained from the 2013 survey mentioned above for 1 center and from a publication for the other (15).

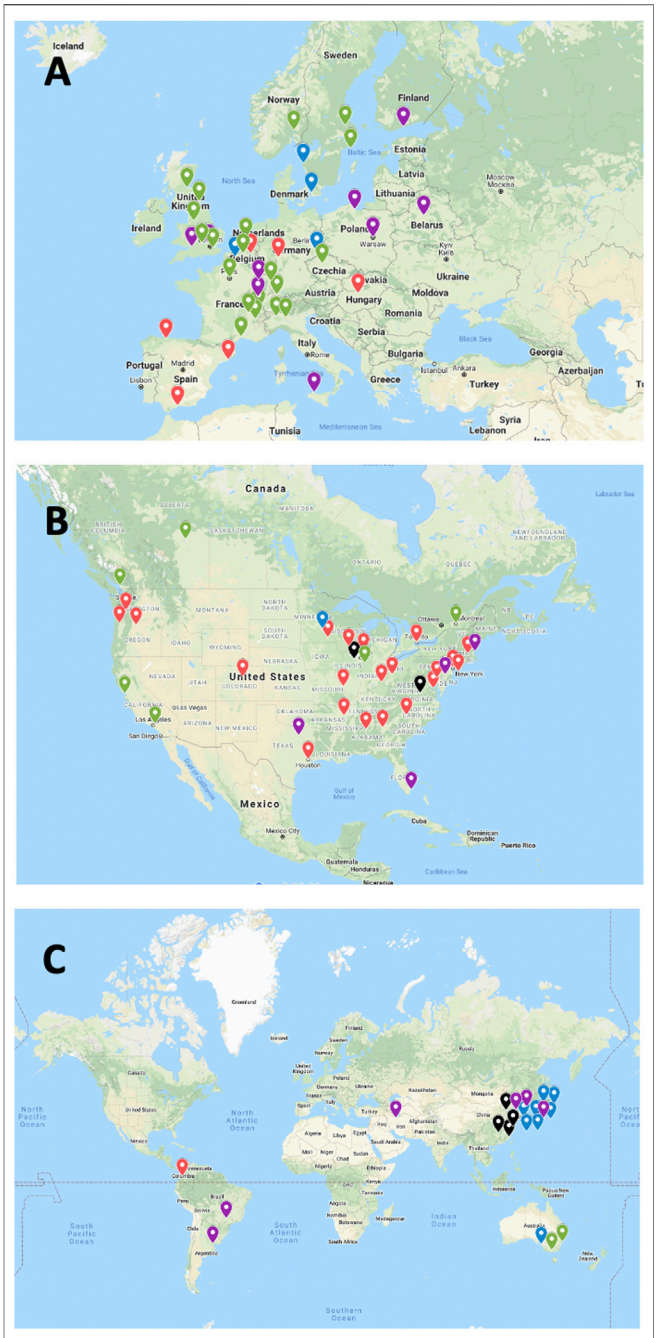


FIGURE 1 | Geographic location and activity status of 94 institutions performing or having performed allogeneic islet transplantation (2000–2020). **(A)** Europe; **(B)** North America; **(C)** "Rest of the World". Green marker: active centers; Blue marker: active centers without activity in 2020; Purple marker: activity on hold; Red marker: activity terminated; Black marker: current status unknown.

Data Analysis

Data are presented and analyzed for each individual center and for each network or consortium. Descriptive statistics were performed using Microsoft Forms and Excel.

TABLE 1 | Islet transplantation networks.

Network	Countries	Number of isolation facilities	Number of transplant centers	Shipment of islets
Japan Islet Transplant Consortium Consortium	Japan	7	7	No
GRAGIL Network	France Switzerland	2	7	Yes
Nordic Network for Clinical Islet Transplantation	Denmark Finland Norway Sweden	2	6	Yes
UK Islet Transplant Consortium (UKITC)	United Kingdom	4[a]	7[b]	Yes
Australian Islet Consortium	Australia	2	3	Yes

[a]*One facility currently on hold or terminated.*
[b]*Two transplant centers (including one with facility) currently on hold.*

RESULTS

Response to the Survey

Invitations to take the survey were sent to 69 program directors, covering 84 centers/5 networks worldwide. We received a response to the survey from 55 (79.7%), covering 65 centers and 5 networks. We obtained partial responses from 6 additional centers (4 terminated, 2 with only an autotransplant program), for a total response rate to the survey of 88.4%.

A list of institutions and networks, with survey response and source of data details is provided in the **Supplementary Table**.

Islet Transplant Centers and Networks

After integration of all data obtained as indicated above, 103 islet transplant centers were identified, of which 94, in 25 countries, had reported allotransplantation activity during the survey period. Fifteen islet allotransplant centers in 4 countries are located in Asia (16%), 39 in 15 countries in Europe (42%), 34 in 2 countries in North America (36%), 3 in 1 country in Oceania (3%) and 3 in 3 countries in South America (3%). Their geographic location is presented in **Figure 1**.

For 85 of 94 centers with relevant data, 45 had a combined allo- and auto-transplant program (53%) and 40 an allo-program only (47%). These proportions varied significantly between North America, Europe and other continents (**Supplementary Figure S1**).

A small majority of programs integrate an islet isolation facility and a local islet transplantation program (35/67; 52%). Thirty islet transplant centers (45%) are organized in 5 networks, built around 17 islet isolation facilities, with different functioning modalities. **Table 1** summarizes the list of networks. Two additional transplant centers (activity terminated) have transplanted islets shipped from another institution in bilateral collaborations (Houston/Miami; Budapest/Geneva).

A list of institutions and networks, with allogeneic and autologous transplantation details, and current activity status is provided in the **Supplementary Table**.

Islet Transplant Activity

Between January 2000 and December 2020, 4,321 islet allotransplants in 2,149 patients were reported worldwide. Islet transplant products pooled from 2 or more islet preparations were counted as a single islet transplant. Most islet transplants were performed in Europe (2,608, 59.7%), followed by North America (1,475, 33.8%), Asia (135, 3.1%), Oceania (119, 2.7%) and South America (28, 0.6%).

A great variation in the levels of activity was observed, 41 centers (44%) having reported <10 transplants and only 12 having (13%) reported ≥100 transplants. Four of 5 islet transplant networks have reported ≥100 transplants. Center-specific activity appears in the **Supplementary Table**. The geographic location of centers or networks according to total activity appears in **Figure 2** and centers and networks having performed ≥100 transplants are listed in **Table 2**. The continental distribution of transplant activities is summarized in **Figure 3**.

Period of Activity

At the beginning of the survey period, 16 of the active centers had performed at least 1 islet allotransplant procedure before 1 January 2000, including 10 in Europe (Milan-San Raffaele, Giessen, Oxford, Brussels-VUB, Geneva, Lille, Grenoble, Strasbourg, Lyon, Besançon, Stockholm), 3 in North America (Minneapolis, Miami, Edmonton), 1 in Asia (Seoul-Samsung) and 1 in South America (Buenos Aires). Additional centers had started and discontinued allogeneic islet transplant programs in the 1990s (2), only one of which (Saint-Louis) resumed its activities in the study period. The number of active islet transplant centers changed continuously during the period, new centers opening and active centers terminating or putting their activity on hold. The evolution over time of the number of active islet centers is presented in **Figure 4**. Of 88 centers with available date, 30 terminated (reportedly or apparently) their activity, and an additional 15 have put their activity on hold. Forty-four centers reported as being active at the time of survey, of which only 26 have performed at least 1 transplant in 2020. Overall, 47% of centers are still reportedly active worldwide, 20% have reportedly put their islet allotransplant program "on hold" and 33% have reportedly or apparently terminated their programs. These proportions vary significantly between world regions (**Supplementary Figure S2**), with a much higher percentage of terminated programs in North America (66%) than in Europe (15%) or the rest of the world (6%).

Types of Transplants Performed

Of 65 centers with relevant data, islet transplantation was performed as islet-after-kidney (IAK) by 89%, islet transplant alone (ITA) by 83% and simultaneous islet-kidney (SIK) by 32%.

FIGURE 2 | Islet allotransplant activity of 94 institutions/networks performing or having performed islet transplantation (2000–2020). **(A)** Europe; **(B)** North America; **(C)** "Rest of the World". Green marker: ≥100 transplants performed; Blue marker: 50–99 transplants performed; Yellow marker: 10–49 transplants performed; Red marker: <10 transplants performed.

IAK is performed in 100%, 82% and 67%; ITA in 80%, 100% and 67%; and SIK in 55%, 13% and 25%, of centers from Europe, North America and the rest of the world, respectively.

To the question about the preferred procedure performed (several answers possible), 44% replied ITA, 24% both ITA and IAK, 16% IAK and 16% SIK, either alone or in combination with IAK, ITA or both, without major continental differences.

Of 5 networks, 2 (Japan, Nordic) perform both ITA and IAK as their only and preferred procedures. In the remaining 3, ITA, IAK and SIK have been performed, but practices vary from center to center.

Eight centers (6 in Europe, 2 in North America) reported performance of islet allotransplantation in other combinations, namely simultaneous islet-lung or islet-after-lung in cystic fibrosis patients (16, 17), simultaneous islet-liver (including simultaneous islet-lung-liver (18) and simultaneous islet-liver-kidney (19)) and simultaneous islet-heart transplantations. The geographic and continental distribution of types of transplant performed are summarized in **Figures 5**, **6**. The center-by-center distribution appears in the **Supplementary Table**.

Fourteen centers have reported performing islet allogeneic transplantation in extra-hepatic sites (20), including 9 in the omentum (21, 22), 4 in the skeletal muscle (23), 3 in the gastric submucosa (24), 2 inside devices (25), 1 in the bone marrow (26) and 1 in the anterior chamber of the eye.

Internal Organization

Allogeneic islet transplant programs (data from 67 centers and 5 networks) are or were directed in majority by surgeons (30 centers, 2 networks; 44%) or diabetes/endocrinology (D/E) specialists (25 centers, 1 network; 37%). The remainder being led by nephrologists (7 centers, 1 network; 10%), a joint team of a D/E specialist and a surgeon (4 centers; 6%), a joint team of surgeon and immunologist (1 network), a joint team of nephrologist and D/E specialist (1 center) or a joint team of lab director and nephrologist (1 center). Program leadership differed significantly between North America (62% surgeons; 24% D/E), Europe (38% D/E; 31% surgeons) and other continents (43% D/E; 36% surgeons).

We obtained data on the co-existence of a pancreas transplant program in the same institution from 64 respondents. Forty-eight institutions had an islet and a pancreas transplant program (75%) and 16 only had an islet transplant program (25%). Of 40 centers with available data, the pancreas and islet transplant programs were described as a joint program in 17 (43%), as separate programs with close interaction in 20 (50%), 2 centers reported separate programs with occasional interaction and 1 center indicated totally separate programs. There were no major continental differences in these organizations.

In 20 institutions reporting a joint program, 16/75%) see their referrals in the same initial visit and discuss them in the same WL conference. For the remainder, referrals are directed to different visits in 2 instances and discussed in separate waiting list (WL) conferences in 2 instances.

Programs described as separate but with close interactions had a similar organization, with a single referral visit and WL conference in 67%. Interestingly, one center described as separate programs with occasional interaction has a unique referral visit, but separate waiting lists. As expected, the remainder had separate referral visits and WL conference.

Of 20 countries in which at least one center responded, 17 (85%) have 2 separate national WLs for islet and pancreas and

TABLE 2 | Islet transplantation centers or networks having reported ≥100 islet allotransplants[a].

Center/Network	Countries	Number of transplants	Number of patients
University of Alberta—Edmonton	Canada	681	293
Nordic Network for Clinical Islet Transplantation	Denmark Finland Norway Sweden	458	199
GRAGIL Network	France Switzerland	457	234
UK Islet Transplant Consortium (UKITC)	UK	331	189
Brussels Free University	Belgium	273	102
Geneva University[b]	Switzerland	205	115
Lille University	France	171	61
University of Uppsala[c]	Sweden	167	60
San Raffaele Institute—Milan	Italy	162	87
University of British Columbia—Vancouver	Canada	142	60
University of Oslo[c]	Norway	127	48
Zurich University	Switzerland	120	54
Australian Islet Consortium	Australia	119	65
IKEM _ Prague	Czech Republic	114	68
Edinburgh Royal Infirmary[d]	UK	113	60
University of Miami	United States	100	56

[a]*Number of islet infusions is counted, regardless of number of islet preparations pooled.*
[b]*Also included in "GRAGIL Network" numbers.*
[c]*Also included in "Nordic Network for Clinical Islet Transplantation" numbers.*
[d]*Also included in "UK Islet Transplant Consortium" numbers.*

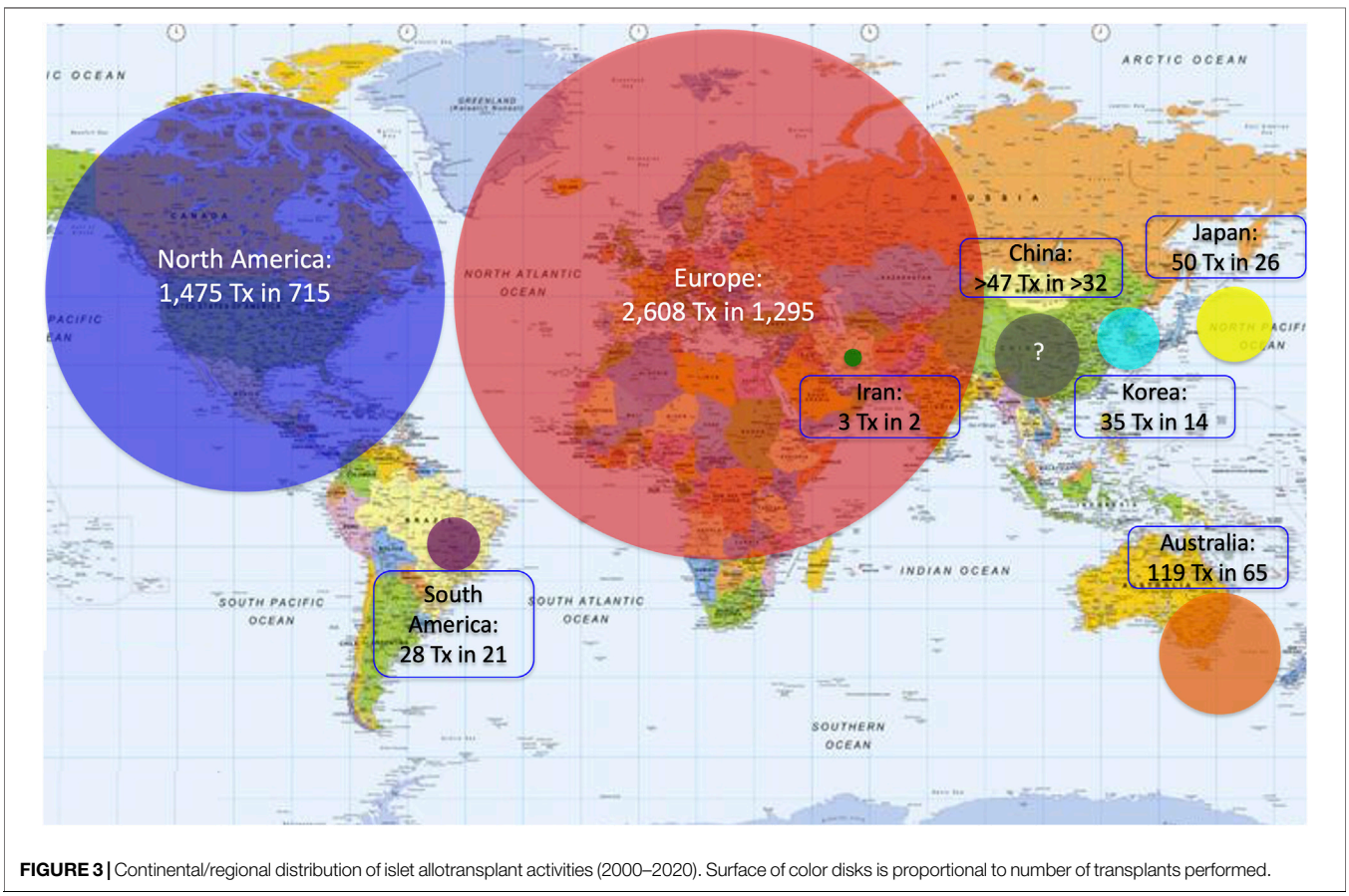


FIGURE 3 | Continental/regional distribution of islet allotransplant activities (2000–2020). Surface of color disks is proportional to number of transplants performed.

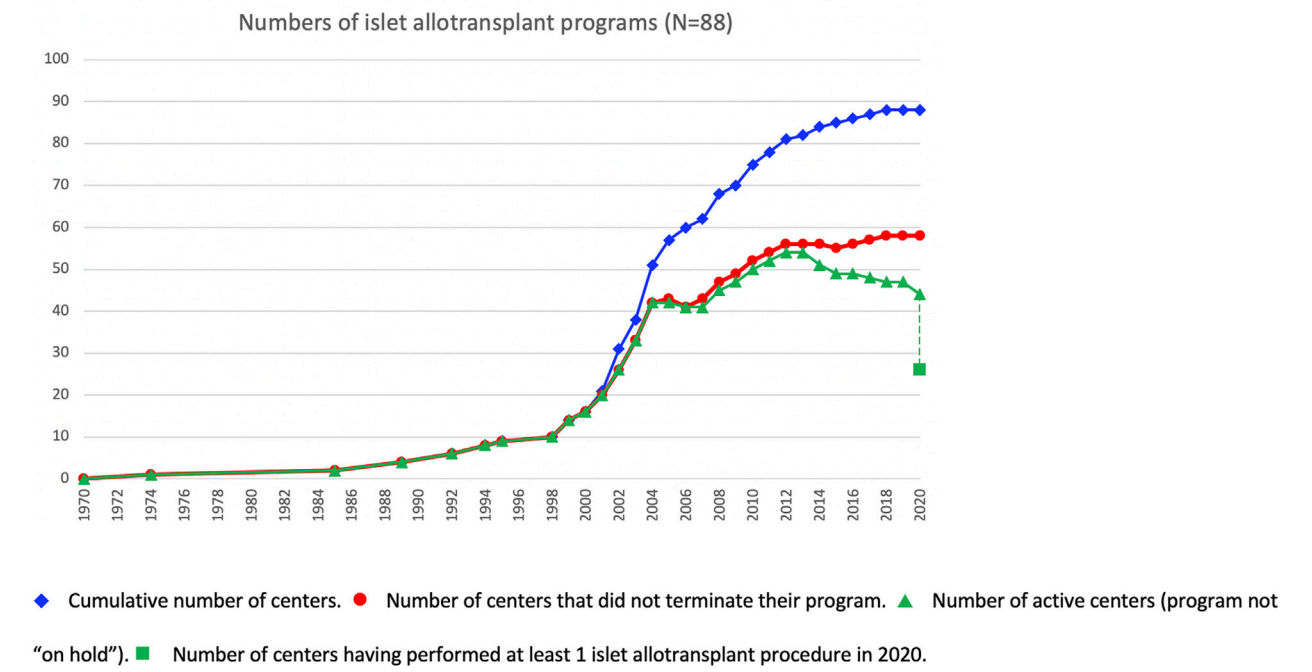


FIGURE 4 | Evolution over time of islet transplant center activities. ◆ Cumulative number of centers. ● Number of centers that did not terminate their program. ▲ Number of active centers (program not "on hold"). ■ Number of centers having performed at least 1 islet allotransplant procedure in 2020.

only 3 (15%) have a single WL (Belarus, Switzerland, United Kingdom).

Health Care Coverage for Islet Allotransplantation

Allogeneic islet transplantation is fully covered by the health care system in 9 countries, namely Australia, Belarus, Canada (Alberta, British Columbia), Finland, France, Iran, Poland, Sweden, Switzerland and the United Kingdom. Reimbursement was reported as partial in Belgium, the Czech Republic, Germany, Italy, Japan, and Norway. The procedure for securing health care coverage was reported as initiated in one further province of Canada (Quebec), but coverage was obtained in the few weeks preceding submission of this report. Islet allotransplantation was reported as not reimbursed, but under evaluation in the Netherlands, and as not reimbursed with no clear perspective in Argentina, Brazil and Korea. Interestingly, for the United States, where islet allotransplantation is not covered, 7/12 survey respondents evaluated the situation as "not reimbursed with no clear perspective", and 5 as "under evaluation" (9).

Impact of COVID Pandemic and Other Factors on Islet Transplant Activity

Of 20 centers who reported a terminated or temporarily on hold program, reasons were regulatory/lack of health care coverage for 6 (4 in the United States, 1 in Korea, 1 in Argentina), logistic for 3, COVID pandemic for 3, institutional decision for 1, and financial unrelated to regulations for 1; six centers did not indicate a reason.

The impact of the COVID pandemic could be assessed for 29 reportedly active centers and 3 temporarily on hold for COVID reasons. Overall, 13 reported a decrease in activity and 13 a temporary or ongoing interruption of their program. Six active centers reported an absence of impact, of which only 3 performed at least 1 allotransplant in 2020. The geographical distribution of these centers appears in the **Supplementary Figure S3**.

DISCUSSION

This study has the merit to present a comprehensive picture of the worldwide allogeneic islet transplant activity in the past 2 decades, i.e., with a starting time point represented by the publication of the seminal Edmonton study (1). It does not only reveal raw activity numbers, but also unveils certain differences between North America—mainly the US- and other continental regions of the world in terms of indications, organization and practices. The major strength of the study is the high rate of response to the survey. One limitation is that it does not provide outcome data, a deliberate choice made by the authors to ensure a maximal response to the survey.

Despite the limitations inherent in a survey, all transplant numbers are accurately reported for almost every country in the world, with the exception of the United States and China. Activity numbers of US centers who did not reply to the survey or could not be contacted were obtained from the CITR, which captures nearly all

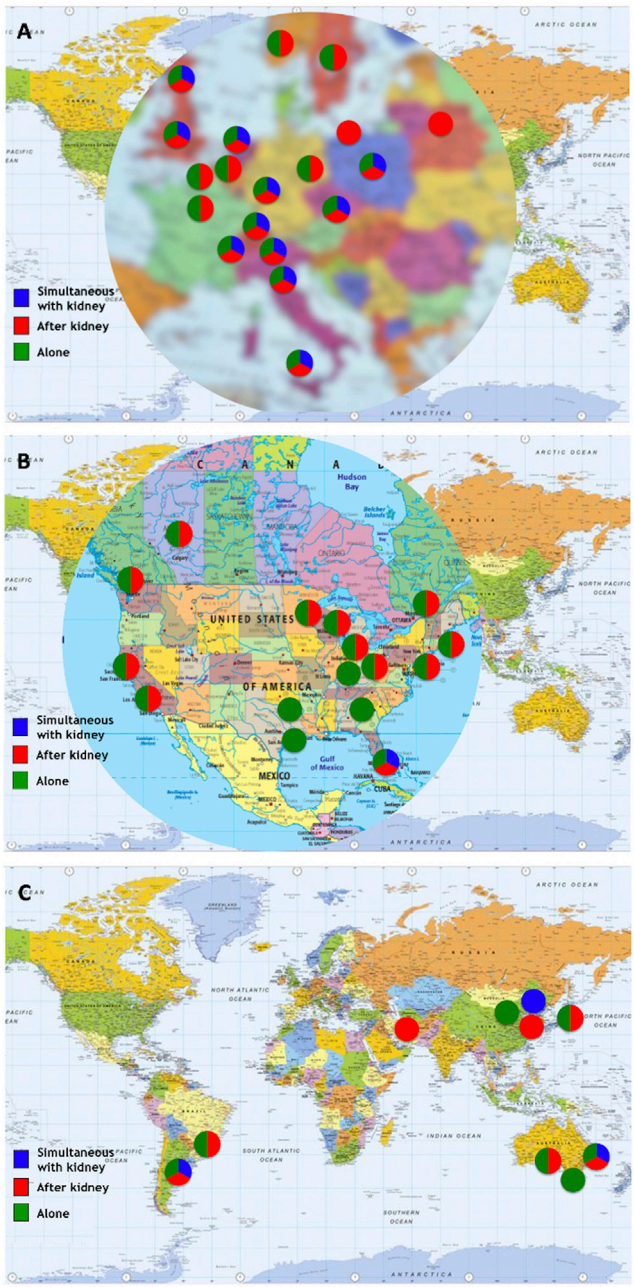


FIGURE 5 | Types of transplants performed by center/network. **(A)** Europe; **(B)** North America; **(C)** "Rest of the World".

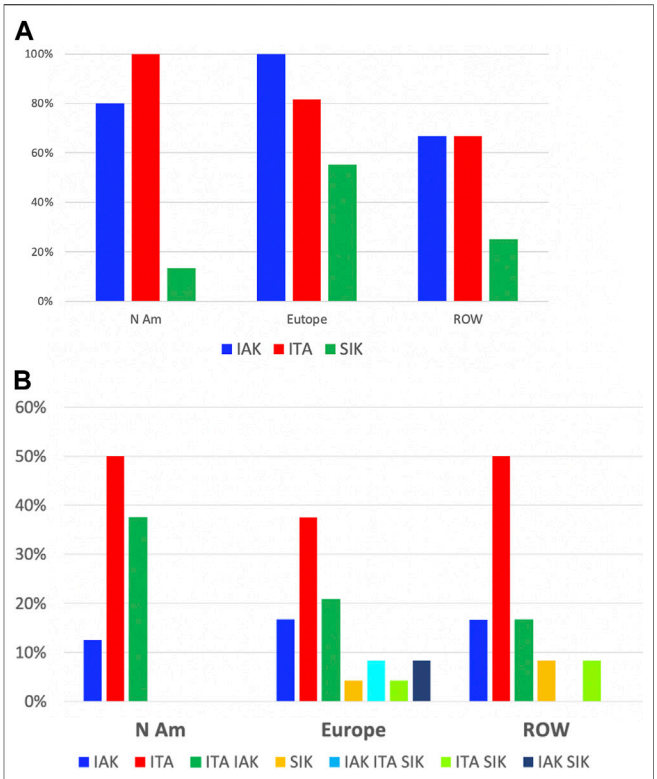


FIGURE 6 | Types of transplants performed: continental distribution. **(A)** Proportion of centers in each region having performed IAK (blue), ITA (red) or SIK (green). **(B)** Proportion of centers in each region by overall preferred types of transplants; blue: IAK only, red: ITA only, green: ITA and IAK, ochre: SIK only, turquoise: IAK, ITA and SIK, light green: ITA and SIK, black: IAK and SIK.

allogeneic islet transplant activity in the US, which has remained dependent on NIH and JDRF support of clinical trials, with mandatory reporting in the absence of biologic licensure. It should be mentioned here that a certain level of underreporting is expected, but is likely to be minimal, especially since most of these centers had terminated their activity, which included <10 transplants in all but 2 institutions. For China, none of the 4 institutions contacted replied to the survey. We obtained information on transplants performed from a 2013 publication (15) or from a survey previously performed in 2013 (see Methods), for one center each, but it is unknown whether these centers have an ongoing allogeneic islet transplant activity or not. Therefore, Chinese data are an underestimation based on partial data. Finally, islet transplant centers were identified based on current and previously existing registries (2, 3, 12, 13), literature searches (11) and personal connections. It is therefore possible that not all active centers were really identified, but missing institutions are unlikely to have significantly contributed in terms of activity volumes.

The survey allowed to identify 94 programs having performed allogeneic islet transplantation in the study period. The decision to start the survey study period in 2000 was arbitrarily chosen because it coincided with the publication of the seminal "Edmonton protocol" paper, which was widely considered to be a game changer in the field at the time (1). The success of the "Edmonton protocol" was not so much a quantum leap as a particular step -albeit a significant one-in a history of continuous progress, from the first demonstration of diabetes reversal in rodent experiments by Paul Lacy in Saint-Louis, to the first clinical islet transplants by D. Sutherland and J. Najarian, the invention of the automated method of islet isolation by Camillo Ricordi and further advances in Europe and North America (27).

Of the 94 programs active in these 2 decades, only two pioneering institutions had performed >10 procedures, namely the University

of Pittsburgh (26 procedures), who eventually elected to focus on autotransplantation, and Washington University in Saint Louis, who resumed an allogeneic program in 2000 (2). Several institutions active in the field in the 1990s had terminated their activities, at least temporarily by the start of the study period (2).

Looking at transplant figures, the good news is that there has been a steady increase in activity. In a previous survey-based report presented at the 2013 IPITA congress, 2,349 transplants in 1,178 patients had been reported, as compared to 4,322 in 2,150 in the present study, i.e., a near-doubling in 7 years.

The improvement of islet transplant outcomes reported by the Edmonton protocol has somewhat overshadowed the paradigm shift represented by the focus on problematic hypoglycemia and ITA as the foremost indication. Indeed, in the 1990s, ITA represented <5% of all islet transplant procedures, and SIK >50% (2). The Edmonton protocol has been much more impactful in North America than in the rest of the world, where IAK and SIK have been much more commonly performed. In this regard, it should be pointed out that technology has been improving exponentially as islet programs have been progressing. The sole hypoglycaemia unawareness indication for ITA may have led to a reduction in referrals as sensor-augmented pumps tend to become the norm (4). Broader indications and inclusion criteria have therefore been explored and implemented.

Another notable difference is the organization in islet transplant consortia, albeit with variations in the types and levels of interaction, that has been embraced in Europe, Australia and Japan, but not in North America. National, or even regional (GRAGIL, Nordic Network), networks, with transplant centers located around a centralized islet production laboratory, facilitate access to islet transplantation and ease the burden of traveling to a distant islet center for the patients. These models undoubtedly have a positive impact on finances and equity of access (28), and national health policymakers should consider promoting and implementing their construction in countries where they do not exist.

In the survey period, allogeneic islet transplantation activity has been mostly performed in Europe, and the differences have accentuated in the past 7 years. High activity levels have progressively shifted from North America to Europe, both in terms of patients transplanted, but also active islet transplant centers. It is striking that more than half of North American transplants have been performed in Canada (833 transplants in 360 patients, versus 642 in 255 for the United States). This is an unsurprising and expected result of the regulatory framework in the United States, in which allogeneic islets are considered a biologic drug, with the ensuing difficulties met by academic institutions to comply with the tremendous logistic and financial consequences (9, 29). This situation seems to be unique to the US, in contrast to Canada and most countries in the rest of the world, where allogeneic islets are considered as cell therapy products and fall under organ transplant regulations (30, 31). The US regulation implies that a Biologics License must be obtained for an institution or a company to be authorized to manufacture and administer allogeneic islets to patients with type 1 diabetes and to secure third party reimbursement. No such license has been granted so far, resulting in the absence of health care coverage in the United States. Many other countries currently have islet transplantation considered as standard-of-care and reimbursed, but there are still several instances in which the classification of allogeneic islets as "basic" cell therapy products has not led to their recognition as standard-of-care treatment of type 1 diabetes and/or to full or even partial insurance coverage.

Over the study period, new centers have constantly opened, and established centers terminated, or put on temporary hold, their activities. Overall, after a regular net increase in the first decade of the century, a net drop in the number of active centers, from a peak of 56 in 2012, can be observed in the past decade. As shown by the data, this is mostly due to the closure of US centers for the reasons outlined above. An interesting point is the important shift of practice adopted by US centers in response to this deadlock situation, and a majority have focused their activity on islet autotransplantation programs.

The impact of the COVID pandemic cannot be overestimated. Activity has decreased or even been interrupted because of the COVID situation in a vast majority of reporting centers, and is accountable for a 40% drop in transplant activity (in terms of active centers) in 2020 with respect to the previous year.

This comprehensive survey was able to quantify islet transplant allotransplantation in the past 2 decades and to identify differences in activity and practices in different regions of the world. Although a steady activity has been reported over the study period, absence of heath care coverage and the COVID-19 pandemic have significantly hampered transplant activity in 2020. This survey gives a rather accurate description of the activity in a critical period in time, but is only a snapshot, that cannot replace data from a comprehensive worldwide registry, unfortunately unavailable at this time. Although the CITR is an extraordinary source of valuable data, this survey indicates that it does not capture a major part of the international islet transplant activities and outcomes (6 of the 16 centers or networks having performed >100 transplants do not report to CITR). The ANZIPTR and NHS-BT registry are publicly available registries containing a wealth of data on islet and pancreas transplantation in Australia/New Zealand, respectively the UK, including outcomes (12, 13). The European Pancreas and Islet Transplant Registry (EPITR) is a current effort from ESOT/EPITA aiming at covering these needs for Europe (32). Similar coordinated efforts should be made in other parts of the world and integrated into a truly international islet transplant registry capturing all the activity.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TB and AA: participated in research design, scientific organization of expert panel, performance of the research, data contribution, data analysis, and writing of paper. MB, EdK, TK, PJ, TL, MR, HS, PS, and SW: participated in data contribution, data analysis and critical review of the paper.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10507/full#supplementary-material

Supplementary Figure S1 | Proportions of islet allotransplant centers harbouring an autotransplant program.

Supplementary Figure S2 | Proportions of islet allotransplant centers according to current activity status.

Supplementary Figure S3 | Impact of the COVID-19 pandemic on islet allotransplantation activity. **(A)** Europe; **(B)** North America; **(C)** "Rest of the World".

Supplementary Table S1 | List of islet transplant centers.

Supplementary Datasheet S1 | Appendix: Survey form.

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BRIEF RESEARCH REPORT
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Islets-on-Chip: A Tool for Real-Time Assessment of Islet Function Prior to Transplantation

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Islet transplantation improves metabolic control in patients with unstable type 1 diabetes. Clinical outcomes have been improving over the last decade, and the widely used beta-score allows the evaluation of transplantation results. However, predictive pre-transplantation criteria of islet quality for clinical outcomes are lacking. In this proof-of-concept study, we examined whether characterization of the electrical activity of donor islets could provide a criterion. Aliquots of 8 human donor islets from the STABILOT study, sampled from islet preparations before transplantation, were characterized for purity and split for glucose-induced insulin secretion and electrical activity using multi-electrode-arrays. The latter tests glucose concentration dependencies, biphasic activity, hormones, and drug effects (adrenalin, GLP-1, glibenclamide) and provides a ranking of CHIP-scores from 1 to 6 (best) based on electrical islet activity. The analysis was performed online in real time using a dedicated board or offline. Grouping of beta-scores and CHIP-scores with high, intermediate, and low values was observed. Further analysis indicated correlation between CHIP-score and beta-score, although significance was not attained ($R = 0.51$, $p = 0.1$). This novel approach is easily implantable in islet isolation units and might provide means for the prediction of clinical outcomes. We acknowledge the small cohort size as the limitation of this pilot study.

Keywords: islet transplantation, transplant assessment, electrophysiology, diabetes mellitus, islet, multielectrode array

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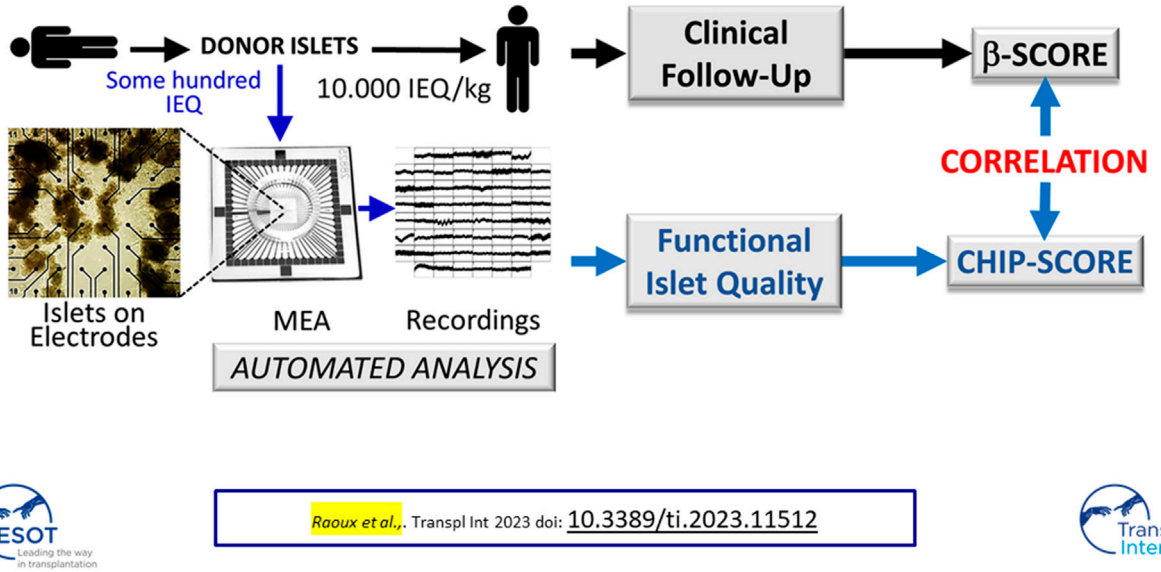
Abbreviations: cAMP, cyclic AMP; GLP-1, Glucagon-like peptide 1; GSIS, Glucose-induced insulin secretion; HbA_{1c}, Glycated hemoglobin; MEA, Multielectrode array; SP, Slow potentials.



GRAPHICAL ABSTRACT |

INTRODUCTION

The incidence of type 1 diabetes is increasing worldwide and pancreatic islet replacement has emerged as a therapy, especially in the case of recurrent severe hypoglycemic events [1–4]. Transplantation of donor islets, obtained by mechanical and enzymatic dissociation of the pancreas, provides sustained improvement of glycemic control with an efficient prevention of severe hypoglycemia in the large majority of recipients, thus improving patients' quality of life [5]. Moreover, islet transplantation has been demonstrated to prevent the progression of chronic diabetes complications [6–8].

Islet graft function can be assessed as clinical outcome by several methods, such as the β-score [9] or the Igls criteria [10]. The islet grafts' potency can be assessed *in vivo* by transplanting a set fraction of the islet preparation into immunodeficient rodents, however, the read-out is retrospective [11, 12]. We are still lacking predictive criteria for evaluating islet quality immediately prior to transplantation [12], an issue that has been called for early on [13]. This issue will also be of considerable importance for potential future therapies using stem cell-derived surrogate islets [12].

Therefore, we investigated whether donor islet quality could be ranked according to their electrical activity. Considerable knowledge in the electrophysiology of human islets has been acquired during the last decade and allows to define meaningful electrophysiological parameters to evaluate their function and establish a ranking score [14]. Indeed, changes in ion fluxes are the first integrative signals of islet activity. Slow potentials (SPs), as recorded here by dynamic multi-electrode arrays (MEAs), reflect the physiological important coupling between islet β-cells, are tightly linked to insulin secretion and exhibit the same biphasic profile, a hallmark of islet activation [15, 16]. Moreover, these SPs are regulated by relevant hormones, such as adrenalin or incretins, and are deteriorated during aging and glucotoxic condition [15, 17]. Finally, these recorded electrical activities can regulate glucose homeostasis *in silico* in the FDA-approved simulator of glucose homeostasis in type 1 diabetes patients [18, 19]. In view of these characteristics of the recorded electrical islet signatures, we hypothesized that a detailed and dynamic electrophysiological analysis may reflect donor islet quality. Moreover, the use of extracellular electrophysiology applied here only requires routine expertise in cell culture, which is available in most clinical laboratories. The analysis of recorded data can either be automated and performed online or offline with commercial software or after electronic data exchange with expert groups.

MATERIALS AND METHODS

Study Design and Scores

Islet CHIP (authorization number NCT03067324) was a pilot study derived from the clinical islet transplantation STABILOT randomized control trial (authorization number NCT02854696) [20]. Eight islet transplant recipients were investigated (see **Table 1**; **Supplementary Table S1**) from the study. Thirty patients were initially eligible for the study but 22 had to be excluded subsequently (donor research opposition, 11; receiver's consent unknown, 5; COVID-related problems in patient follow up, 4; logistic problems, 2). For each recipient, an aliquot of islets (1,000 IEQ) was sampled from the islet preparation used for the first infusion and examined for glucose-stimulated insulin secretion (GSIS) and electrophysiology. Recipients were

TABLE 1 | Characteristics of the study population (N = 8) and islet donors (N = 8).

A. Recipient population	
Baseline characteristics	**Mean (SD)**
Age (years)	48.3 (±4.8)
BMI (kg/m^2)	22.8 (±2.5)
Daily Insulin Dose (UI/kg/day)	0.47 (±0.1)
Glycemia (mmol/L)	13.1 (±3.2)
HbA1c (%)	7.9 (±0.8)
(mmol/mol)	63.0 (±6.4)
Basal C-Peptide (ng/mL)	0.05 (±0.06)
After the first islet infusion	**Mean (SD)**
Daily Insulin Dose (UI/kg/day)	0.36 (±0.1)
Glycemia (mmol/L)	6.3 (±1.3)
HbA1c (%)	7.9 (±1.1)
(mmol/mol)	63.0 (±9.7)
Basal C-Peptide (ng/mL)	1.5 (±0.4)
Before the 2nd islet infusion	**Mean (SD)**
Daily Insulin Dose (UI/kg/day)	0.18 (±0.1)
Glycemia (mmol/L)	6.0 (±0.6)
HbA1C (%)	6.0 (±0.4)
(mmol/mol)	42.0 (±2.3)
Basal C-Peptide (ng/mL)	1.3 (±0.5)
B. Donor population	
Baseline characteristics	**Mean (SD)**
Age (years)	49.1 (±7.3)
BMI (kg/m^2)	29.8 (±7.1)
GSIS Index	2.4 (±0.4)
GSIS with theophylline Index	6.0 (±1.7)
Purity (%)	73.0 (±21)

transplanted as described [20]. The immunosuppression protocol was as follows: thymoglobulin administration 2 days before islet transplantation; 1 hour before the first thymoglobulin infusion, 2 mg/kg methylprednisolone was administered intravenously and pentoxifillin (400 mg twice a day for 5 days) was started. A second thymoglobulin infusion (1 mg/kg body weight) was administered the day before the transplantation, a third thymoglobulin infusion (1.5 mg/kg body weight) was administered on the day of transplantation and again 2 days after transplantation. Etanercept (50 mg intravenously) was administered on the day of islet infusion, and subsequently administered subcutaneously (25 mg) on days 3, 7, and 10. Heparin (35 UI/kg) was injected into the portal vein just before islet infusion, followed by intravenous heparin infusion for 2 days, and finally subcutaneous application until day 8 after islet infusion. Tacrolimus (1 mg twice a day) was started and then adjusted according to residual tacrolimus blood concentrations with a target between 9 and 13 ng/mL for 3 months after transplantation, and subsequently decreased to a target between 6 and 10 ng/mL. Mycophenolic acid (1 g, twice daily) was administered the day before the first islet infusion. Detailed information is given in **Supplementary Table S1**.

To rank patients' clinical outcomes after islet transplantation, the β-score was used [9]. This score gives 2 points each for normal fasting glucose (≤5.5 mmol/L), HbA_{1c} (≤6.1% (43 mmol/mol)), stimulated and/or basal C-peptide (≥0.3 nM), and absence of insulin or oral hypoglycemic agent use. No points are awarded if the fasting glucose is in the diabetic range, HbA1c >6.9%, C-peptide secretion is absent on stimulation, or daily insulin use is >0.24 units/kg. One point is assigned for intermediate values. The graft function is considered optimal for a β score of 7 or 8, suboptimal for values between 6 and 4, and poor if 3 and lower. Clinical metabolic data were collected and β-scores were determined at inclusion in the study, 1 month after the first infusion, and before the second infusion (between 1 and 3 months after the first infusion). CHIP-scores from 1 (lowest) to 6 (highest) were attributed to donor islet preparations after exposing them to various physiological conditions (for details see Results).

Human Islet Preparation

Human islets were isolated at the Geneva Cell Isolation and Transplantation Center from pancreata obtained from braindead multiorgan donors through the Swiss Transplant Agency and the French Biomedicine Agency (Agence de la Biomédecine). Islets were isolated using the automated method described by Ricordi et al. [6], with local modifications as previously reported [21] and glucose-induced insulin secretion (GSIS) measured as described [21]. GSIS is defined as the fold increase in static insulin secretion between 2.8 and 16.7 mmol/L of glucose (in the absence or presence of the cAMP raising agent theophylline). Detailed information is given in **Supplementary Table S1**.

Electrophysiology

Aliquots sampled from islet preparations dedicated for transplantation were shipped to Bordeaux, seeded on multi-electrode arrays (MEAs, 60MEA200/30iR-Ti-gr, MCS, Reutlingen, Germany) coated with Matrigel (2% v/v; BD Biosciences, San Diego, CA, USA) by application in 10 µL and gentle concentric rotation in the middle of the MEA chip, and cultured at 37°C (5% CO_2, >90% relative humidity) using CMRL-1066 medium (5.6 mmol/L glucose, 10% vol./vol. FBS, penicillin-streptomycin and L-glutamine) [15]. Solutions were replaced by pipetting during dynamic recordings. MEA recordings were performed at 37°C and pH 7.4 in solutions containing (in mmol/L) NaCl 135, KCl 4.8, $MgCl_2$ 1.2, $CaCl_2$ 1.8 (or zero, when indicated, to inhibit any electrical signals), HEPES 10 (pH 7.4 adjusted with NaOH) and glucose and drugs as indicated [22]. GLP-1 solutions (Bachem Bioscience, King of Prussia, PA, USA) were prepared *extempore*, adrenalin and glibenclamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Extracellular field potentials were acquired at 10 kHz, amplified and band-pass filtered at 0.1–3,000 Hz with a USB-MEA60-Inv-System-E amplifier (MCS; gain: 1200) controlled by MC_Rack software (v4.6.2, MCS) [15, 22, 23].

Analysis

Dynamic electrophysiological recordings were either analyzed on-line in real time [23, 24] or off-line as described [15, 22, 25]. Correlation analyses were performed using SAS v9.4 (SAS Institute, Cary, NC, USA) and confirmed by SPSS® Statistics (IBM, New York, NY, USA). Plotting was performed using Prism 7.

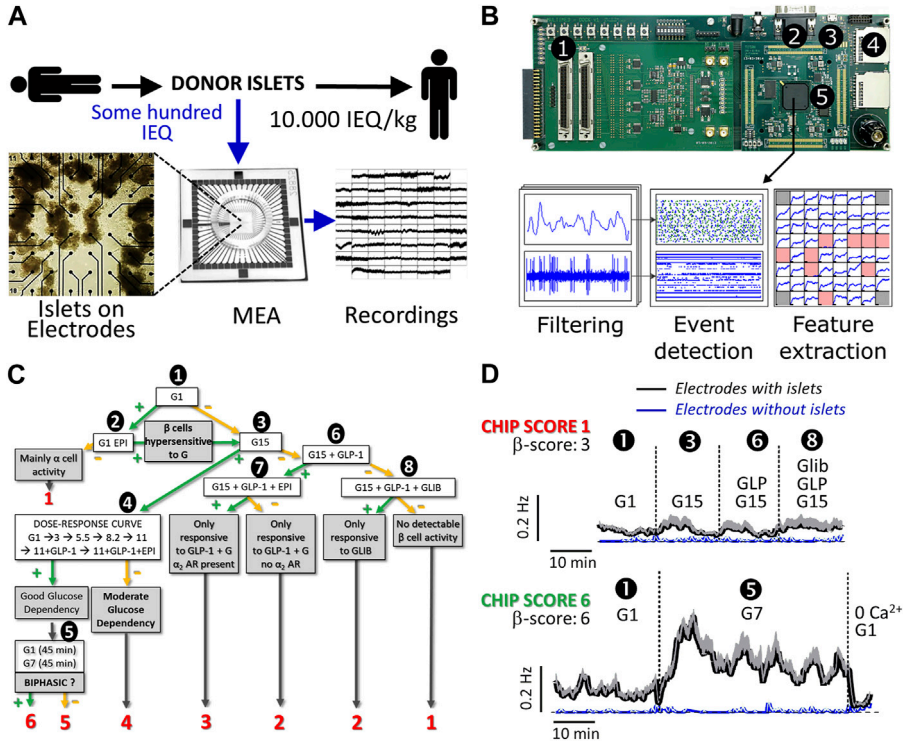


FIGURE 1 | Flow process and examples of donor islet evaluation by micro-electrode arrays. **(A)** General work flow: a small number of donor islets were seeded on microelectrode arrays (MEAs) to record the electrical activity. **(B)** Custom electronics for real-time processing of islet signals. ① Signal inputs (x60); ② VGA monitor output for live display; ③ USB port for board configuration and recording control; ④ SD memory card slots for recording of islet cell signals and processed data; ⑤ Digital signal processing board for real-time filtering, event detection, and measurement of activity markers; **(C)** Algorithm for gradual islet ranking via CHIP-score; decision points are given in bold text and final ranking outcome from 1 to 6 in red. CHIP-score represents the highest rank attained by an islet preparation. Absence or presence of effect after test stimuli is indicated by—(green) or—(orange) symbols. First, the activity was recorded at 1 mmol/L glucose (G1, ①) when β-cells should be silent. If activity is present (>0.5 Hz), the use of epinephrine (EPI, 5 µmol/L, ②) permits the distinction between hyperactive islets (silencing) and islets containing mainly α-cells (enhanced activity). In that case, islets were not further analyzed. Subsequently, islets were exposed to 15 mmol/L glucose (G15, ③); if significant increases in slow potentials were observed, islets were submitted to a full physiological range of glucose concentrations (1, 3, 5.5, 8.2, 11 mmol/L) as well as GLP-1 (testing for incretins) and EPI (for silencing) ④. In the case of a dose-dependent glucose response, islets were tested for the presence of a biphasic response ⑤, a hallmark of islet activity. If islets did not respond to G15 (③), they are exposed to G15 in combination with an incretin (GLP-1, 50 pmol/L, ⑥). When the incretin had a stimulatory effect, a physiological stress hormone (epinephrine, EPI, 5 µmol/L, ⑦) was added to verify the inhibition, i.e., the presence of functional α_2 adrenergic receptors (α_2AR), as expected in β-cells. In case of absence of response to GLP-1, the sulfonyl urea glibenclamide was added (GLI, 100 nmol/L, ⑧) to test for the functional presence of K_{ATP} channels. Depending on the path in this decision tree and on the final point of arrival, the CHIP-scores indicated in red were attributed to each islet preparation. **(D)** Analysis of two donor islets (slow potential frequencies ±SEM) representative of the lowest and highest CHIP-scores (1 and 6, respectively). The test steps are given and numbers (①, ③, ⑥, ⑧ and ⑤) correspond to steps indicated in **(C)**. Means of recorded slow potential frequencies are given in black (SEM in grey) and noise (electrodes without islets) are given in blue (SEM in grey).

RESULTS

Islet signals were recorded using electrodes that measure extracellular islet field potentials due to ion channel activities. In our approach approximately 100 IEQ donor islets suffice to analyze their quality (**Figure 1A**) after seeding on a commercial microelectrode array (MEA); thus, only a minute aliquot of islets was necessary from a single donor as compared to the 10,000 IEQ per kilogram body weight required for transplantation. The recorded signals were amplified, digitized, and processed in real-time using dedicated hardware that applies a series of filters and detection algorithms to extract SP frequencies, which are representative of islet activity (**Figure 1B**) [15, 22]. The analysis can be performed directly online in real-time using custom electronics as given in **Figure 1B**, which provides automated filtering of recordings, detection of electrical signals (events) and feature extractions [23, 24].

As extracellular electrophysiology is non-invasive without rundown, even over several days, repetitive dynamic measures are possible over physiologically meaningful time spans. This allows to test a series of physiological relevant parameters in a dynamical fashion, as opposed to simple glucose-induced increase of islet activity [15, 18, 22]. To rank the performance of donor islets according to SP frequencies, we established a number of criteria and ensuing testing scenario. The score rankings were established prior to actual recordings ranging from CHIP-scores 1 to 6, reflecting the least physiological performance, i.e., glucose insensitivity, to the most

physiological performance, i.e., glucose concentration dependency and biphasic activity pattern [15, 17] (see **Figure 1C**; **Supplementary Table S1**). The criteria applied here are based on well-known islet physiology [14, 26]: at low glucose, islet β-cells show no or only minor spontaneous activity; the neurohormone epinephrine inhibits β-cell activity, an important feature during physical activity or stress; increasing levels of glucose over its physiological range considerably enhances electrical activity and in the best case, this electrical activity is biphasic; the stimulation by glucose is further augmented postprandially by the incretin hormones, such as GLP-1, at physiological levels of 50 pmol/L and less [14, 15, 22]; and finally, sulfonyl-urea drugs such as glibenclamide stimulate islet β-cells independent from glucose via pharmacological closure of K_{ATP} channels. The absence of glucose, GLP-1 or glibenclamide induced activity was ranked as least performant with a score of 1 (**Figure 1C**, red numbers) and those islets were not further investigated. If at least glibenclamide or GLP-1 effects were observed, a score of 2; if responses to GLP-1 and to the stress hormone adrenalin were observed, a score of 3; if glucose concentration dependency was evident only at high glucose concentrations (15 mM vs. 1 mM), a score of 4 was attributed; if glucose concentration dependency was observed over the physiological range of 5.5 mM–11 mM glucose, a score of 5 was given; finally, if biphasic glucose-induced activation was observed, the (highest) score of 6 was assigned. Islets were consequently tested for basal and non-β cell activity using adrenaline known to inhibit β- and stimulate α-cells (**Figure 1C**; ❶,❷), responsiveness to elevated glucose (**Figure 1C**; ❸), glucose concentration dependency (**Figure 1C**; ❹), biphasic activity at physiological glucose concentrations (**Figure 1C**; ❺), and the effects of drugs such as the sulfonylurea glibenclamide or hormones (GLP-1, adrenaline) on glucose-induced activity (**Figure 1C**; ❻,❼,❽).

Two MEA recordings are given in **Figure 1D** (black traces) as examples of lowest (CHIP-score 1; maximum frequency 0.095 ± 0.014 Hz) and highest CHIP-score (CHIP-score 6; maximum frequency 0.409 ± 0.034 Hz) and their corresponding β–scores are provided in **Figure 1D**. Recordings from electrodes not covered with islets were provided (**Figure 1D**, blue traces) and show the high signal-to-noise ratio of the MEA approach. Recordings with the lowest CHIP-score (1; upper panel) showed neither clear glucose-dependency nor any effect of GLP-1 or even glibenclamide and were not submitted to further tests. Recordings with the highest CHIP-score of 6 (lower panel) passed successfully steps 1 (as shown), as well as 3 and 4 (traces not shown) and exhibited a clear biphasic increase in SP frequency in step 5 (as shown). The peak of the first phase occurred after 5 minutes, in-line with reported electrical behavior of human islets [14, 15, 17] and exhibited even typical 5–10 min oscillations in the second phase.

The Islet CHIP study included 8 recipients from the STABILOT clinical islet transplantation study (4 men and 4 women; mean diabetes duration, 34 ± 11 years; **Table 1**; **Supplementary Table S1**). The evolution of primary graft function evaluated using the β-score prior to the second infusion is shown in **Figure 2A**. Groups of CHIP-score vs. β-score are apparent: patients who improved rapidly after the 1st infusion and further before the 2nd infusion (**Figure 2A**, green), patients who improved only partially after the 1st infusion

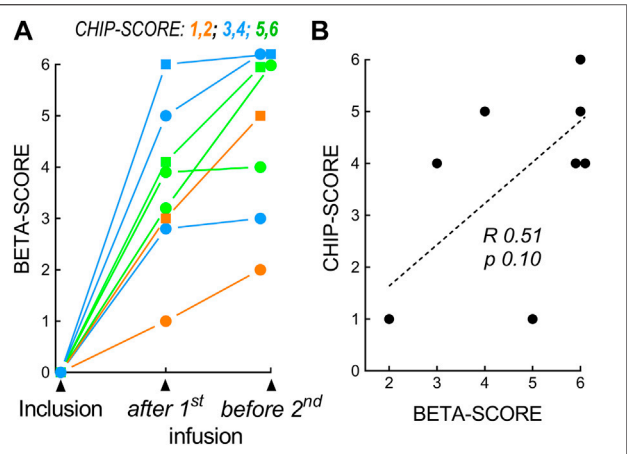


FIGURE 2 | Correlation of CHIP-scores and β-scores. **(A)** β-scores at inclusion, after the 1st islet infusion, and before the 2nd islet infusion. Three subgroups were identified in relation to the β-score and CHIP-score. Orange, CHIP-score 1 to 2, no detectable β-cell activity; blue, CHIP-score 3 to 4, moderate glucose dependency; green, CHIP-score 5 to 6, excellent activity. **(B)** Correlation (R, Spearman) for CHIP-Score and β-score (before 2nd infusion).

(**Figure 2A**, blue), and patients who changed little after the 1st infusion and progressed little afterwards (**Figure 2A**, orange). There was a correlation between the β-score established prior to the second islet infusion and the CHIP-score (**Figure 2B**; $\rho = 0.51$, $p = 0.1$), as well as between HbA_{1c} levels and CHIP-score; $\rho = -0.556$, $p = 0.08$) but statistical significance was not attained in either case. Islet purity, GSIS or total amounts of insulin secreted did not correlate with the β-score, HbA_{1c} level, or CHIP-score as reported previously. CHIP-score did not correlate with main donor criteria such as age, sex, BMI, cause of death, warm or cold ischemia time. These latter parameters as well as volume or amount of IEQ infused were also not correlated with β-scores.

DISCUSSION

The most relevant score in terms of success is given by the β-score and final clinical outcome will evidently depend on numerous parameters including recipients' characteristics. It is thus reasonable to expect that donor organ quality may only be one of the many factors influencing the clinical outcome [27]. Obviously, determining the quality of the main therapeutic agent remains a major issue as in any clinical intervention. Function of human islet transplanted in nude mice correlates well with clinical outcomes. Although this highlights the importance of quality of islets to be transplanted, the read-out of this assay is only retrospective [11]. New approaches to quality control are required and they may also provide means for better comparison of results between transplantation cohorts in view of the diverging criteria applied in donor selections [28].

Previously, a number of islet parameters were tested for their predictive value in animal transplantation studies [29].

Mitochondrial markers have been reported as significant indicators in the case of allo- or auto-transplants [30, 31]. However, islets were generally of lower purity, and the clinical endpoints used were either insulin dependency or independence, which is quite different from the currently used scaled β-score. Studies on the size of islets used for transplantation have not shown any correlation with clinical outcome [32]. Investigation of donor long noncoding RNA repertoire revealed that *MALAT1* expression predicts the quality of human islets prior to their isolation [33] but a potential correlation of this signature with clinical outcomes has not been published.

Similar to our study, insulin secretion of donor islets prior to transplantation has been reported to correlate poorly, if at all, with outcome in animal transplantation studies [29, 34]. In contrast to GSIS, the CHIP-score described here relies on combining a proven technology [15, 17, 18, 22, 23] with parameters such as a range of glucose concentrations, as compared to a single concentration of high glucose, relevant hormones and direct assessment of K_{ATP}-channel function, central to islet activity. Moreover, the potential clinical relevance of the electrical signals recorded here by MEAs is underscored by the observation that they can be used in an FDA approved simulator of human metabolism of patients afflicted by type 1 diabetes to control *in silico* glucose homeostasis via insulin delivery [18, 19]. For those reasons the static evaluation of insulin secretion by GSIS may not provide sufficient details on islet function, including physiologically relevant parameters, such as biphasic activity and β-cell coupling [14]. Additional parameters as used here in the electrophysiological characterization (hormones and detailed glucose concentration dependency), may *per se* also be determined in classical dynamic secretion assays. However, this would considerably increase the workload and costs as compared to an automated electrophysiological analysis. Moreover, dynamic measurements of insulin release do not inform *per se* about the important physiological parameter of islet β-cells, which is coupling. Finally, the MEA technology required is fully compatible with the expertise of a clinical laboratory.

As expected from a small sample sized pilot study, the limitation of our study is the absence of statistical significance, despite a good correlation, that may also reflect the influence of multiple confounding clinical factors.

In conclusion, to the best of our knowledge, this pilot study is the first to correlate donor islet functional quality and clinical outcomes prior to human allotransplantation. Biomimetic potency tests have been strongly advocated for islet transplantation, and recent progress in islets-on-chip may provide solutions [12, 35]. Our observations indicate a potential usefulness of our islets-on-chip system presented here in evaluating islets before grafting and might consequently improve clinical outcomes. The approach used here may also be developed as microfluidic device thus further reducing the number of islets required [15, 23, 36, 37]. In the long run, such a system might also qualify for evaluation of stem cell-derived pseudo-islet organs prior to their implantation [12].

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving humans were approved by Islet CHIP (authorization number NCT03067324) was a pilot study derived from the clinical islet transplantation STABILOT randomized control trial (authorization number NCT02854696). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The study was designed by BC, JL, MR, SL, TB, and P-YB; BC, JL, SR, P-YB, MR, and AW obtained funding; JL, MR, SL, FL, P-YB, AP, TB, DB, BC, and MJ researched and analyzed the data; JL, SL, MR, P-YB, BC, AP, and SR cowrote the manuscript; All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11512/full#supplementary-material

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REVIEW
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Bioengineering the Vascularized Endocrine Pancreas: A Fine-Tuned Interplay Between Vascularization, Extracellular-Matrix-Based Scaffold Architecture, and Insulin-Producing Cells

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Intrahepatic islet transplantation is a promising β-cell replacement strategy for the treatment of type 1 diabetes. Instant blood-mediated inflammatory reactions, acute inflammatory storm, and graft revascularization delay limit islet engraftment in the peri-transplant phase, hampering the success rate of the procedure. Growing evidence has demonstrated that islet engraftment efficiency may take advantage of several bioengineering approaches aimed to recreate both vascular and endocrine compartments either *ex vivo* or *in vivo*. To this end, endocrine pancreas bioengineering is an emerging field in β-cell replacement, which might provide endocrine cells with all the building blocks (vascularization, ECM composition, or micro/macro-architecture) useful for their successful engraftment and function *in vivo*. Studies on reshaping either the endocrine cellular composition or the islet microenvironment have been largely performed, focusing on a single building block element, without, however, grasping that their synergistic effect is indispensable for correct endocrine function. Herein, the review focuses on the *minimum* building blocks that an ideal vascularized endocrine scaffold should have to resemble the endocrine niche architecture, composition, and function to foster functional connections between the vascular and endocrine compartments. Additionally, this review highlights the possibility of designing bioengineered scaffolds integrating alternative endocrine sources to overcome donor organ shortages and the possibility of combining novel immune-preserving strategies for long-term graft function.

Keywords: type 1 diabetes, beta cell replacement, bioengineering, vascularized endocrine pancreas, 3D-bioprinting, extracellular matrix, biomaterials, alternative endocrine sources

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INTRODUCTION

Type 1 diabetes (T1D) is characterized by autoimmune-driven destruction of insulin-producing β-cells, which leads to altered control of glucose homeostasis and induction of hyperglycemia. The first line treatment is the exogenous insulin administration *via* multiple daily injection (1–4). An alternative strategy to insulin injection is to replace the endocrine mass by transplanting allogeneic pancreas or pancreatic islets in T1D patients experiencing insulin-dependent hypoglycemia unawareness, severe hypoglycemia, and unstable glycemia (5–9). To date, pancreas transplantation is more frequently used in clinical practice than islet transplantation, although it has more important surgical procedures. Indeed, islet transplantation is an easy and poorly invasive procedure that avoids post-surgery burdensome effects on patients (10). Islet transplantation has a high success rate in alleviating hypoglycemic events and improving the quality of life of patients. However, only a small percentage of recipients acquire insulin independence after intrahepatic islet transplantation. A gradual loss of both graft function and insulin independence was observed within 5 years of islet implantation (7,8,11). Despite the short-term function, the results derived from the recipients demonstrated that reestablishing endocrine pancreatic function has the potential to restore fine endogenous control over glucose homeostasis, which cannot be precisely mimicked by closed-loop artificial pancreas devices (12,13).

The inability to achieve long-term function of the intrahepatic islet graft must be sought 1) in the inflammatory processes in the peri-transplantation phase, leading to early graft loss, 2) in the missed prompt vascularization, and 3) in allo-immune reaction and autoimmune recurrence (14–16). In particular, instant blood-mediated inflammatory reaction (IBMIR) leads to a loss of approximately 50%–70% of the total infused islet mass within the first few hours to days after transplantation. Additionally, at the hepatic site, tissue reperfusion-related damage and thrombotic events further increase the inflammatory state, leading to poor engraftment efficiency (17,18). Furthermore, the delay in functional graft vascularization dangerously exposes islets to hypoxic stress and lack of nutrients for at least 2 weeks after transplantation, causing islet cell death and apoptosis (19). To balance this intrinsic procedure limitation, a high number of islets are infused, with at least 10000 islets equivalents (IEQ)/kg body weight generally obtained from two or three donor pancreata, increasing the overall organ demand (20,21). On the other hand, to avoid immunological reactions against the graft in the post-transplant phase, life-long immunosuppressant administration is provided, which in turn can provoke kidney failure and increase cancer risk and infection (15). In recent years, alternative transplantation sites have been proposed to increase the success rate of allogeneic islet transplantation; however, to date, no one has shown superior outcomes compared to the intrahepatic site (6,7,11).

In this scenario, to overcome the current limitation and improve outcomes, several points need to be achieved: 1) the identification of an alternative site with a microenvironment architecture that may improve endocrine function; 2) fostering prompt vascularization, able to ensure an adequate exchange of oxygen, nutrients, and hormones to support endocrine pancreatic cells in effectively sensing blood glucose changes; 3) the identification of a method to mitigate the innate immune reaction to avoid early graft loss; 4) the definition of alternative strategies granting long-term graft immune protection; and 5) the identification of a renewable source of insulin-secreting cells to widen the treatment to a larger cohort of T1D patients (22). To achieve these goals, tissue engineering (TE) approaches can provide new insights, especially in increasing vascularization at transplantation sites through biomaterial-based strategies. Indeed, the intention of the last years has been to recreate a vascularized site to accommodate endocrine cells in order to accelerate graft revascularization and shorten the hypoxic phase. Although these approaches have been largely investigated in clinical trials, research in this field is moving towards the design of systems resembling the endocrine native niche, especially considering its organization, in terms of supporting cell type and microarchitecture. Introducing these two components into bioengineered systems may support structural and functional integration between the endocrine and vascular compartments, which is fundamental for recreating the physiological microenvironment of the endocrine niche and improving the biocompatibility of the graft with the host tissue (23).

TE technologies may also give the chance to recreate endocrine pancreas using alternative endocrine sources, such as pluripotent stem cells (PSCs) or xenogeneic source appropriately modified, favoring the exploration of their function and the feasibility of the approach in clinical practice (11,24). Finally, the flexibility of TE technologies might help overcome the systemic administration of immunosuppressive drugs by combining novel immunosuppressive strategies to locally achieve an immune-privileged transplantation site (15,23,25,26). To overcome the limitations of classical β-cell replacement, bioengineered endocrine pancreas systems need to be inspired by the native niche. Therefore, we will first define the native endocrine niche architecture and functional components and subsequently address TE strategies for tuning and reshaping.

THE ENDOCRINE NICHE

Human pancreas is a unique and complex organ that contains both exocrine and endocrine tissues. The exocrine part accounts for 98% of the organ parenchyma and secretes pancreatic juice into the duodenum for correct digestion and assimilation of nutrients (27). The endocrine compartment represents the remaining 2%. The endocrine side is organized into independent cluster units (27) scattered throughout the exocrine parenchyma, best known as the islet of Langerhans (27,28). They are embedded within a capsule consisting of an extracellular matrix (ECM) and fibroblasts, in which endocrine cells are non randomly aggregated. Islets are independently fed by a dense network of highly fenestrated capillaries, which allows

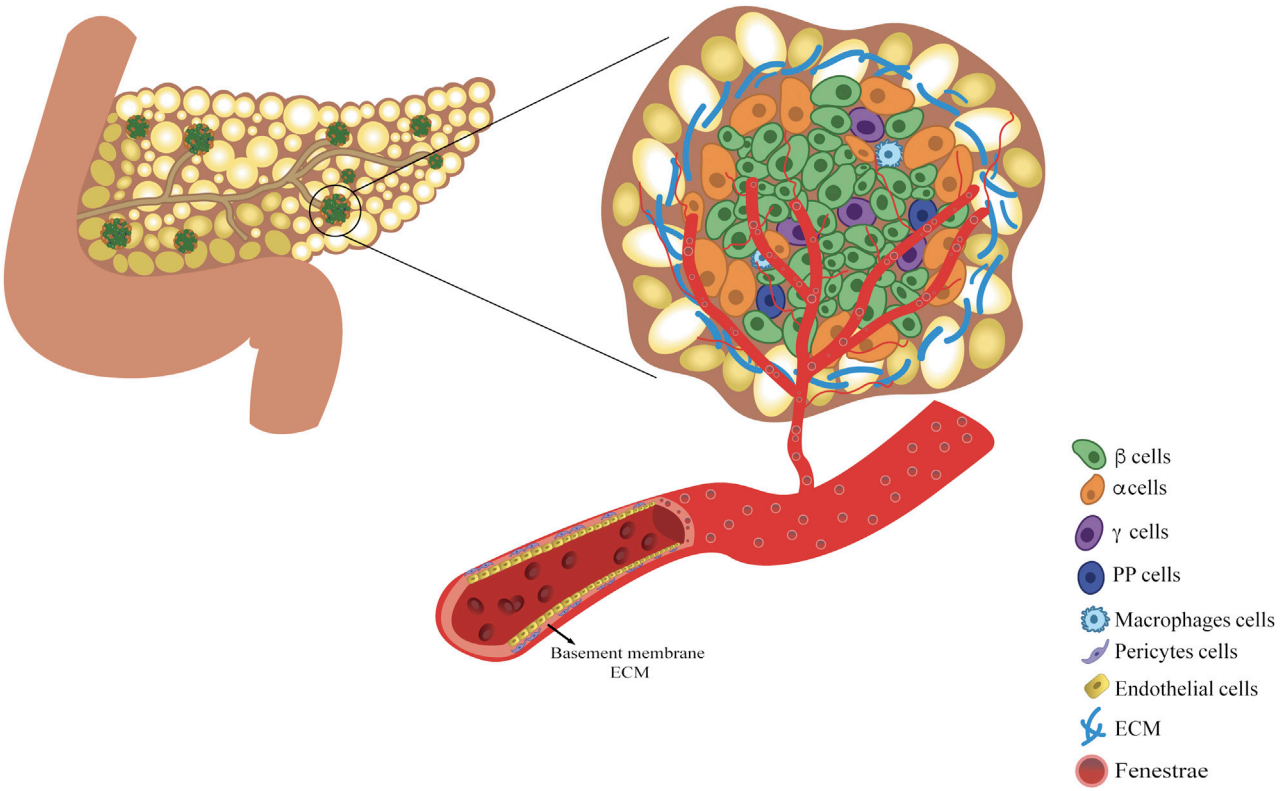

FIGURE 1 | The vascularized endocrine niche within the pancreatic tissue. Pancreatic endocrine niche is enclosed within the pancreatic exocrine tissue and it is constituted by three main components: the extracellular matrix, islet of Langerhans and fenestrated vascular network. Islet is mainly composed of insulin-secreting β-cells, glucagon-secreting α-cells, somatostatin-secreting δ-cells, pancreatic polypeptide-secreting PP-cells, and macrophages. The microvasculature within the endocrine cell cluster is fundamental both for sustaining the endocrine cells viability and for accomplishing for their function.

each endocrine cell to be in close contact with the blood (28). Specific organization of the ECM, cells, and microvasculature consitutes the endocrine niche (**Figure 1**). Owing to the evaluation of the endocrine niche, it was possible to identify the fundamental features useful for bioengineering endocrine pancreatic tissues. Thus, the role of each component will be briefly reviewed, identifying it as an essential part of the niche microenvironment that synergistically supports endocrine functions.

Cell Roommates of the Endocrine Pancreatic Niche

Among endocrine pancreatic cell types, β-cells are the most abundant, accounting for 60%–75% of islet cells, constituting the sole source of cells capable of secreting insulin and amylin. α-cells are the second most abundant cells (20%–30%), secreting glucagon as an insulin counter-regulatory hormone. Other endocrine cells are δ, ε, and pancreatic polypeptide cells (PP), which release somatostatin, ghrelin, and PP hormones, respectively (28). According to the work of Bonner-Weir et al., differences in cell composition based on islet dimensions have been observed: large islets have a lower content of β-cells compared to medium-sized islets (~60% vs. ~75%) (29). Additionally, most medium- and small-sized islets have a non-random organization. They present a layer of β-cells between the two layers of α-cells. Large islets display a more random organization owing to their low β-cell percentage (29,30).

All endocrine cells work together to establish a complex paracrine network that ensures proper control of blood glucose levels (31,32). In addition, interactions between endocrine cells and other roommate microenvironments, such as vascular and innate immune cells, are essential for the correct development and function of the endocrine network (33).

Vascular cells, such as endothelial cells (EC) and pericytes, generally constitute the cellular part of the *tunica intima* of vessels, while the structural part is the basement membrane (BM), which is constituted by a specific ECM. In the endocrine pancreatic niche, ECs form a fenestrated endothelium, guaranteeing high permeability (ten times more fenestrae compared to exocrine vessels) and a greater capacity for nutrients, hormones, oxygen, and metabolic waste exchange (34,35). ECs can directly affect β-cell function by upregulating insulin secretion and promoting β-cell survival via the secretion of soluble factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF)-A (36) and ECM proteins (37).

Although the contribution of EC to islet endocrine function has been well described in the literature, the role of PC has recently been emphasized (35). Pericytes are abluminal mural cells embedded in the BM-ECM of blood vessels and play a key role in regulating endocrine niche homeostasis and function (35,37). Indeed, Landsman et al., in a series of ablation experiments, reported the role of pericytes in β-cell expansion during the neonatal stage and in the maintenance of β-cell maturation and function in adulthood (38–40), regulating the production and deposition of islet ECM components and promoting the expression of β-cell genes including Ins1, Mafa, and Glut2 (41). Additionally, pericytes are directly involved in β-cell function through nerve growth factor (NGF) pathways, activating the release of insulin granules in the presence of high glucose levels (42).

Along with vascular cells, macrophages reside in the endocrine niche (43) and participate in maintaining tissue homeostasis and/or dysfunction (44). Studies on mice have revealed that resident macrophages are present in the prenatal stage, constituting a pool of tissue-resident macrophages maintained by local proliferation (45). Two different subsets have been identified by immune profiling: F4/80^{lo}CD11c^{+} macrophages present within the islet structure and F4/80^{hi}CD11c^{-} macrophages largely residing in the peripheral islet area (46). Both subsets are in close contact with vasculature and endocrine cells and act as sensors; they sense and respond to cues modulating their activation state and release proliferative factors, such as tumor necrosis factor-alpha, interleukin (IL)-6, IL-10 (47), insulin growth factor-1 (48) and transforming growth factor-beta, which have been demonstrated to sustain β-cell mass viability (43). Saunder et al. confirmed the synergistic network between roommates, demonstrating a coordinated interaction between EC and resident macrophages in promoting β-cell regeneration. They also highlighted the key role of ECM-mediated signaling and remodeling (49). Taken together, each roommate presents peculiar features and tasks in efficiently supporting the endocrine compartment and its function, which makes it an optimal candidate to consider and integrate in bioengineering a platform.

Islet ECM Composition and Architecture

Physiologically, the ECM provides mechanical and physical support to cells and affects cell migration, proliferation, and differentiation (50,51). It is a three-dimensional network composed of fibrous-forming proteins, such as collagens, laminins, glycoproteins, elastin, and glycosaminoglycans (51,52). In the pancreas, BM is predominant: it surrounds the acinar cells of the exocrine pancreas, duct vessels, and pancreatic islets (53). More specifically, pancreatic islets are embedded in ECM-based structures with a specific and balanced protein composition, hierarchical organization, and determined architectural features, which are strictly related to the correct endocrine function (54–57). Islet ECM can be subdivided into an external and incomplete peripheral capsule, the peri-islet ECM, and an internal ECM, the inner matrix (IM) (58). They are secreted from different cell types; the former is secreted by exocrine cells (59), while the latter is the vascular BM secreted by vascular cells (35). As endocrine cells are not able to secrete ECM proteins, VEGF-A secretion from β-cells recruits EC to induce ECM deposition and maintain homeostasis (57,60,61). The islet inner ECM of humans has unique features: endocrine cells and islet capillaries are separated by double leaflets of vascular BM (30,62,63). The ECM composition of the endocrine niche varies during human development, as different protein isoforms are expressed from early tissue precursors to mature human pancreas (64). Although there is no consensus on islet ECM composition, the predominant proteins are collagen type IV, laminin, and fibronectin with various prevalence (65). Collagen type IV contributes to BM formation (66) and favors the maintenance of the capsule architecture. Collagen IV binds $\alpha_1\beta_1$ integrin expressed on β-cells, inducing essential signals for islet development, enabling migration of fetal β-cells, and forming normal islet architecture (67). It also enhances islet adhesion, proliferation, and insulin secretion (68). Laminin exists in several isoforms in the islet niche, and although the cell responsible for producing each isoform is still unclear, some studies have defined temporal and spatial expression. Laminin-111 is the primary isoform expressed during pancreatic development that promotes β-cell differentiation (69). During islet maturation, laminin-111 is completely replaced by the laminin-511, -521, -411, and -421 isoforms. In mature endocrine tissue, the BM leaflet towards the endocrine cells displays laminin-511, while the leaflet of the vascular lumen also laminin-411, -421 and 521, besides laminin-511 (62). Laminins bind to different integrin and non-integrin receptors on β-cells, such as β1 integrin, αV integrin, and dystroglycan (58). As a result, the interaction in β-cells activates several signaling cascades aimed at enhancing insulin secretion, inducing the expression of islet-specific transcription factors such as PDX1, Ins1, Ins2, glucagon, somatostatin, and GLUT-2 (70) and promotes β-cell survival and proliferation (59). Fibronectin is a multifunctional component of ECM that facilitates cell adhesion. It interacts with the arginine-glycine-aspartic acid (RGD) receptor to improve islet function, β-cell proliferation, and glucose-stimulated insulin secretion. The interaction with fibronectin-RGD induces the expression of differentiation markers for endocrine tissues, such as PDX1 and Ins2 (70) and improves islet cell survival, boosting the expression of anti-apoptotic protein BCL-2 (71). This evidence supports the idea that ECM components play beneficial roles towards endocrine pancreatic cells. In conclusion, the ECM was originally thought to exist to solely provide structural support to cells and it is now recognized as a reservoir of information contributing to tissue homeostasis and function (51).

Vasculature

Although the endocrine compartment represents 2% of the pancreatic mass, it receives about 15%–20% of the pancreatic blood flow (9). Islets have a highly specialized network of arterioles, capillaries, and venules, known as the microvasculature. Owing to the high density and fenestration of capillaries, endocrine cells are bathed by blood, allowing a rapid exchange of nutrients and hormones, which is essential to correctly control blood glucose levels. Depending on their

dimensions, each islet is in contact with 1-5 arterioles, which are divided into capillaries enveloping the islet and generating a structure similar to a renal glomeruli (72–75). If small islets have their own microvasculature organization, large islets have been proposed to be organized in small endocrine subunits, independently fed by proper but similar microvasculature (30). Several hypotheses have been proposed to model islet blood flow and its correlation with endocrine function, given its importance in the rapid sensing of blood glucose fluctuations and the corresponding counterbalancing hormone outflow (76–78). Three models of islet flow, which are not mutually exclusive, have been proposed and supported by studies on mice. In the first model, peripheral-to-center blood flows from the exterior to the interior of the islet. According to this model, islets are composed of a β-cell core surrounded by an α-cell layer, which is the first layer exposed to blood flow. Thus, α-cell secretagogues might directly influence the function of the β-cell core (76). In the second model, center-to-periphery, the blood flow reaches the β-cell core and then flows to the periphery where the α-cells are located. Products from β-cells can directly influence α-cells (77). In the third model, the pole-to-pole arterioles simultaneously contact all cell types in different islet regions (78). However, it is worth underlining that the architecture of the islet varies across species and β-cells are not always confined to a central core, as in humans, and in some species, a totally opposite islet organization can be found (79).

TUNING THE ENDOCRINE NICHE

In the field of β-cell replacement, recreating the endocrine niche *ex vivo* might be advantageous, as it could overcome the current limitations of clinical treatments in T1D. Deep investigations of the physiology of the native endocrine pancreatic niche have helped to understand the principal features useful for bioengineering vascularized endocrine pancreas. In addition, other evidence has been derived from the comprehension of the mechanisms involved in the failure of engraftment upon transplantation at different sites (7,11).

Vascularization and Oxygenation of Transplantation Site

The endocrine niche is not only deeply vascularized, but the vascular architecture is also based on hierarchical vessel distribution, which rules oxygen diffusion, nutrient distribution, and hormone secretion, affecting the physiological endocrine function (6,9). These features can explain the sensitivity of endocrine pancreatic cells to hypoxic environments and a lack of nutrients (80). After the isolation process and in the early transplantation phases, islets are completely deprived of vascularization and the correlated oxygen and nutrient supply until engraftment within the host tissue, which occurs upon the re-establishment of functional vascularization in 1–2 weeks (19). Based on this evidence, β-cell replacement strategies are focused on finding vascularized sites, evaluating alternative transplantation sites compared to the liver, or preconditioning strategies that increase vessel density at the implantation site. Owing to the failure to find alternative transplantation sites, the preconditioning strategy has gained ground, especially by exploiting biocompatible materials (7,11,81,82). In particular, engineering a transplantation site to increase vascularization is thought to be suitable for ameliorating the engraftment and function of endocrine pancreatic grafts (83) (**Figure 2**). Implantation of nylon catheters or cylindrical stainless-steel mesh tubing in rodents helped to create vascularized pouches in 1 month, exploiting the foreign body response (FBR) without inducing scar formation in different tissues. After removal, syngeneic islets or human islets were easily implanted, showing the ability to reverse diabetes in the respective appropriate rodent models (84), while islets infused in the not-preconditioned pouch were not able to restore normoglycemia (85–89). Similarly, poly-D,L-lactide-co-ε-caprolactone (PLCL)-based scaffolds were used to pre-vascularize the subcutaneous space after 1 month. The islets were positioned in channel structures, which were closed using polyethylene tubing. The system restored normoglycemia in recipient mice with a similar trend as that in kidney capsule recipient mice (90). Exploiting the capability of materials to induce vascularization of the implantation site through FBR, clinical studies have been performed on vascularizing systems. Another study demonstrated the possibility of creating a subcutaneous cavity using a non-degradable Silon monofilament mesh in a murine immune-deficient diabetic model for islet implantation. This study showed that rat islets, in combination with additional supporting cells, were able to engraft and restore normoglycemia for up to 4 months. However, additional investigations are required to further validate this promising approach (91).

However, devices investigated in clinical trials do not consider the pre-vascularizing phase, such that the second surgery for islet positioning is avoided. ViaCyte Encaptra (NCT02239354, NCT03163511) and Sernova Cell Pouch (NCT03513939) are devices that encapsulate insulin-producing cells that induce vascularization of the subcutaneous site upon implantation. Particularly, ViaCyte (VC-01), which is based on Theracyte technology, was composed of a semipermeable membrane to allow oxygen and nutrient exchange but at the same time to isolate transplanted cells from the recipient immune system. The trial was suspended because of poor survival and engraftment of the transplanted cells due to FBR, which clogged the membrane, preventing nutrient exchange and vascularization (92). Additionally, the presence of an immune-isolating membrane hinders the recipients' capillaries formation, which, therefore, are not able to recreate the native islet perfusion and connection. In 2017, ViaCyte started a second trial using a modified encapsulation device (VC-02). The system did not provide complete immune isolation but allowed vascular permeation through the presence of dedicated pores on the device surface. The upgraded design of the device showed a great improvement in the final outcome by the detection of secreted C-peptide in T1D patients within the first year (63%) (93,94). The Sernova cell pouch was similarly aimed to recreate a suitable subcutaneous microenvironment for islet implantation, and a clinical study is

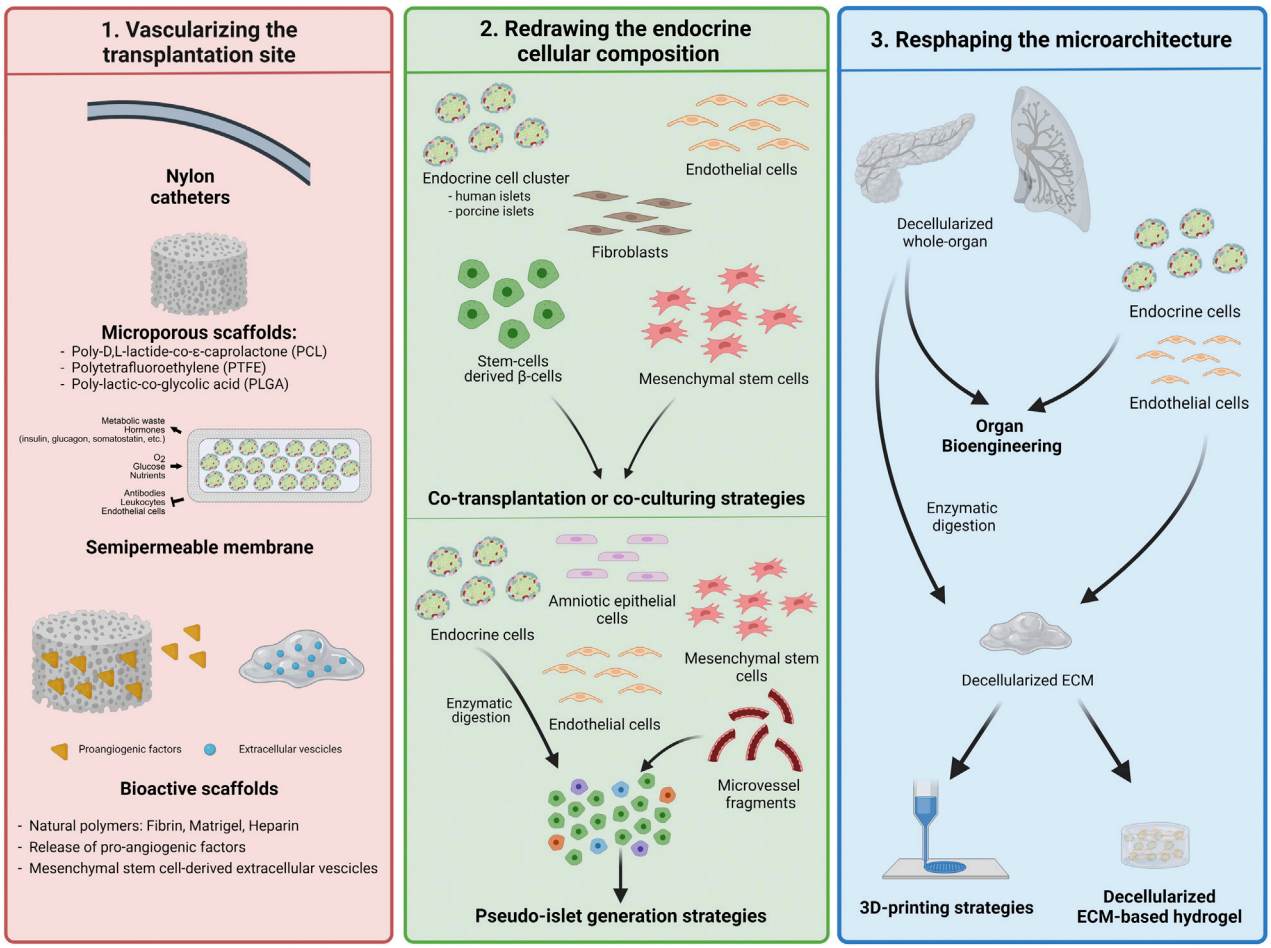

FIGURE 2 | Bioengineering the vascularized endocrine pancreas—building blocks assembly. Strategies mostly used for recreating the endocrine niche in order to improve the endocrine cells viability, their engraftment and function. All of them are aimed to accelerate the vasculature-building block to shorten the hypoxic with different approaches.

still ongoing with patients in an immunosuppressive regimen (7). However, further studies are needed to evaluate the long-term functions of these devices (**Figure 2**).

The aforementioned systems use materials with inert features and are unable to actively interact with the surrounding tissue because they are neither provided by recognition sites for cells nor by biological stimuli. The introduction of bioactive molecules and/or bioactive materials enables the finalization of several mechanisms for device vascularization and colonization of host cells (25). Natural polymers have been widely investigated to enhance the vascularization of transplantation sites (**Figure 2**). Kuppan et al. used a poly lactic-co-glycolic acid (PLGA)-based fibrous scaffold modified with gelatin polymer, which was implanted in the abdominal subcutaneous space for 4 weeks to induce site vascularization. Implantation of xenogeneic islets has reversed the hyperglycemia within 20–25 days, similarly to mice with islets implanted at the kidney capsule site (95). Fibrin has been widely used as the Food and Drug Administration (FDA) approves its clinical use (25). It is a fibrous protein derived by the self-assembly of fibrinogen molecules upon cleavage by thrombin during the coagulation process, and helps the revascularization during wound healing as it presents RGD domains, which induce cell survival and migration (25,96,97). Fibrin hydrogel was previously used to encapsulate islets, which were then transplanted into the omental pouch of diabetic rats or diabetic *Cynomolgus* monkeys. The hydrogel reversed hyperglycemia, gradually reducing the exogenous insulin need, and efficiently supported optimal graft revascularization (98). The promising results in preclinical models led to an ongoing clinical study of the BioHub platform (NCT02213003) (98,99). However, the results at 1 year follow up after transplantation showed decreased graft function. According to the authors, the recipients lost insulin independence over time due to a switch in immunosuppressive regimen from tacrolimus to sirolimus administration (99,100). Fibrin is also involved in IBMIR and prudence is therefore required to avoid the presence of complement proteins in fibrin batches.

Among the commercially available native biomaterials, murine sarcoma-derived hydrogel-Matrigel™ and heparin were also used. They were positioned in a silicone cylinder tubing at the mouse groin, which was closed at the distal end with inguinal fat

and completely sealed with bone wax. The study showed the ability of the chamber to obtain a microvascularized network after 28 days and to sustain the engraftment and function of syngeneic islets after 10–14 days (101,102). However, the use of Matrigel in clinical procedures has some shortcomings related to its poorly defined chemical composition (25).

Vascularization strategies using synthetic or natural materials have shown the ability to create a microenvironment that is more comfortable for islet accommodation at transplantation sites, as highlighted by previous reports. For preconditioning strategies, there is the disadvantage of a second surgery for positioning insulin-producing cells. Additionally, the triggering of FBR is due to the recruitment of neutrophils, macrophages, and mast cells, which normally react against a material-based implant, generating a fibrotic capsule around it, isolating the endocrine pancreatic graft, and finally inducing the formation of an unorganized vessel network that is not properly functional in a fully vascularized graft (103). However, these strategies still present positive and relevant aspects, considering the delay of technological improvement, which hinders the development of new strategies clinically relevant for β-cell replacement.

The ViaCyte experience has highlighted the crucial role of prompt and complete vascularization upon implantation. Therefore, to provide a scaffold with optimal proangiogenic capability, several studies have evaluated the addition of growth factors such as VEGF, angiopoietin-1 and 2 (Ang1 and Ang2), platelet-derived growth factor (PDGF)-BB, and fibroblast growth factor (FGF)-2 (104–109). The controlled and sustained release of single or multiple factors from the scaffolds has been demonstrated to induce both angiogenesis and the formation of mature vessels with respect to a random mix of growth factors within the hydrogel or direct injection at the implantation site (110–112) (**Figure 2**). In fact, the tailored release of growth factors allows the creation of gradients that attract recipient ECs towards the implantation site (113). In previous studies, VEGF has been released in a sustained manner through chemical binding to polymeric scaffolds or by exploiting the growth factor-binding ability of heparin. Sustained release of VEGF over time improved islet engraftment because of higher cell penetration, which allowed the formation of new capillaries than islets embedded into free VEGF-polymeric scaffolds (104,113–115). Similarly, multiple or sequential release of different proangiogenic factors from the implanted scaffold might be another approach for increasing new vessel formation at the graft site. The release of VEGF followed by PDGF or FGF-2 has been shown to increase the maturation of vessel networks compared to VEGF alone. FGF-2/VEGF co-release has been proposed to mimic physiological secretion in the vascularization process during wound healing. Polylactic acid (PLA) fibrous scaffolds modified with heparin-binding amphiphilic peptides could store and slowly release VEGF and FGF-2. Recipient mice receiving the modified fibrous scaffolds with islets reversed the hyperglycemia faster than control mice receiving the bare fibrous scaffold, thus suggesting the ability of the modified scaffold to sustain islet engraftment and function (116). To this end, platelet-rich plasma (PRP) is suitable for multiple factors release for revascularization. It has a growth factor composition in a ratio that is useful for efficient vascularization. Indeed, it is used for chronic wound healing treatment (117) and cell transplantation as a coating of a PLA-based chamber to induce vascularization in the subcutaneous space of mice (118).

Recently, with the idea to promote angiogenesis in a biomimetic manner, several studies have focused on introducing multiple proangiogenic stimuli mimicking the multi-combinatorial aspects of physiological processes (109,119–121). Knowing that islets physiologically are able to secrete factors for recruiting ECs, Staels et al. considered the possibility of enhancing the capability of transfecting islets with an mRNA encoding VEGF, showing that vessel formation was increased (119). Xing et al. proposed the use of mesenchymal stem cell (MSCs)-derived extracellular vesicle (EVs) chemo-selectively immobilized onto a collagen-based scaffold. This strategy induced higher host cell infiltration and improved angiogenesis, including vascular ingrowth and macrophage recruitment, compared to scaffolds without immobilized EVs (121). Similarly, Najjar et al. proposed the use of a fibrin-based gel complexed with a recombinant human fibronectin fragment containing integrin and binding domains for VEGF and PDGF. Thus, although the gel had minimal doses of VEGF and PDGF-BB and was loaded with a marginal mass of syngeneic islets, the interaction of both VEGF and PGDF receptors with integrin $\alpha_5\beta_1$ through fibronectin domains enhanced revascularization. The induced vascularization showed a higher ability to reverse hyperglycemic conditions compared to the non-complexed and unloaded hydrogels upon implantation in the epididymal fat pad in preclinical models of diabetes. This was positively correlated with the prompt revascularization induced by the fine assembly of the gel with encapsulated VEGF and PGDF (109,120).

Overall, these results highlight that releasing multiple factors in a biomimetic manner might enhance the recruitment of proangiogenic cells, accelerating vessel formation. However, it is not enough to recapitulate either the physiological mechanisms of angiogenesis or the impact of ECM components on vascular regeneration through cell-cell and cell-matrix interactions. Therefore, structural support is required to achieve more efficient and physiological vascularization (25) (**Figure 2**).

A different strategy to support β-cell viability and function upon implantation reduces the physiological latency of the vascularization process using oxygen-producing devices. A clinical study evaluated the β-Air bio-artificial pancreas, which had a daily refillable oxygen chamber between two layers of alginate encapsulating the islet to maintain an adequate oxygen supply (NCT02064309) (98,112,122). β-Air improved cell viability and supported graft function, which were detected for 10 months without immunosuppression. However, this strategy still cannot ensure adequate glucose sensing and insulin release kinetics in the islets (122). Another study designed an encapsulation system that generates oxygen starting from metabolic waste products such as carbon dioxide through an inverse breathing chemical reaction. The device uses the gas-solid reaction of carbon dioxide with lithium peroxide to produce oxygen, whose pressure remains constant. However, there are

concerns related to lithium peroxide toxicity and the finite oxygen supply of the device (123).

Supporting encapsulated islets through oxygen-dispensing techniques in the post-transplant phase may be useful to support their viability and long-term function, without the need to induce vascularization. However, endocrine function, especially glucose sensing, can be hindered by the lack of vascularization, which is fundamental for an efficient endocrine graft function (122).

Redrawing the Endocrine Pancreatic Cellular Composition

Alternative approaches in the field of β-cell replacement aim to combine additional cell types with endocrine cells to foster functional vascularization and engraftment of endocrine cells in a physiological and biomimetic fashion. MSCs, EC, and fibroblasts have been used *ex vivo* and *in vivo* to reshape endocrine cell cluster composition (25,124). EC are directly involved in reconstructing the vessel as they compose the endothelial barrier and sustain the mechanism through paracrine signals, whereas MSCs and fibroblasts are known to participate in the vascularization process by supporting the EC (125–127). Additionally, MSCs enhance angiogenesis by remodeling the ECM, secreting VEGF, Ang1 and 2 and stabilizing vasculature (128) (**Figure 2**).

Co-transplantation of porcine islets with MSCs in diabetic mice or primates has been shown to support vascularization and normoglycemic restoration (25). Following the same strategy, other reports showed that the combination of human or rodent islets with MSCs and/or fibroblasts loaded in a collagen-fibrin hydrogel implanted in recipient diabetic mice demonstrated the positive impact of accessory cells to promote higher vascularization, earlier graft function, and better control on glucose homeostasis compared to islets alone (129–131). Additional studies have characterized the MSC subtypes and their different roles in supporting cells in the β-cell replacement approach. Forbes et al. showed that human islets co-transplanted under the kidney with MSC derived from the perivascular tissue capsule had better glycemic control than human islets implanted alone and restored normoglycemia conditions within 5 days. Comparing this work with other reports in which MSC derived from bone marrow or adipose tissues were used, perivascular MSC seemed to be more effective in rapidly restoring normoglycemia (132–137).

Human umbilical vein endothelial cells (HUVEC) were widely used as an EC model to evaluate their impact on endocrine graft revascularization. Collagen type I hydrogels embedded with rat islets and HUVEC have been shown to restore normoglycemia within 8 days, displaying a higher presence of $CD31^+$ cells and proangiogenic $CD206^+/MHCII^-$ (M2-like) macrophages after 7 and 14 days compared to non-encapsulated islets (138). In addition, 24h-self-aggregation of human or mouse islets with HUVEC and human MSCs promoted both good endocrine pancreatic graft function and a massive improvement in post-transplant engraftment, suggesting the beneficial activity of the supporting cells. The authors also highlighted the role of MSCs and HUVEC in producing ECM, in particular laminin and collagen IV of the BM, which was highly observed along the EC within endocrine tissues (134). Blood outgrowth endothelial cells (BOEC) have also been used as EC model to ameliorate graft vascularization. They showed their ability to reduce β-cell death and induce good vascularization of the graft, producing metabolic benefits in diabetic immunodeficient murine models (139). Other studies used ECs or MSCs to coat islet preparations, and independent of the methods used, coated islets showed better engraftment due to enhanced vascularization. Finally, other authors have proposed specifically coating islets with proangiogenic cells rather than co-culture or co-transplantation (135–137) (**Figure 2**).

More recent strategies have been developed to reshape endocrine cell composition. Starting from the possibility of creating pseudo-islets by re-aggregating enzymatically digested islet cells into homogenous cell clusters, several groups have suggested combining islet cells with other cell types to enhance the pseudo-islet endocrine function (140–144). Digested rat islets in single cells were reassembled in new type of endocrine-like cluster co-aggregating HUVEC and human amniotic epithelial cells (hAEC), obtaining heterotypic spheroids with homogeneous size. While HUVEC were added to sustain the vascularization of the cellular clusters, hAEC, known as cells expressing a pluripotent and immune-modulating repertoire (140–142), were introduced to shield endocrine cells and modulate the response of the host immune system. The assembled spheroids demonstrated an enhanced *in vitro* function and, upon implantation into the epididymal fat pad of a diabetic immunocompromised murine model, faster engraftment and vascularization when compared to undigested rat islets (142). Similarly, a previous study evaluated the impact of spatial aggregation of the human β-cell line EndoC-bH3 and EC. Heterotypic pseudo-islets composed of a core of islet-derived cells surrounded by an outer layer of EC showed increased insulin secretion and, therefore, β-cell functionality, emphasizing that the spatial distribution and cell-cell interactions are features to be considered to reconstitute the organization of the pancreatic islets (143). Digested islet cells along with EC and MSC embedded in collagen type I-based hydrogel rods, further coated by other EC, were able to restore normoglycemia within 2 weeks after subcutaneous implantation in streptozotocin-induced diabetic mice, which also showed good control of glucose metabolism (144) (**Figure 2**).

The introduction of supporting cells into the re-shaped endocrine pancreatic constructs has been shown to increase vascularization by their direct involvement in vessel formation or by recruiting host cellular counterparts. However, their random addition to the system imperils the rapid formation of organized vessels, retarding the anastomosis with host vessels and hampering the gain of graft function. This provokes a lag in the integration of the endocrine graft in the host tissue, dangerously exposing insulin-producing cells to hypoxic stress. To further shorten engraftment time, Nalbach et al. fused murine islet-derived cells to murine epididymal fat pad micro-vessel fragments, which consist of EC-lined lumen covered by stabilizing α-smooth muscle actin (αSMA)-positive cells with

preserved micro-vessel structures. The resulting organoids displayed reduced hypoxic stress, increased insulin secretion *in vitro* and faster hyperglycemia-reversing ability due to rapid revascularization compared to non-pre-vascularized organoids and fresh islets (145). These results highlight the importance of preformed structures to obtain a pro-vascularizing architecture within insulin-producing elements, which have been shown to increase oxygen penetration within the 3D organoid structure and accelerate revascularization *in vivo*.

The Role of ECM and Microarchitecture

Pancreatic islet isolation is associated with peri- and intra-islet ECM destruction (55,56,146). Even if intra-islet ECs are still present in the first days after isolation, they undergo gradual death, compromising their islet-ECM replacement ability (56). Upon isolation, laminin significantly decreases after 24 h in *ex vivo* culture, while the remaining collagen IV remain during the culturing phase (14,56,57,147). Loss of ECM leads to β-cell cytotoxicity, apoptosis, and reduced insulin production (56,147–149). Therefore, the use of ECM-based polymers to bioengineer endocrine pancreas is another necessary aspect to consider. ECM proteins, alone or in combination with synthetic materials, have been used to fabricate scaffolds for β-cell replacement (54,56,57,150–155). However, the microenvironment of the pancreatic endocrine side is characterized by a specific ECM design with a fine balanced protein composition; therefore, a simple mixture of ECM-derived polymers may not be sufficient to reproduce the complexity of the mechanobiology involved (156). This specific "intrinsic design" is not only structurally strategic for cell-to-cell interactions but also functionally relevant for tuning endocrine function. Several studies decellularized whole organs through detergent perfusion, preserving the entire ECM organization as well as the microarchitecture with the aim of taking advantage of the native organ ECM structure and composition (**Figure 2**). Decellularized lungs (157–159), kidney (160), spleen (161), liver (162–164) and pancreas (165–172) were recellularized by exploiting the pre-existing macro- and micro-architecture of native organs to recapitulate the complexity of the native endocrine microenvironment (173,174). As expected, decellularized scaffolds preserved native ECM composition, confirming that decellularization did not alter the chemical and physical properties of the native organ (170). However, glycosaminoglycan loss can occur, depending on the decellularization protocol, leading to an increase in both the stiffness and Young's modulus of the decellularized scaffolds (175). Seeding insulin-secreting cells within these scaffolds, regardless of the organ source, improved insulin expression and efficiency in response to high-glucose stimuli *in vitro*. Furthermore, the implantation of recellularized scaffolds with insulin-producing cells at the subcutaneous site was effective in decreasing blood glucose (<15 mM) after 10 days, suggesting successful engraftment sustained by vascularization (169,172). The use of a native scaffold allowed the exploitation of pre-existing vessel structures within the decellularized organs to achieve reconstruction of the vasculature side of the endocrine pancreas. In fact, among these studies, only a few reports have seeded HUVEC within the native decellularized organs vasculature structure, achieving a successful reconstruction of the endothelial barrier *in vitro* and obtaining an *ex vivo* vascularized organ (**Figure 2**) (159,170). More interestingly, the dynamic culture of bioengineered devices has been shown to support the reconstitution of vasculature and to ameliorate the insulin secretion efficiency and viability of insulin-producing cells compared to those cultured in standard conditions, suggesting a successful *ex vivo* engraftment of endocrine cellular components (159,170). Our group used a decellularized rat lung left lobe to recreate a vascularized islet organ (VIO). HUVEC were seeded through pulmonary artery and vein, and the vasculature of the native lung was successfully recreated. Through the trachea, rodent islets were co-seeded with an additional amount of HUVEC, allowing them to reach the native decellularized alveolar structure, where they were retained. At this site, rodent islets receive metabolic support owing to the dense capillary network surrounding the alveoli, which are already vascularized (159). The relevant aspect was appreciated when the VIO platform was implanted in an immunocompromised diabetic murine model. In fact, its function was detected in almost 80% of recipient mice 5 days after subcutaneous implantation, demonstrating the importance of restoring vascularization *in vitro*. This allows a rapid vascular connection *in vivo*, shortening the hypoxic phase and limiting the loss of insulin-producing cells (159). These results demonstrate the tremendous impact of the ECM-shaped native-like architecture to favor pre-vascularization and engraftment *ex vivo*, which accelerates anastomosis and endocrine function *in vivo*. In this scenario, both endocrine pancreatic components and vascularizing elements were functionally and structurally intertwined owing to the coupled effect derived from the tailored ECM composition and microarchitecture of the decellularized organ.

To date, the use of native ECM to fabricate hydrogel scaffolds for tissue engineering is rapidly expanding because of the ease of decellularization. Upon decellularization, native ECM can be enzymatically digested to obtain smaller peptides that are useful for thermal-triggered hydrogels (dECM) (176–182). Hydrogels derived from porcine pancreas dECM have a beneficial role towards encapsulated rat islets, which secrete higher amounts of insulin than those encapsulated in alginate- or collagen-based hydrogels (183,184). Moreover, a recent study compared the impact of dECM derived from different porcine tissues (bladder, lung, and pancreas) on human and rodent islets. *In-situ* islet encapsulation within 3D-ECM hydrogels derived from the bladder and pancreas improves functional stability over standard culture conditions and enhances the retention of islet-resident EC (185). However, the resulting hydrogels gradually lost some of the native components, had poor mechanical properties, and were subjected to rapid degradation *in vivo*, without sustaining vascularization, leading to graft loss. Therefore, they are coupled with bio-inert materials showing poor degradability and higher stiffness, resulting in more suitable mechanical properties (112,186–188). Alginate capsules were generated to encapsulate insulin-producing cells dispersed within the dECM derived from human adipose tissue or porcine pancreatic tissue. Chemical modification of alginate with poly-L-

lysine has also been proposed to increase the tolerance of the capsule by the immune system. This platform supported cell viability and differentiation and significantly improved insulin delivery, whereas *in vivo*, dECM-encapsulated cells were shown to be non-immunogenic and to significantly improve glycemic control in a diabetic preclinical model (188–191).

Hydrogels based on dECM showed good potential in terms of manipulation and production but were strongly limited by the loss of structure and microarchitecture. Hence, the control and reproducibility of spatial cell distribution are lost, as well as the ability to achieve total functional integration between endocrine and vascular compartments. Therefore, among the decellularized platforms, whole-organ engineering remains advantageous, as the native structure and composition might be completely exploited. Indeed, the results obtained from whole decellularized organs owing to their specific features have emphasized their promising capability in efficiently transplanting endocrine cells by recreating the native endocrine pancreatic niche. Furthermore, the flexibility of such systems might allow 1) their integration with valid alternative sources to human islets, and 2) exploitation of local immune-protection strategies for creating an immune-privileged endocrine site. With these important advances, bioengineered endocrine pancreas based on whole-organ decellularized scaffolds has the potential for clinical translation. Animal origin concerns might be overcome by using transgenic animal sources, limiting the xeno-reaction, and standardizing the procedures to obtain endotoxin-free scaffolds according to good manufacturing practices. Indeed, the use of animal tissue-derived ECM products has already been approved by the FDA and is commercially available for orthopedic surgery, and cardiovascular and skin repair (182).

Reshaping the Architecture: 3D-Bioprinting Strategies

Lessons learned from whole-organ bioengineering highlighted that to recreate a microenvironment that is able to sustain the viability and function of both endocrine pancreatic and vascular compartments, the bioengineered scaffold should be provided with 1) a vascularized network and 2) a determined ECM composition with a hierarchical organization and microarchitecture (22). The use of ECM-derived components along with a wise design of bioengineered systems might be a good alternative strategy to match and functionally integrate these two features into unique bioengineered scaffolds, allowing the amelioration of both graft vascularization and endocrine viability and activity (22,112). 3D bio-printing technologies can be suitable for achieving tailored bioengineering devices for β-cell replacement, offering the following opportunities: 1) tuning the 3D spatial deposition of different cell types simultaneously, 2) encapsulation of cellular components within different hydrogel preparations, and 3) customizing the scaffold architecture according to the requested function (22,98,192) (**Figure 2**).

The 3D-printers available for this purpose differ with respect to the deposition methods (**Table 1**). Inkjet bioprinters are based on piezoelectric or thermal-driven mechanisms, allowing the deposition of a few microliters of a polymeric solution (193). They are poorly used because of the low cell density achievable in the structure compared to the physiological condition, as a high cell number may obstruct the nozzle. Additionally, the polymeric solution suitable for this system should not have a high viscosity, and thus the resultant structures are characterized by weak mechanical properties (194–196). Extrusion-based bioprinters consist of one or more nozzles that dispense the polymeric solution, namely bioinks, through pneumatic systems (air pressure or mechanical pistons) (193,197). These types of instruments are widely used in this research field as they are able to distribute an appropriate cell density in three-dimensional space, providing optimal structural integrity. With this system, the polymeric solution might have a wide range of viscosities, and the bioinks could be loaded with bioactive compounds, different cell types, cell aggregates, organoids, and tissue fragments (25,194,198). Additionally, it has a low printing speed and low spatial resolution, and, according to the nozzle diameter used, the pressure could affect cell viability (98). Finally, light-based printing strategies, such as stereolithography, exploit lasers to induce polymerization and deposition with high-resolution photo-crosslinkable polymers. Light-based printers accept a limited range of bioinks. Additionally, it considers encapsulating a finite number of cells resistant to the presence of a photoinitiator, which potentially exposes them to cell damage due to the generation of heat during polymerization (22,199). Among these setups, extrusion-based 3D printers are mostly used because of their flexibility, good biocompatibility, and minimal risk of damage to cellular components.

3D-bioprinting is still in an exploration phase in β-cell replacement; therefore, research is focused on recreating not the whole pancreas, but the fundamental unit of the endocrine pancreatic tissue: insulin-producing and vasculature components supported by an ECM-based bio-mimicking scaffold. 3D-bioprinted scaffolds based on alginate/gelatin bioink encapsulating human islets showed improved islet viability *in vitro* (200). However, insulin secretion analysis was conditioned by the high viscosity and reduced porosity of the hydrogel, which hindered glucose and insulin diffusion (200). To favor vessel formation, PCL was 3D-bioprinted in a porous ring scaffold, which was superficially modified with VEGF-binding heparin with a high degree of functionalization, while the human islet-encapsulating alginate solution was positioned in the ring hollows. The high surface-to-volume ratio provided by the specific porous structure, along with the slow release of VEGF, augmented the vascularization capability of the system *in vivo*. However, this study did not show the efficacy of the technology in sustaining endocrine function *in vivo* (104). These previous studies were limited in their ability to investigate and reconstitute the endocrine compartment and vasculature reconstruction without considering the functional and structural integration that exists in physiological conditions. Following this idea, another report used coaxial extruders, which allowed the fabrication of 3D-bioprinted alginate/methacrylate-gelatin (GelMA) strands with a core-shell structure, encapsulating EC and murine islets in the shell and core, respectively, obtaining uniform distribution of both cellular

TABLE 1 | Summary of the 3D bioprinting strategies and their possible advantages in β-cell replacement field.

3D printing strategies	Technical characteristics	Benefits for β-cell replacement
Inkjet-based bioprinting	Release of few microliters of hydrogel solution based on thermal or piezoelectric mechanisms Use of low-viscous polymeric solutions Low cells density	No published works exploiting this technique
Extrusion-based bioprinting	Extrusion of hydrogel solution through air pressure or mechanical pistons Adjustable cells density Use of polymeric solutions with different viscosity Adjustable 3D spatial distribution	Use of different type of cells Possibility to provide a fine microenvironment composition and 3D structure Spatial deposition for recreating pro-vascularizing structures
Light-based printing	Deposition of polymers exploiting photo-initiators Photo-crosslinkable polymers Low cells density Risk of cells damage	Possibility to provide 3D pro-vascularizing structures

components in the bioengineered compartments (201,202). This system supported the islet viability, but the small pore size made it difficult to analyze insulin secretion (202). Given the key features of dECM in sustaining endocrine pancreatic components, recent studies have focused on the development of dECM-based bioinks for 3D-bioprinting. Porcine native pancreatic ECM (pdECM) embedded with insulin-producing cells and HUVEC was 3D-bioprinted, supporting the viability and function of β-cells and the pro-vascularizing ability of HUVEC *in vivo*, inducing optimal insulin secretion efficiency. This report validated the efficacy of pdECM as a source of bioink, demonstrating the possibility of recapitulating tissue-specific conditions in 3D constructs (183). Another study developed alginate/pdECM and alginate/fibrin bioinks to encapsulate porcine islets and HUVEC with MSCs. Alginate/pdECM hydrogel composition has been shown to sustain the viability and insulin secretion activity of porcine islets, whereas alginate/fibrin hydrogel supported the viability of HUVEC, inducing them to acquire sprouting morphology. Moreover, scaffolds with three different configurations were successfully fabricated, indicating that the complexity of the 3D-printed scaffold could be easily increased (203).

Despite the great advantage of precisely controlling cell deposition, it is worth emphasizing that cell organization changes over time through self-assembly mechanisms. Thus, there is a need to understand the underlying physiological processes behind in order to exploit them for the effective development and maturation of bio-mimicking structures, finalized to graft survival, integration, and function. Evidence from decellularized organs have highlighted the advantages of pre-vascularizing scaffolds in terms of insulin-producing cell viability and rapid *in vivo* revascularization and engraftment. 3D-bioprinted constructs designed for β-cell replacement should follow this strategy by creating channel or tubular architectures. Several bio-fabrication protocols have proposed different approaches for this purpose: use of coaxial nozzles to obtain hollow tubular strands; introduction of sacrificial polymers in extrusion-based bioprinting, such as Pluronic F127 or gelatin, which can be removed by changing the temperature, pH, or through enzymatic degradation, leaving hollow structures; and fabrication of perfusable light-based printed structures, which can be embedded in the 3D printed scaffolds (22,25,204–207). All these structures can be *in vitro* re-endothelialized to recreate a functional vasculature using a medium flow connected to a perfusable system, allowing a dynamic culture. This may promote cell infiltration and prompt revascularization *in vivo* (22). However, systems with this complexity have not been fabricated for β-cell replacement so far, not only because of the technological limitations of the 3D-bioprinting field, but also because of the biological issues concerning the sensitivity of insulin-producing cells and the intricacy of recreating the physiological mechanisms. To date, although investigation aimed at developing a bioengineered artificial endocrine pancreas through this type of technology is an attractive solution in the field of β-cell replacement, the advantageous use of a bioengineered decellularized whole organ for recreating the endocrine pancreatic niche remains a concrete and clinically relevant strategy for the treatment of T1D (**Table 2**).

INSULIN-PRODUCING CELLS: FINDING AN ALTERNATIVE SOURCE TO DONOR'S ISLETS

The identification of alternative and unlimited insulin-producing cell sources compatible with human implantation might fix donor organ shortage and broaden the clinical application of the treatment to a larger cohort of patients (208). To this aim, several solutions have been investigated developing differentiation protocols to β-cells derived from PSCs, as pluripotent embryonic stem cells (ESCs) or inducing-pluripotent stem cells (iPSCs) or evaluating xenogeneic sources.

PSCs as a Source for β-Cell Replacement in T1D (ESC and IPSC)

ESCs and iPSCs are PSCs that are able to develop all three germinal layers of the embryo and therefore can differentiate

TABLE 2 | Summary of the bioengineering strategies aimed to improve the β-cell replacement.

Bioengineering strategies	Pros	Cons
Vascularizing the transplantation site	Increase the vascularization exploiting foreign body response Release of proangiogenic factors Endocrine cells encapsulation grants the substitution of device upon exhaustion Encapsulation grants also immune-protection	Delay of graft vascularization Passive and disorganized vessels formation Encapsulation hinders the ingrowth vessel formation
Redrawing the endocrine cellular composition	Introduction of other cellular components for achieving biomimetic mechanisms to • Increase vascularization • Grant immune-protection • Increase the viability and/or function of the endocrine cells Making insulin producing components homogenous in size to • Increase their viability and/or function • Facilitate clinical procedures	Delay of graft vascularization Disorganized vessels formation Scarce insights about the real immune-protection
Reshaping the microarchitecture	Introduction of ECM components to provide the endocrine cells with suitable microstructures Evidences on viability and function increasing thanks to ECM proteins Biomimetic cell-cell and cell-ECM interactions *Ex-vivo* pre-vascularization *In vivo* rapid graft vascularization	Batch-to-batch differences Need of standardized protocols *For 3D printing strategies*: lack of fidelity in recapitulating physiological structure and composition

into all cell types and tissues of the body (209). They can be guided to a specific cell fate by exposure to a defined combination of physical, chemical, and biological stimuli that can activate and/or inhibit specific signaling pathways that mimic human development. However, their high pluripotent hallmark represents a double-edged sword, as it is difficult to efficiently control their differentiation towards a specific cell fate. ESCs are isolated from the inner cell mass of blastocysts during embryonal development (210). Despite these advantages, there are some issues concerning their clinical translation, such as ethical concerns regarding their origin and allogeneic features. In 2006, Yamanaka et al. introduced the concept of reprogramming terminally differentiated somatic cells to an induced-PSC, forcing the expression of four key transcription factors, specifically Oct 4, Klf4, Sox2, and C-myc (211). iPSCs show similar features to ESCs, with the same morphology and proliferative rate, similar telomerase activity, normal karyotype, and the same *in vivo* teratogenous potential (ability to give rise to a teratoma, a germ layer tumor containing several types of tissues). Contrary to ESC, they had fewer ethical concerns and no allogeneic-related issues as they could be isolated from the patient itself. These features highlight the great potential of iPSCs for being used in clinical applications.

In the last 15 years, several authors have proposed protocols to reproduce step-by-step human pancreatic development *in vitro* to generate functional β-like cells from both ESCs and iPSCs (212). D'Amour et al. defined the first protocol to produce *in vitro* definitive endoderm from human PSCs, while later Kroon et al. demonstrated that ESC-derived pancreatic progenitors could further differentiate into glucose-responsive insulin-secreting cells after implantation into immune-deficient mice (213,214). Since then, several efforts have been made to understand and define a protocol for generating functional SC-derived β-cells *in vitro* that can secrete insulin in response to glucose stimuli. Pagliuca et al. were the first to report a scalable protocol to generate high numbers of functional SC-derived β-cells from both ESC and iPSCs of non-diabetic patients, with an average efficiency of 33% for β-like cells (215). Rezania et al. demonstrated the *in vivo* reversal of diabetes after the transplantation of SC-derived insulin-secreting cells. Although these insulin-secreting cells are similar to mature β-cells in terms of marker expression and insulin secretion, the differentiation protocol could not obtain cells fully equivalent to mature β-cells (216). In addition, the yield efficiency is still too low for clinical applications. In 2016, Millman et al. reported a scalable differentiation protocol to generate syngeneic β-cells from T1D of patients with iPSCs (217). Some years later, the same group demonstrated that differentiation towards β-like cell fate is guided by small molecules and growth factors and by cell-biomaterial interaction, which changes the cell cytoskeleton configuration and affects cell differentiation (218). Cells sense the microenvironment through integrin proteins that interact with the ECM, altering and/or promoting specific cellular processes. Thus, exploiting biomaterials to mimic ECM features, such as composition, stiffness, and geometry, might further improve differentiation protocols. After publication of these milestone differentiation protocols, several others came out with slight modifications, enhancing the quality of β-like cells generated *in vitro* and obtaining higher percentages of mono hormonal and insulin-expressing β-like cells (219–221). To achieve a successful clinical translation of PSC, there are several important challenges to be faced: 1) the lack of knowledge about the mechanisms that fully control cell differentiation towards all endocrine cell types, and adjustment of the ratio between β and non-β cells in the cluster to mimic the complexity and heterogeneity of human islet function, and 2)

poor efficient strategies to protect PSC from immune rejection. Several clinical trials using ESCs and iPSCs have proposed different strategies to overcome these limitations. Viacyte investigated hESC-pancreatic progenitor cells transplanted within different encapsulation devices, VC-01 and VC-02, used in NCT02239354 and NCT03163511 trials, respectively (92–94). The VC-02 trial showed that hESC-pancreatic progenitor cells were successfully tolerated without teratoma formation. Moreover, they acquire a mature β-cell phenotype, as suggested by the analysis of explanted grafts (93,94,213). Finally, patients had increased fasting C-peptide levels and increased glucose-responsive C-peptide levels (93,94).

Vertex is another company that started a phase 1/2 clinical trial (NCT04786262), where the safety efficacy and tolerability of insulin-producing cells (VX-880) infused through the portal vein were evaluated. Recently, Vertex reported results from the first patient, who had a successful increase in fasting C-peptide and a decrease of exogenous insulin need by 91% over 90 days after implantation with half the target dose (222,223).

Finally, two other products, MailPan® and Seraxis, use insulin-producing cells of different origins embedded in an immune-protective membrane. In conclusion, several efforts have been made to find an alternative source of islets, as indicated by numerous developed products and ongoing clinical trials (224,225).

Xenogeneic Sources

The use of xenogeneic sources is another valid strategy for overcoming donor organ shortage. Previously, xenogeneic insulin from pigs has been adopted for human diabetes treatment for more than 60 years because of the amino acid similarity of porcine insulin to human insulin. The idea of using porcine islets as an alternative source of human islets was also derived from biological evidence. Porcine islets have the ability to respond to glucose stimuli within the same physiological range as human islets. Another advantage is the easy and reproducible isolation procedure. In contrast to human islet isolation, procedures adopted for porcine sources allow the preparation of high-quality porcine islets with good predictability and without being compromised by comorbidities, brain death, and ischemia. Additionally, porcine islets might be potentially used for highly allosensitized patients who present circulating antibodies against human leukocyte antigens (HLA), limiting the donors' pool of compatibility with those patients (226).

Initially, the clinical use of porcine sources encountered some relevant limitations, especially related to the risk of zoonosis and more specifically, to the risk of porcine endogenous retrovirus (PERV) transmission (227). This can be overcome by genetic modification of the donor pigs. In a recent study, Yang et al. demonstrated the production of pigs with genetically inactivated PERVs using a combination of CRISPR-Cas9 and transposon technologies (228). Therefore, the use of animal sources coupled with advanced gene editing and cloning strategies has provided the opportunity to obtain genetically modified endocrine pancreatic sources, which can potentially cancel these concerns and improve their function. In this scenario, the low risk-benefit ratio of exploiting porcine islets as an alternative source to human islets makes them a promising option for the treatment of T1D (226).

Two fundamental aspects need to be considered when choosing the optimal porcine islet source: age and strain. Adult pigs can supply mature and large islets with the potential to efficiently secrete insulin within a few minutes or hours after transplantation, and the number of islets isolated from a sole adult pig might be sufficient for T1D patients (229). However, the disadvantages are principally related to the high costs of pig housing for an extended period before pancreas excision, and considering the need for endocrine sources owing to the wide diffusion of T1D disease, the costs can further increase. Moreover, islets from adult pigs have difficulties in isolation procedures and are fragile during culture (230). In contrast, neonatal islet-like cell clusters (NICCs) and fetal porcine islet-like cell clusters (FICCs) are easy and less expensive to isolate, as they have a relatively low cost of herd housing. In addition, isolation from fetal or neonatal porcine sources ensures procedures with a low contamination risk because of the ease of isolation in pathogen-free facilities. NICCs and FICCs do not present totally differentiated cells; therefore, they are prone to proliferation (231,232). In 1996, Korbutt et al. reported a simple, inexpensive, and reproducible method for isolating a large number of NCCIs (233). NCCIs consist of differentiated endocrine pancreatic cells and precursor cells, which showed *in vitro* and in preclinical studies to have the potential for proliferation and differentiation (232). Although NCCIs implantation in mice required at least 6–8 weeks to correct diabetes (233), when implanted in allogeneic pigs (234) or non-human primates (235), they demonstrated reversal of diabetes symptoms within 2–3 weeks. Therefore, they have the potential to increase endocrine cluster volume and, once matured, functionality after transplantation (236). In addition, they are more resistant to hypoxia, hyperglycemia, and pro-inflammatory cytokines than adult pigs (233,237). Elliot et al. performed a clinical trial of NCCIs transplants (NCT00940173). To date, 14 non-immunosuppressed patients with T1D have been treated with alginate-encapsulated NCCIs to alleviate and avoid the onset of hypoglycemic events (238). Separately from the metabolic improvement, none of the recipients showed signs of porcine viral infection, thus demonstrating the safety of the procedure. Nevertheless, increasing insulin production by NCCIs through genetic modification of genes involved in insulin granule exocytosis may be beneficial for their function. In particular, enhancing the response to glucose- and calcium-dependent depolarization *via* adenoviral transfer-mediated transgenic methods has been shown to increase the insulin stored within the granules and its secretion. This improved islet secretory function *in vitro*, bringing it closer to that of human islets and making them more efficient in controlling host glycemia in both preclinical and clinical trials, without the need to transplant a high number of islets (239,240). Another difference affecting the properties of old and young islets is related to ECM expression. In particular, islets from older pigs are isolated with higher ECM content than those from younger pigs (241). This may be reflected in the function of islets (242). Indeed, as for human islets, porcine islets are positively affected by ECM interaction, promoting islet

cells survival, proliferation and efficient insulin secretion (243). More specifically, ECM proteins have been shown to be involved in modulating the differentiation of immature cells to mature cells (244). ECM content can also change depending on the pig breed. For example, German Landrace pigs have higher ECM protein expression and deposition in islet capsules than Deutsches Edelschwein pigs, facilitating isolation and permitting islets to be healthier for transplantation (245).

To date, the main disadvantages of all porcine endocrine cell clusters are their function-onset delay after transplantation and the high expression of oligosaccharide moieties, which trigger stronger cell and humoral-mediated immune rejections than allogeneic immune responses, rapidly leading to total xenograft rejection (246). Among the oligosaccharide groups, the most abundant are Galα1–3Galβ1–4GlcNAc-R (α-Gal), which is physiologically lower in adult pig islets (246,247) and is synthesized by α-1,3- galactosyltransferase (GGTA1), N-acetylneuraminic acid (Neu5Gc) synthesized by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), and an Sd(a)-like glycan made by β-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2) (248). Genetic engineering methodologies may facilitate the xenogeneic source compatibility with human. To abolish these carbohydrate antigens from porcine islets, pigs with knockout (KO) mutants of GGTA1, CMAH, and B4GALNT2, or a combination of these were generated. GGTA1-KO/CMAH-KO pigs did not show alterations in islet architecture or function. After transplantation of islets from these pigs into CMAH-deficient mice, no antibodies against Neu5Gc were detected (249). In addition, deletion of all three oligosaccharide antigens leads to considerably reduced human antibody binding to pig cells *in vitro* (250). In addition to delete xenogeneic genes, there is also the possibility to induce the expression of human genes, like CD55 and CD59 in α-Gal-deficient pig islets, which led to significantly high compatibility to the innate and adaptive immune system in humans. This strategy efficiently attenuates IBMIR after intraportal transplantation into immunosuppressed non-diabetic baboons *in vivo* (251).

IMMUNOSUPPRESSIVE STRATEGIES

The success of β-cell replacement is hampered by the poor engraftment capability of the graft in the peri-transplant phase and by the immunological reactions against the graft upon implantation. After intrahepatic transplantation, islets are exposed to the following: 1) IBMIR and innate immune reactions in the peri-transplant phase, 2) allogeneic immune recognition, and 3) recurrent autoimmune responses due to pre-existing adaptive immune memory (17). Thus, T1D patients receive life-long immunosuppressive treatment to prevent immune rejection (15,252,253). However, these treatments, although specific for the depletion of CD8 T-cells, are not able to completely target CD4 memory T-cells, accounting for autoimmunity recurrence (254–256). Additionally, chronic administration of immunosuppressive drugs results in severe systemic drawbacks and organ failure. In addition, some immunosuppressive drugs, such as tacrolimus and sirolimus, are toxic to β-cells (15). In this scenario, bioengineering approaches are not exclusively aimed at reshaping the endocrine pancreatic niche, but also at designing innovative strategies to overcome the immunological bottleneck.

Owing to the limitations of immunosuppressant strategies, the possibility of reconstituting the endocrine pancreatic niche by assembling the building blocks—insulin-producing cells, vasculature structure, and ECM-based microarchitecture—might be prone to the introduction of components able to locally immune-preserve the graft or modulate the host immune response, granting a long-term function (15). Bioengineering of an immune-protected vascularized endocrine device is challenging. It should not hamper the generation of vascular connections with the host, while it should promote both endocrine and vascular viability and function. This can be achieved by hiding the graft through encapsulation strategies or release of anti-inflammatory molecules, or by introducing components physiologically involved in immune-regulating mechanisms, making immune-stealth the endocrine pancreas device. Several strategies have been exploited for this purpose including the use of semipermeable membranes to physically immune-isolate the graft, chemical modification of the scaffold with anti-inflammatory or immune-modulating molecules, and the use of gene-edited cells expressing immune-modulating proteins.

Immuno-Hiding the Endocrine Pancreatic Graft

The dimension of insulin-producing cells allows their encapsulation within biomaterial-based structures, which is useful for masking immunogenic antigens on cell surfaces and avoiding direct recognition by the host immune system (257–259). Currently, encapsulation with semi-permeable polymeric membranes is clinically investigated with the aim of hiding the bioengineered endocrine pancreas and blocking host immune cell infiltration and immunoglobulin and cytokine penetration, as well as allowing the diffusion of glucose, oxygen, and hormones (15,92–94). PTFE (92–94,225,260), alginate (122,261–265), agarose (266–268) and polyethylene glycol (PEG) (259,269–271) polymers have been used because of their anti-fouling and immune-inert features, ease of manipulation, and inclination towards chemical modifications. Various polymers and geometric configurations provided protection to islets in rodent models but failed to show benefits in large-scale animal models and clinical trials, especially because of FBR, which hampered the vascularization of the endocrine graft (122,264,272–274). Carefully tailoring the physicochemical and biological features of biomaterial-based encapsulation devices might reduce FBR mechanisms. The pore size plays an important role and therefore needs to be finely designed by adjusting the polymer molecular weight, concentration, composition, crosslinking degree, and porogen properties (273,275,276). Gradual degradation of the encapsulated biomaterials might provide a minor host

immune response, allowing a more biomimetic graft integration process (277–279). Hence, innate immune cells and antigen presenting cells (APC) may switch towards a more tolerogenic phenotype, reducing the activation of adaptive immune response and potentially positively affecting long-term endocrine graft viability and function (280,281). Therefore, scalable engineering projects, comprehensive screening of FBR-inducing materials in preclinical models, and careful transplant site selection are required to strengthen translational effectiveness (122,272).

As of now, alternative bioengineered platforms aimed at immuno-hiding insulin-producing cells after implantation are designed either to integrate immune-instructive materials or to introduce immune-modulating cells or to deliver immune-modulating compounds for interfering with the locally inflamed microenvironment, reducing immunosuppressant side effects (282,283). Immunosuppressive molecules, such as mTOR and calcineurin inhibitors or mycophenolate mofetil (MMF), which are routinely systemically administered after clinical islet transplantation, can be locally delivered by the bioengineered devices for immuno-modulating the host response, decreasing their side effects (284–286). For instance, alginate-based beads modified with a "clickable" chemical group, complementary to another chemical moiety attached to rapamycin, have been implanted in the subcutaneous space of immunocompromised mice. Once consumed, it is refilled with complementary rapamycin, potentially providing continuous local immunosuppressive activity (284). However, some of these immunosuppressive drugs, such as fingolimod, which did not show adverse effects in preclinical studies upon systemic administration, may exhibit toxicity towards β-cells when locally delivered (287). Immune-modulating chemokines can be used for this purpose. CXCL12 linked to alginate scaffold-encapsulating islets has been shown to impair host T cell effector populations, granting graft long-term viability and function (288,289).

Making Immune-Stealth the Endocrine Pancreatic Graft

Recently, the continuous understanding of immunological processes, such as immune tolerance, has opened the way for their potential exploitation in suppressing the host response after organ transplantation or triggering the host immune system against the tumor mass in cancer treatment (290,291). Immune tolerance involves a range of active processes that modulate or prevent potentially harmful immune responses and differ from immune ignorance, in which the immune system does not notice or recognize danger signals (292). Immune tolerance can be divided into two main categories: central and peripheral, with multiple layers of active regulation (293). Central tolerance refers to the mechanism by which immature T-cells are educated in the thymus. This selection induces apoptosis of T-cells with either too low affinity for HLA or too high reactivity to self-proteins expressed in the thymus. Finally, the selected T-cells can recognize peptides presented by HLA but do not respond to self-peptides (294). However, these central tolerance mechanisms are not impeccable, and self-reactive T-cells against islet autoantigens are frequently found in the circulation of healthy individuals, even if they do not manifest autoimmune disease (295). Therefore, the difference between healthy individuals and patients with autoimmune diseases must be researched in the role of these types of cells in peripheral tissues as well as in the efficacy of peripheral tolerance-regulating mechanisms (295). On the other hand, peripheral tolerance occurs in mature CD4 T and B cells, which are normally inhibited upon recognition of self-antigens (296). In addition, depending on the density of the antigen in peripheral tissues, immune cells may not respond to immunomodulatory co-stimulation, resulting in their inactivation (297). The players involved in peripheral tolerance induction are immune-modulating molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death (PD)-1, PD-ligand 1 (PD-L1), and Fas-ligand (FasL), which can decrease immune cell activation and correct the immune response.

Within the context of bioengineered systems, the use of immune tolerance induction could be capitalized on making devices with immune-stealth properties. The combination of endocrine pancreatic cells with cells expressing these proteins, such as MSCs or hAEC, which are physiologically involved in immunomodulation, has been exploited in previous studies, suggesting that they can potentially prevent graft rejection (140–142,298). Additionally, the identification of these specific proteins has opened the way for a combinatorial approach by modifying the bioengineered platform to generate an immune stealth device. For instance, PD-L1 directly linked to the islet surface increased graft survival in 90% of recipients, while, when it was linked to alginate, only 58% of recipients showed long-term graft function (299,300). Despite these results, exploiting this strategy in combination with material-based devices might facilitate its clinical translation. FasL is a molecule that causes T-cell apoptosis when linked to the cell surface or ECM, while its soluble form is anti-apoptotic. FasL materials have been fabricated and have demonstrated a positive impact on long-term graft function (301,302).

CONCLUSION

Endocrine cells are structures enclosed by a BM-ECM layer that separates them from exocrine tissue and is fed by independent vasculature formed by a dense network of capillaries. The specific organization of the three building blocks, including the vasculature, ECM-based architecture, and insulin-producing cells, is essential for the physiological function of the endocrine pancreatic niche. In recent years, understanding their importance has become crucial to ameliorate β-cell replacement strategy outcomes, especially in improving the engraftment efficiency of insulin-producing cells. Bioengineering the transplantation sites using inert biocompatible materials to increase vascularization and shorten the hypoxic phase has been the most investigated approach in current clinical trials. However, these studies were principally focused on immune-preserving endocrine grafts and secondarily on increasing vascularization. In fact, semi-

permeable membranes have been shown to hinder host immune system penetration and impede the migration of vascular cells, delaying the re-establishment of functional vascularization. The consequent loss of the graft highlights the necessity to develop strategies to trigger prompt graft vascularization rather than to grant graft immune protection, at least during the first phase of implantation. The use of more biomimetic approaches, such as introducing proangiogenic molecules or cells, redrawing the endocrine cellular composition with the addition of accessory cells, has been shown to ameliorate the rate of vascularization and consequently the treatment outcomes in preclinical studies; however, they did not fully reproduce the endocrine pancreatic native niche complexity. The missing part in those studies was the consideration of the endocrine pancreatic micro-architectural features because they allow the structural and functional integration between the vasculature and the endocrine components, as demonstrated by evidence from the positive results with the dECM organ used for bioengineering the vascularized endocrine pancreas. The preserved vessel structures of native organs allowed vasculature *in vitro* reconstruction. Additionally, the ECM-based microarchitecture, along with its specific composition, promotes full intertwining between the endocrine system and vasculature, ensuring rapid engraftment and function onset *in vivo*. In this scenario, the method for bioengineering a vascularized endocrine pancreas is paved as it should integrate insulin-producing cells, pro-vascularizing elements, and ECM-based scaffolds mimicking the endocrine pancreatic native niche. The use of 3D-bioprinting technologies might help to condense the building blocks in a fine-tuned bioengineered vascularized endocrine platform, exploiting its ability to finely fabricate a scalable microstructure encapsulating different cell types simultaneously. However, nowadays, the use of dECM organs is more ready for a possible clinical translation, as demonstrated by other dECM-based devices already used in clinical practice.

Bioengineering a vascularized endocrine device may also take advantage of alternative sources to human islets, overcoming donor organ shortages. PSC and xenogeneic source are valid alternative that can be easily integrated in bioengineered devices. Although PSC have been used in clinical studies, differentiation protocols are still not completely optimized. On the other hand, xenogeneic sources are endocrine cellular elements naturally assembled and prone to accomplish endocrine function, and additionally, they are easy to isolate. Concerns are related to their human immune-compatibility, which are easily surmountable with *ad-hoc* gene-editing strategies, as recently reported by gene-edited xenogeneic kidney and heart transplantation in human. Finally, bioengineered vascularized endocrine pancreas platforms are suitable for integrating novel immune-preserving strategies to ensure local immune modulation. Delivering immune-modulating molecules from the device or introducing immune-modulating cells are feasible strategies owing to the flexibility of tissue engineering fabrication methodologies.

AUTHOR CONTRIBUTIONS

CP and AC conceived the study. CP, FC, AN, LP, and AC wrote the manuscript. All the authors contributed to the review and approved the submitted manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Bio-Engineering of Pre-Vascularized Islet Organoids for the Treatment of Type 1 Diabetes

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Lack of rapid revascularization and inflammatory attacks at the site of transplantation contribute to impaired islet engraftment and suboptimal metabolic control after clinical islet transplantation. In order to overcome these limitations and enhance engraftment and revascularization, we have generated and transplanted pre-vascularized insulin-secreting organoids composed of rat islet cells, human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs). Our study demonstrates that pre-vascularized islet organoids exhibit enhanced *in vitro* function compared to native islets, and, most importantly, better engraftment and improved vascularization *in vivo* in a murine model. This is mainly due to cross-talk between hAECs, HUVECs and islet cells, mediated by the upregulation of genes promoting angiogenesis (*vegf-a*) and β cell function (*glp-1r*, *pdx1*). The possibility of adding a selected source of endothelial cells for the neo-vascularization of insulin-scereting grafts may also allow implementation of β cell replacement therapies in more favourable transplantation sites than the liver.

Keywords: regenerative medicine, tissue engineering, β cell replacement therapies, prevascularized iset organoids, human amniotic epithelial cells, HUVECs

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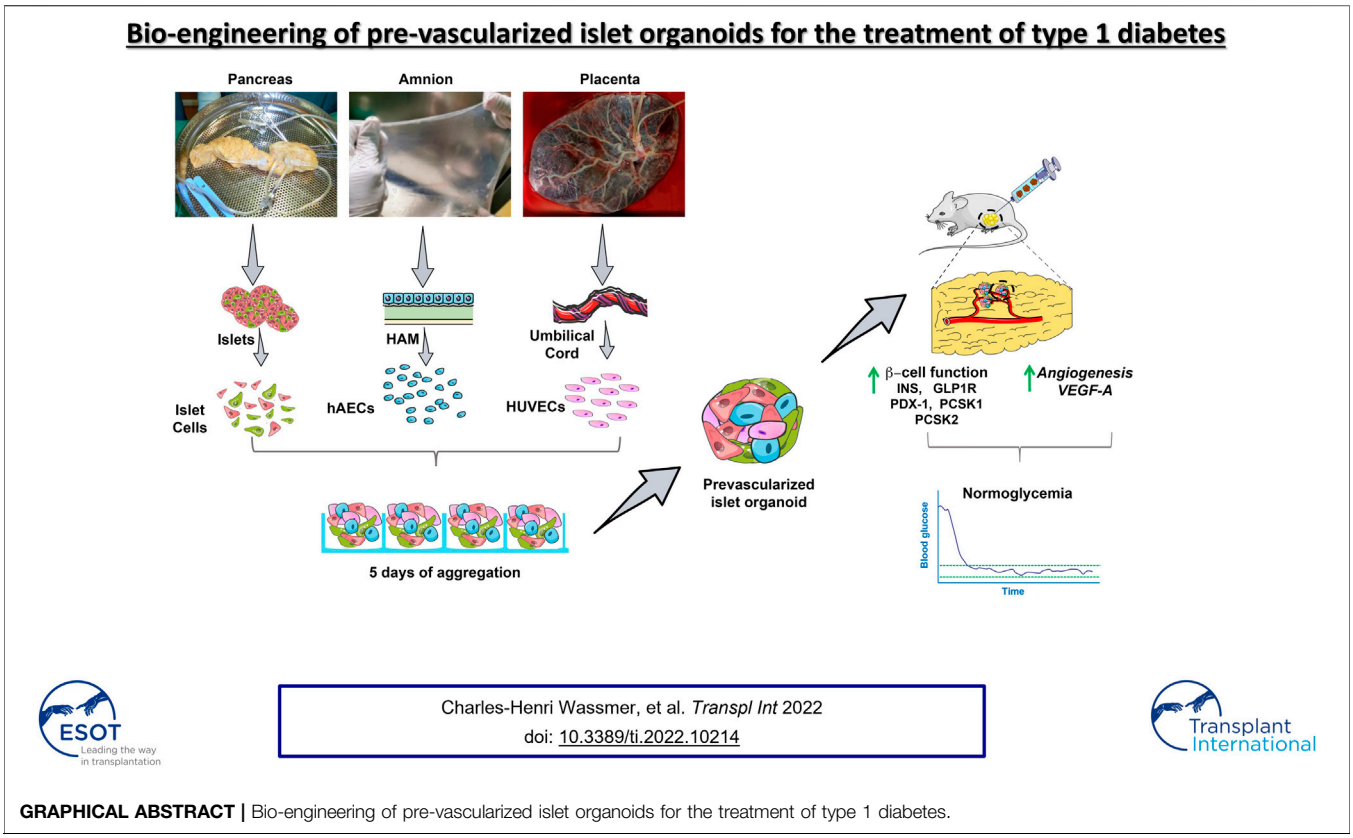
INTRODUCTION

Allogenic transplantation of pancreatic islets is a cell therapy option that holds great promise in the treatment of type 1 diabetes. The development of the Edmonton protocol has drastically increased the success rate of islet transplantation, and has proven to be able to achieve insulin independence in patients with type 1 diabetes (1). Most importantly, pancreatic islet transplantation confers a significant improvement in glycemic control and prevents life-threatening severe hypoglycaemia (2). Despite its efficacy, clinical islet transplantation is facing a number of challenges that limit achievement of steady functional success comparable to whole organ transplantation (3). One of the major challenges is the suboptimal long term graft function caused by the loss of the large portion of intraportally transplanted islets due to the IBMIR reaction, pro-inflammatory microenvironment, low oxygen tension in the liver, impaired vascularization and immunosuppressive drug toxicity (3). Therefore, the search for a suitable alternative transplantation site is a major focus of research in the



GRAPHICAL ABSTRACT | Bio-engineering of pre-vascularized islet organoids for the treatment of type 1 diabetes.

field. Other limiting factors hampering the widespread application of islet transplantation are shortage of donor organs and need for lifelong immunosuppression (4). Xenogenic islets and stem cell-derived beta cells are the two major potentially unlimited sources of insulin-producing tissue (5).

In recent years, substantial progress has been made in generating and characterizing functional stem cell-derived beta cells, which will undoubtedly change the way we will treat type 1 diabetes (6). The first attempts of clinical application of microencapsulated porcine islets or stem cell-derived endocrine tissue incorporated into macrodevices have already taken place (7, 8) and re-enforce the need to identify a site as functional as portal vein infusion but allowing easy graft removal—a site that to date this remains clinically elusive.

Despite the fact that islets represent only 1–2% of pancreatic tissue volume, they receive 10–15% of the total pancreatic blood flow (9). Each islet possesses 1 to 3 pre-arterioles (10), depending on islet size, that rapidly branch out into a multitude of fenestrated capillaries and form an important intra-islet micro-circulation that is five time denser than in the exocrine tissue (11). The cross-talk between endocrine and endothelial cells is vital for proper islet development, configuration and vascularization. Islet cells secrete vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1 in order to recruit endothelial cells (ECs) that are necessary for islet development, survival and function. On the other hand, ECs are involved in cell differentiation, insulin gene expression and cell segregation during embryogenesis (12, 13). In addition, they secrete components of the intra-islet basement membrane that are crucial for proper endocrine function (11).

Islet isolation and culture lead to the disruption of the islet capillary system, with significant loss of ECs due to de-differentiation or necrosis (14). In addition, islets vary in size, ranging from 50 to 400 µm in diameter. In the immediate post-transplantation period, avascular islets are supplied with oxygen and nutrients solely by diffusion until re-establishment of the blood flow, a process that can take about 2 weeks (9). Because of that, larger islets fail to engraft due to insufficient vascularization and subsequent necrosis (15). Significant efforts have been made to develop new strategies to minimize hypoxia-induced β cell death.

Several scientific groups, including our own, have demonstrated that re-aggregation of islet cells in combination with other cell types into homogeneous, round shaped and size-controlled spheroids leads to improvement of function and viability, thanks to heterotypic cell–cell interactions and reproduction of the complex natural morphology of the islet (16–20). In our previous studies, we have shown that incorporation of human amniotic epithelial cells (hAECs) into insulin-secreting organoids protected islet cells from oxidative stress *in vitro*, subsequently improving ß cell viability, function and engraftment (17, 20). Here, we propose an improved approach, in which we engineer pre-vascularized organoids that provide both control over their size and composition, and prompt re-establishment of the cross-talk between ECs and islet

cells, thereby facilitating graft revascularization after transplantation.

MATERIALS AND METHODS

Reagents and Antibodies

All reagents and antibodies used in this study are listed in **Supplementary Tables S1–S3**.

Animals

Animal experiments were performed in accordance with the Geneva veterinary authorities and approved by the Institutional Animal Care and Use Committee of the University of Geneva. Ten-week-old, pregnant female, Lewis rats were purchased from Janvier Laboratory (Le Genest St-Isle, France) and bred in our animal facility at the Geneva University. Fifteen-to 21-week-old male rats were used for pancreatic islet isolation. Six-to 9-week old male B6.129S7-Rag1^{tm1Mom}/J (abbreviated NOD–*Rag1*^{null} bred at Charles River Laboratories, Saint-Germain-Nuelles, France) mice were used as transplantation recipients. All animals were kept under conventional housing conditions with free access to water and food.

Human Tissues

Studies involving human tissues were approved by the Commission Cantonale d'Ethique de la Recherche (CCER; protocol PB_2017-00101), in compliance with the Swiss Human Research Act (810.30).

Placentas were obtained from women undergoing elective caesarean section of uncomplicated, term pregnancies. Informed, written consent was obtained from each donor prior to tissue collection.

Isolation and Culture of Human Umbilical Vein Endothelial Cells and Human Amniotic Epithelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated using a method adapted from a previously published protocol (21). Briefly, the umbilical vein was rinsed, then distended with Collagenase A solution (2 mg/ml) and incubated at 37°C for 12 min. Released cells were then collected by flushing the vein with cold HBSS supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. Isolated HUVECs were plated in a 75 cm^2 flasks and cultured at 37°C, 21% O_2 and 5% CO_2 in M199 medium supplemented with 20% FBS, 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution), Fungin 0.1%, 30 µg/ml endothelial cell growth supplement and 100 µg/ml heparin. HUVECs from passage 2 to 7 were used in this study.

hAECs were isolated, cultured and characterized as described previously (10, 14). Freshly isolated hAECs were cultured in DMEM/F-12 medium, supplemented with 10% FBS, 2 mmol/l L-Glutamin, 100 U/ml Penicillin, and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution, 1 mmol/L sodium pyruvate, 1% MEM NEAA 100X, 0.1% fungin, 0.05 mmol/L 2-mercaptoethanol, 10 ng/ml human recombinant epidermal growth factor (EGF). Only cells at passage 1 were used in this study.

Medium was changed every 48 h. Confluent cells were recovered by mild trypsinization and were cryopreserved for later utilization.

Rat Islet Isolation and Dissociation

Rat islets were isolated by enzymatic digestion (collagenase V) and purified using a discontinuous Ficoll gradient (22–24). Isolated islets were cultured (37°C, 5% CO_2) in DMEM medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, 0.1 mg/ml 1 mmol/L sodium pyruvate and 11 mmol/L glucose for 24 h. Islets were then dispersed into single islet cells (ICs) by incubation in 0.05% trypsin-EDTA (16).

Characterization of Human Umbilical Vein Endothelial Cells and Human Amniotic Epithelial Cells

HUVECs and hAECs were analyzed for expression of previously reported endothelial cell surface markers or specific amniotic epithelial cell surface markers by flow cytometry.

For analysis, cells (2.5×10^5) were stained by incubation for 30 min with primary or isotype control antibody in 100 µl PBS with 0.2% BSA, washed twice with PBS, and analyzed. Antibodies used for HUVECs were: AlexaFluor 657-conjugated anti-CD144 (1:40 dilution), PE-conjugated anti-CD31 and PerCP-Cy 5.5-conjugated anti-CD45 (1:25 dilution). Antibodies used for hAECs were: FITC-conjugated anti-human CD105 (clone 266), BV421-conjugated anti-human CD326 (clone EBA-1), PerCP-Cy5.5 conjugated anti-SSEA4 (clone MC813-70) (1:50 dilution), PE-Cy7 conjugated anti-human CD90 (clone 5E10; 1:100 dilution), PE-conjugated anti-human HLA-E (clone 3D12) and APC-conjugated anti-human HLA-G (clone 87G; 1:20 dilution).

Flow cytometry analysis was performed on a Gallios cytometer using the Kaluza Analysis software.

HUVECs were further characterized by immunostaining. Immunofluorescent assessment was performed on the cells cultured on gelatine-coated glass coverslips. Fixed cells were washed, permeabilized and stained with the following primary antibodies: mouse anti-CD31 (1:50 dilution), rabbit anti-von Willebrand factor (1:100 dilution) and mouse anti-vimentin (1:50 dilution). Cells were then incubated with corresponding Alexa Fluor and FITC-conjugated secondary antibodies. For nuclear counterstaining samples were mounted with aqueous solution containing 4,6 diamidino-2-phenylindole (DAPI).

Functional Assessment of Human Umbilical Vein Endothelial Cells *In Vitro*: Tube Formation Assay

The tube formation assay was performed according to manufacturer's protocols of Corning® Matrigel® Matrix.

Briefly, Matrigel thawed overnight at 4°C was mixed with VEGF (200 ng/ml) and 250 µl of matrix was added to each well osf 24-well plates. After 1 h of incubation at 37°C, cells (8×10^4) were seeded onto the Matrigel and tube formation of HUVECs was observed and photographed using an inverted phase-contrast microscope during 6 h.

Lentiviral Transduction

Lentiviral vector carrying the green fluorescent protein (GFP) under the control of an endothelial specific promoter Vascular endothelial cadherin (VEC/Cdh5) (LV-VEC.GFP) was provided by Prof. A. Follenzi (Università del Piemonte Orientale). HUVECs were transduced with LV-VEC.GFP at passage 3 using a multiplicity of infection (MOI) of 10 (MOI = 10). Transduction efficiency was assessed by fluorescent microscopy and flow cytometry and considered successful when at least 80% of cells showed expression of GFP.

Generation of Pre-Vascularized Islet Organoids

Pre-vascularized islet organoids (PIO) were generated on AggreWell™400 24-well plates by seeding mixture of ICs, HUVECs and hAECs at a ratio of 5:4:1 (800 cells/organoid). Undissociated native islets (NI), ICs spheroids (400 ICs/spheroid), hereafter referred to as pseudo-islets (PI), and IC: HUVEC spheroids (ratio 1:1, 800 cells/spheroid), hereafter referred to as IC + HUVEC served as controls. PIO, PI and IC + HUVEC were cultured for 4 days to allow cell aggregation at 37°C, 21% O_2 and 5% CO_2.

Culture medium for PIO was prepared by mixing equal volumes of complete DMEM, DMEM/F12 and M199 medium, hereafter referred to as organoid medium. IC + HUVEC were cultured in the mixture of complete DMEM and M199 medium at the ratio 1:1. Finally, PI and NI were cultured in complete DMEM medium. Culture medium was changed every other day. Mean diameter of NI, PIO and PI were calculated on the images taken on light microscope using ImageJ software.

In order to observe PIO composition and cell distribution during culture, fluorescent carbocyanine dyes CM-DiL (red) prelabeled hAECs and GFP transduced HUVECs were used. Pictures were taken using an epifluorescent microscope (DMi8 manual microscope).

PIO, PI and NI were collected fixed in formalin and embedded in paraffin. Serial sections of 5 µm were cut and processed for immunofluorescent staining. Slides were stained with the following primary antibodies: guinea pig anti-insulin (1:100), chicken anti-GFP (1:500), and rabbit anti-CK-7 (1:100). The following secondary antibodies were then applied: donkey anti–guinea pig Alexa 555 Fluor-conjugated (1:300), donkey anti–guinea pig FITC-conjugated (1:200), donkey anti-mouse AMCA-conjugated (1:50), goat anti-chicken Alexa Fluor 488 (1:500).

Organoids Sprouting Assay

One hundred PIO were resuspended in a collagen solution, transferred into prewarmed 24-well plates and allowed to gelify for 30 min. Next, 0.1 ml organoid medium supplemented with VEGF-A at the concentration of 200 ng/ml was pipetted on top of each hydrogel containing PIO. The hydrogels were cultured for 24 h at 37°C, 5% CO_2, and 100% humidity. As control, one hundred IC + HUVEC spheroids and PI were cultured in the same way in the hydrogel.

In Vitro Functional Assessment

To assess functional capacity, 300 NI and an equivalent number of PIO and PI, were incubated in duplicates for 1 h at 37°C in Krebs–Ringer solution containing low glucose (2.8 mmol/L) in order to equilibrate the samples. After a change of medium, islets and aggregates were incubated at 37°C for another hour in Krebs–Ringer solution containing low glucose (2.8 mmol/L), followed by 1 h at high glucose (16.7 mmol/L). Supernatants were collected and stored at −20°C. Insulin concentration in supernatants was measured using a rat insulin ELISA kit and normalized to the total insulin content. Results are expressed as the ratio between insulin secreted in high glucose to low glucose, referred to as stimulation index (SI). In addition, total insulin content per IC was measured by dividing the total insulin content by the number of ICs present in the NI, PI and PIO.

Diabetes Induction and Xenogeneic Transplantation

Three days before transplantation mice were subjected to intraperitoneal injection of STZ (180 mg/kg). Non-fasting blood glucose levels were then checked daily using a portable glucometer. Only mice with blood glucose levels over 18 mmol/L for 3 consecutive days were used in this study. Glycemia readings over 28 mmol/L, indicated as "high" on glucometer, were recorded as 30 mmol/L.

A marginal mass of 300 islet equivalents (IEQ) for NI and 1200 PIO, PI and IC + HUVEC were transplanted. Number of organoids was based on the average number of islet cells per IEQ, previously estimated as 1,560 ICs/IEQ (25).

At the day of transplantation, NI and engineered constructs were recovered from culture, packed in PE50 tubing and transplanted into the epididymal fat pad (EFP) of diabetic mice. Non-fasting glucose was assessed daily during the first week and 3 times per week thereafter. Normoglycemia was defined as two consecutive blood glucose levels under 11.1 mmol/L.

Graft Metabolic Function Assessment

Graft capacity to clear glucose *in vivo* was assessed dynamically by intraperitoneal glucose tolerance test (IPGTT) at 30 days after transplantation. Mice were fasted for 6 h and intraperitoneally injected with 2 g of glucose/kg. Blood glucose measurements were taken at 0, 15, 30, 45, 60 and 120 min.

Lectin Injection

Functional graft vasculature was assessed by infusing DyLight 594-conjugated Lycopersicon Esculentum (Tomato) lectin into the beating left ventricle of mice hearts. Mice were injected with 100 µl of undiluted lectin. Lectin was allowed to circulate for 1 min. Then, the right ventricle was cut to allow blood flow decompression and a volume of 3 ml of PBS was injected into the

left ventricle, followed by 1 ml of 4% PFA. The graft bearing EFPs were collected and fixed overnight in 4% PFA at 4°C. They were then maintained in 30% sucrose at 4°C until used for histology.

Immunohistological Assessment of Recovered Grafts

Grafts were recovered, fixed in formalin and embedded in paraffin. Serial sections of 5 µm were cut and processed for immunofluorescent staining. Tissue samples were permeabilized with 0.5% Triton X-100/PBS for 30 min, followed by 1-h incubation in 0.5% BSA/PBS at room temperature to block unspecific sites. Slides were then incubated with the following primary antibodies: guinea pig anti-insulin (1:100), rabbit anti-CD34 (1:2,000), chicken anti-GFP (1:500), and rabbit anti-VEGF (1:100). The following secondary antibodies were then applied: donkey anti–guinea pig Alexa 555 Fluor-conjugated (1:300), donkey anti–guinea pig FITC-conjugated (1:200), donkey anti-rabbit Alexa 555 Fluor-conjugated (1:300) and goat anti-chicken Alexa Fluor 488 (1:500). Both primary and secondary antibodies were diluted in PBS-0.5% BSA. Finally, slides were mounted with aqueous mounting medium containing DAPI for nuclear staining. Slides were processed on a Zeiss Axioscan.Z1 slide scanner and a Zeiss Axiocam. To analyse vascularization, six pictures per condition were taken and the number of $CD34^+$ cells were counted and normalized by the graft area.

Morphometric analysis was performed using Zen 2.3 Blue Edition software.

Real-Time Quantitative PCR

Graft bearing EFPs recovered at 3 and 30 days after transplantation were processed for PCR analysis. RNA was extracted using the RNeasy minikit and reverse transcribed with a High Capacity cDNA Reverse transcription kit. Gene amplification was performed by RT-PCR using TaqMan Fast Advance Master Mix. Primers used for amplification are listed in **Supplementary Table S4**. *RPLP1* was used as a housekeeping gene to normalize gene expression values. Data were calculated using the comparative cycle threshold Ct method ($2^{-\Delta Ct}$ method) and are expressed in arbitrary units.

Statistical Analysis

Continuous variables are expressed as mean ± SEM. Multiple comparisons were analyzed using one-way ANOVA followed by Dunnett multiple comparisons test while two-way comparisons were analyzed using the Student's t-test. Cumulative number of animals reaching normoglycemia was compared using the log-rank (Mantel-Cox) test. A p value ≤0.05 was considered statistically significant. All statistical analyses were performed with the Prism software 8.0.

RESULTS

Human Umbilical Vein Endothelial Cell Characterization and Transduction

HUVECs reached 80% confluence within 5 days with initial seeding density of 6,000 cells/cm^2. Morphologically, cells displayed typical elliptic shape (**Figure 1A**) and were positive for von Willebrand factor and CD31 (**Figure 1B**). Endothelial origin of the cells was additionally confirmed by flow cytometry. Cells were positive for CD31 and CD144 (97.8% ± 0.7 and 98.1% ± 0.6, respectively) and negative for CD45 (95.8%) (**Figure 1C**).

When cultured on Matrigel, HUVECs formed well-shaped vascular-like structures over a period of 6 h (**Figure 1D**).

To track HUVECs within organoids both *in vitro* and *in vivo*, cells were transduced with LVs carrying green fluorescent protein (GFP) gene under the control of the VEC promotor. HUVEC positivity for GFP was observed during culture and confirmed by flow cytometry 3 days after transduction with 86.6% of GFP+ cells (**Figure 1E** right and left panel, respectively).

Human Amniotic Epithelial Cells Characterization

hAECs used in this study were isolated from six different placentas. Flow cytometry analysis demonstrated strong positivity of hAECs for the embryonic cell surface marker SSEA-4 (88.4 ± 5.0%) and the epithelial cell adhesion molecule (CD326; 95.9 ± 1.3%). HLA-E and HLA-G were expressed in 16.9 ± 4.7% and 48.6 ± 12.3% of the cells, respectively. Finally, expression of CD105 and CD90 by hAECs were 17.6 ± 5.6%, 50.1 ± 7.1, respectively. The results of each hAEC preparation are described in **Supplementary Figure S1**.

Cellular Composition, Endocrine Function and Angiogenic Activity of Pre-Vascularized Islet Organoids

Generation of PIO and PI is described in **Figure 2A**. Aggregation and incorporation of the different cell types occurred within 4 days (**Figures 2B,C**). Mean diameter of NI, PI and PIO was 144.4 ± 6.6, 105.8 ± 1.2 and 134.3 ± 2.3 µm, respectively (**Figure 2D**). NI showed the biggest heterogeneity in size. PI exhibited a significantly smaller mean diameter in comparison with PIO ($p < 0.0001$), due to fewer cellular content. Cellular composition observed by fluorescent microscopy showed that all 3 cell types were present in the PIO (**Figure 2E**). The functional capacity of the constructs was evaluated by glucose-stimulated insulin secretion (GSIS) assay. PI and PIO demonstrated significantly improved insulin secretion in response of glucose stimulation (SI = 7.8 ± 1.5 and 7.7 ± 1.2), compared to NI (SI = 2.0 ± 0.5, $p = 0.013$ and $p = 0.014$, respectively). No significant difference was observed between PI and PIO (**Figure 2F**). In addition, total insulin content/IC was measured and compared between the three groups. PI and PIO demonstrated an increased insulin content/IC (0.01 ± 0.003 and 0.008 ± 0.002 pmol/L, respectively) in comparison with NI (0.002 ± 0.0004 pmol/L). These dramatic enhancement of static GSIS secretion in our constructs compared to unmodified native islets indicate that better oxygen and nutrient access, and improved transport of glucose and insulin, enhanced survival and function of PI and PIO. Our findings are consistent with previous reports on better *in vitro* performance of smaller pseudoislets (26, 27).

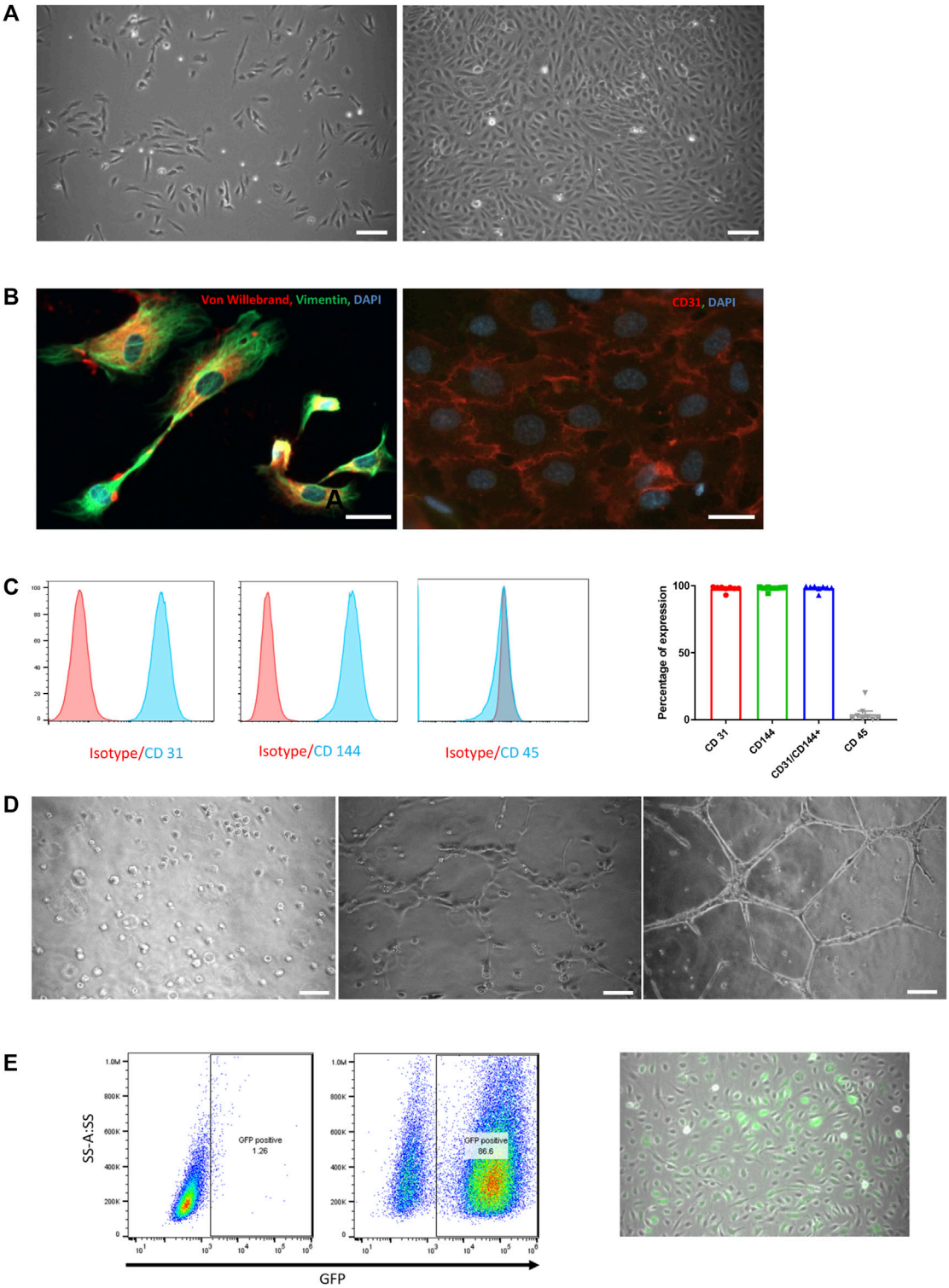


FIGURE 1 | HUVEC characterization and *in vitro* functional assessment. **(A)** Phase-contrast microscopic pictures of HUVEC in culture at day 1 and day 5. Scale bar = 50 µm. **(B)** Immunofluorescence staining of cultured HUVEC with von Willebrand (red) and Vimentin (green, left panel) and CD31 (red, **right panel**). Nuclei are labelled with DAPI (blue). Scale bar = 25 µm. **(C)** FACS analysis on HUVEC for CD31, CD144 and CD45 with their respective isotypes **(left panels)** and expressed as the percentage of positivity of expression on 8 consecutive preparations (mean ± SEM, **right panel**). **(D)** Phase-contrast microscopic pictures of tube formation assessment on Matrigel at 0 h, 2 and 6 h. Scale bar = 50 µm. **(E)** Assessment of GFP transduction success by flow cytometry analysis **(left panel)** and by phase-contrast microscopic images (right panel). GFP-positive cells are spontaneously green, scale bar = 50 µm.

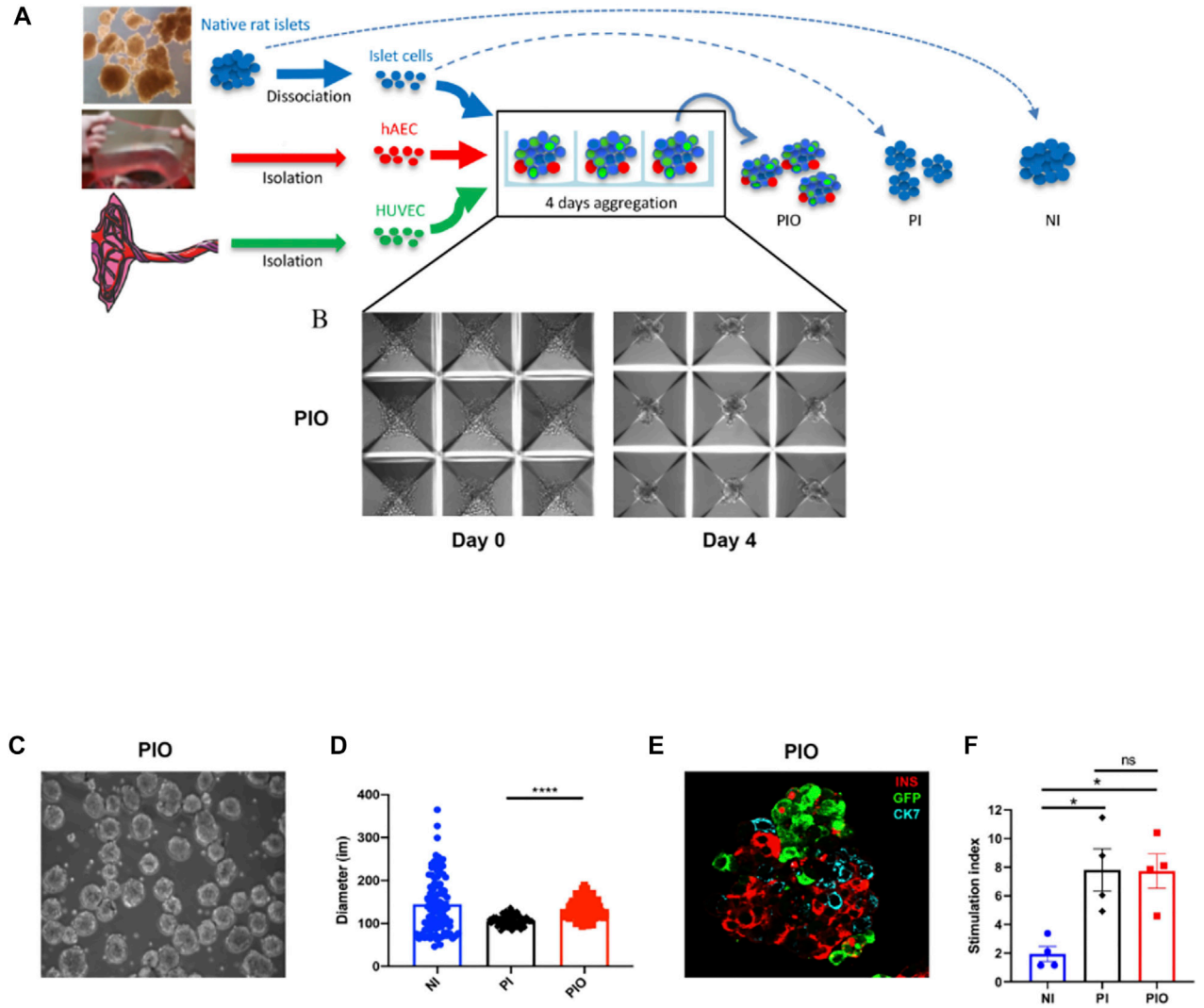


FIGURE 2 | Organoids generation. **(A)** Schematic representation of PI and PIO generation in culture. **(B)** Light microscope pictures of the PIO cultured in AggreWell™400 24-well plates at day 0 and day 4. Scale bar = 100 µm. **(C)** Light microscope pictures of the PIO after collection from the wells. **(D)** Average diameter of each condition calculated at 4 days of culture (n = 100/condition). **(E)** Representative immunofluorescence stainings of PIO. Islet cells are stained for insulin (red), HUVECs for GFP (green) and hAECs for CK7 (blue). Scale bar = 25 µm. **(F)** *In vitro* function assessed by GSIS and represented by the stimulation index (n = 4). All data are expressed as mean ± SEM. *p < 0.01, ***p < 0.001, one-way ANOVA with Dunnett's multiple comparison test.

To investigate the angiogenic potential of the PIO, collagen-based sprouting assays were performed. Our results demonstrated that PIO showed more extensive sprouting in surrounding matrix compared to IC + HUVEC (**Supplementary Figure S2**). In contrast, no sprouting was observed from PI (data not shown). Furthermore, immunofluorescence revealed GFP positive cells confirming their endothelial nature.

Pre-Vascularized Islet Organoids Improve Glycaemic Control in Immunodeficient Diabetic Mice

To assess whether incorporation of hAECs and HUVECs into the islet organoids could promote engraftment and function *in vivo*, diabetic NOD–*Rag1*^{null} mice were transplanted with a marginal mass of PIO (n = 14), NI (n = 13) and PI (n = 9). Mice transplanted with PIO demonstrated significant improvement of glycaemic control compared to both controls. Average blood glucose levels were significantly lower in the PIO group compared to NI and PI (**Figure 3A**). Normoglycemia was reached in 78.6% of animals (11/14) in the PIO group, in comparison with 55.6% (5/9) and 46.2% (6/13) for the PI and NI groups, respectively (**Figure 3B**). Median time to achieve normoglycemia was 6 days in the PIO group, 21 days in the PI group and >30 days in the NI group. To investigate secretory function of the graft, IPGTT was performed at 30 days post-transplantation. Mice transplanted with PIO and non-diabetic controls (NDC) showed lower blood glucose levels

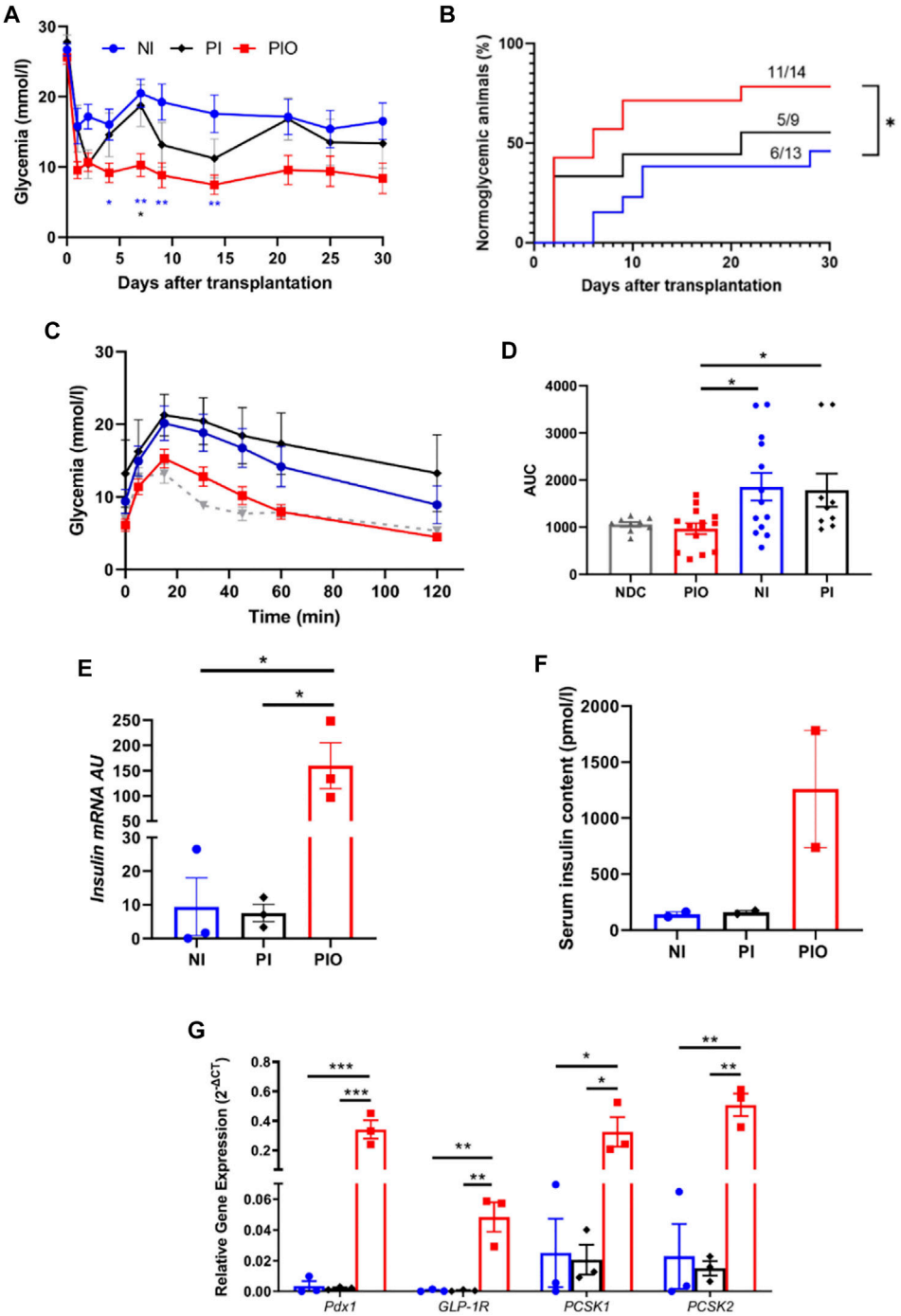


FIGURE 3 | *In vivo* function of organoids in immunodeficient, diabetic mice. **(A)** Glycemia level measured over 30 days in NOD-*Rag1*^{null} mice transplanted with 300 NI (n = 13, blue circle) and their equivalent number of PI (n = 9, black diamond) and PIO (n = 14, red square). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days by a one-way ANOVA with Dunnett's multiple comparison test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(B)** Cumulative number of mice reaching normoglycemia over 30 days. Comparison made using the log-rank (Mantel-Cox) test, *p < 0.05. **(C–D)** Glycemia level of each group during the intraperitoneal glucose tolerance test performed at 30 days post-transplantation **(C)** and their corresponding AUC values **(D)**. Grey triangles represent the non-diabetic control (NDC) group (n = 9). **(E)** Insulin mRNA expressed by NI, PI and PIO at 30 days post-transplantation; insulin mRNA was analyzed by qPCR, arbitrary units (AU) after normalization to housekeeping genes. Data shown are mean ± SEM, *p < 0.05, one-way ANOVA with Dunnett's multiple comparison test, n = 3. **(F)** Insulin concentration measured by ELISA in mice serum at 30 days post-transplantation. All data are expressed as mean ± SEM, one-way ANOVA with Dunnett's multiple comparison test, n = 2. **(G)** *pdx1, glp-1r, pcsk* and *pcsk2* expressed in PIO (red columns), PI (black columns) and NI (blue columns) at 30 days after transplantation, data presented as arbitrary units (AU) after normalization to housekeeping genes. Data shown are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and comparisons were made by a one-way ANOVA with Dunnett's multiple comparison test, n = 3.

when compared to animals transplanted with PI and NI (**Figure 3C**). This is illustrated by the increasing area under the curve (AUC) of the different groups, with PIO (966.8 ± 113.7), PI (1783 ± 351.1, $p = 0.05$ vs. PIO) and NI (1856 ± 294.5, $p = 0.014$ vs. PIO; **Figure 3D**).

We further investigated whether the improved glycemic control in the PIO group was associated with insulin production from the transplanted β cells. Remarkable upregulation of rat insulin mRNA levels in the graft was found in the PIO group in comparison to controls (PIO vs. PI, $p = 0.013$, PIO vs. NI, $p = 0.013$; **Figure 3E**). These results were supported by insulin measurements in the serum taken from the same mice (**Figure 3F**). Although a statistical significance wasn't achieved, a ten-fold increase in insulin levels was detected in the PIO group (1,259 ± 521 pmol/L), in comparison to both controls (NI: 140.6 ± 22.1 pmol/L, PI: 159.8 ± 14.4 pmol/L, p = ns).

Glp-1r, *pdx1* are known to be critical for promoting insulin secretion (28–31). Therefore, we investigated whether these genes were involved in the improved secretory outcomes of PIO. Gene expression analyses revealed upregulation of genes involved in β-cell function (*pdx1*, *pcsk1*, *pcsk2* and *glp-1r*) in PIO at 30 days post-transplantation, compared to controls (*pdx1*: PIO vs. PI, $p = 0.0009$, PIO vs. native islet, $p = 0.0009$; *glp-1r*: PIO vs. PI, $p = 0.002$, PIO vs. native islet, $p = 0.002$; *pcsk1*: PIO vs. PI, $p = 0.02$, PIO vs native islet $p = 0.021$ and *pcsk2*: PIO vs. PI, $p = 0.0005$, PIO vs. native islet, $p = 0.0006$; **Figure 3G**). Interestingly, at an earlier time points (3 days), a similar increase in gene expression was observed in PI and PIO in comparison with NI group, although without reaching statistical differences (**Supplementary Figure S3**). These data indicate that incorporation of accessory cells into the organoids supports long term secretory function of β cells.

Transplantation of Pre-Vascularized Islet Organoids Accelerates Graft Revascularization

To evaluate engraftment and revascularization, graft-bearing EFPs were removed at 30 days post-transplantation and processed for histology. Immunohistochemical staining for CD34, a marker for endothelial cells, showed that vessel density was significantly higher in the PIO samples (22.6 ± 3.5 CD34 + cells/cm^2) than in the NI samples (7.6 ± 0.9, $p = 0.002$; **Figures 4A,B**). Furthermore, in the PIO group, vessels were observed not only around graft, but mainly within β-cell positive area.

To investigate whether the blood vessels formed within the engrafted tissue constructs become functional and contribute to graft perfusion, we used intravascular injection of fluorescently labeled Lectin. Histological assessment of the Lectin-perfused grafts demonstrated the presence of functional Lectin positive vascular network within the PIO, in contrast only few vessels were present within NI (**Figure 4C**).

Next, we examined the mechanisms by which supportive cells (HUVECs and hAECs) contributed to rapid neovascularization of the graft. To this end, we investigated whether these cells might induce the production of angiogenic factors, such as *vegf-a* (**Figure 4D**). We observed, that rat *vegf-a* mRNA expression was significantly higher in PIO group (0.365 ± 0.033 AU) compared to NI (0.038 ± 0.005 AU; $p = 0.0006$) group. This finding was further confirmed by immunohistochemical staining for *vegf-a* of recovered samples, demonstrating higher fluorescent intensity in the PIO compared to NI (**Figure 4E**). These data indicate that incorporation of HUVEC and hAEC into PIO contribute to graft revascularization.

Human Amniotic Epithelial Cells Incorporation Into Organoids Improves Function and HUVEC-Derived Revascularization

Finally, we evaluated whether incorporation of hAECs into the organoids was essential for the engraftment and vascularization of the PIO. To this end, we added an additional group of mice transplanted with spheroids composed of IC: HUVEC (1:1 ratio) to the three existing groups.

Figure 5 summarizes the results obtained with this group. Blood glucose control was significantly lower in the IC + HUVEC group in comparison to the PIO group (**Figure 5A**). The IPGTT performed at 30 days post-transplantation demonstrated a poor glucose clearance in the IC + HUVEC group (**Figure 5B**). Response to increased blood glucose levels was significantly lower than for the PIO group as demonstrated by the AUC (2044 ± 578.1 vs. 966.8 ± 113.7, $p = 0.008$, respectively; **Figure 5C**).

After demonstrating that incorporation of supportive cells into the PIO improved graft revascularization, we investigated the degree to which these cells were contributing to new vessel development in the graft. To easily identify donor-derived new vessels, GFP-transduced HUVECs were incorporated into the PIO. Graft-bearing EFPs were recovered at 30 days post-transplantation and processed for immunohistological analysis. Interestingly, GFP positive cells were found inside the graft in the PIO group, while none was found in the IC + HUVEC group (**Figure 5D**). Both human and mouse vessels were positively stained by anti-CD34 confirming the establishment of anastomoses between donor derived HUVECs and mouse blood vessels. Furthermore, GFP/CD34 double positive endothelial cells were found at the graft periphery, inside capillaries containing erythrocytes, indicating that HUVECs were able to migrate and merge with a murine vascular system, forming functionally perfused blood vessels, as shown in **Figure 5E**. These data indicate that hAECs support HUVECs inside the organoids and thus contribute to accelerated revascularization.

DISCUSSION

Impaired and delayed revascularization of the graft is a major issue in islet transplantation and represents a main limitation to the search for extrahepatic sites for islet transplantation. Common vascularization strategies focus either on the combination of accessory cells with islets (32) or

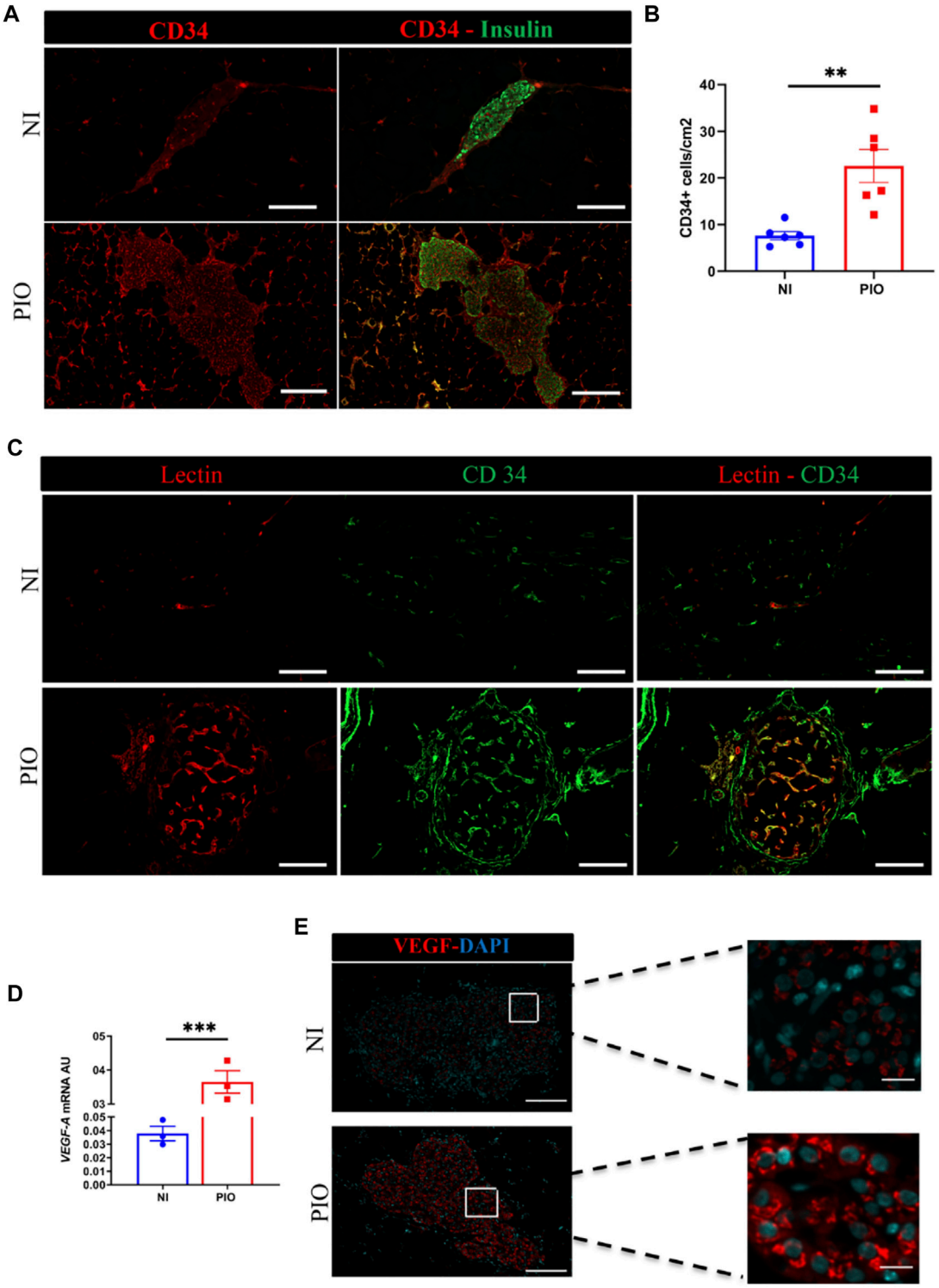


FIGURE 4 | *In vivo* revascularization assessment by immunohistological analysis. **(A)** The blood vessels of the graft detected at day 30 post-transplantation using CD34 (red) and insulin (green) immunostaining. Grafts Scale bar = 50 µm. **(B)** Quantitative analysis of revascularization was achieved by calculating the number of CD34 positive cells in the insulin positive area and the result was divided by the graft surface area. This was realized in two graft regions per mouse and in 3 mice per group. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, comparisons were made by a 2-tail unpaired Student *t* test. **(C)** Assessment of vessel functional capacity by mice injection of 100 µl of lectin. Capillaries are labelled in red and endothelial CD34+ cells in green. Scale bar = 50 µm. **(D)** *vegf-a* mRNA expression analyzed by qPCR at 30-days post-transplantation in PIO and NI groups; data presented as arbitrary units (AU) after normalization to housekeeping genes. Data shown are expressed as mean ± SEM. ***$p < 0.0006$, 2-tail unpaired Student t test, $n = 3$. **(E)** Recovered grafts stained for VEGF-A at day 30 after transplantation. Scale bars = 100 µm.

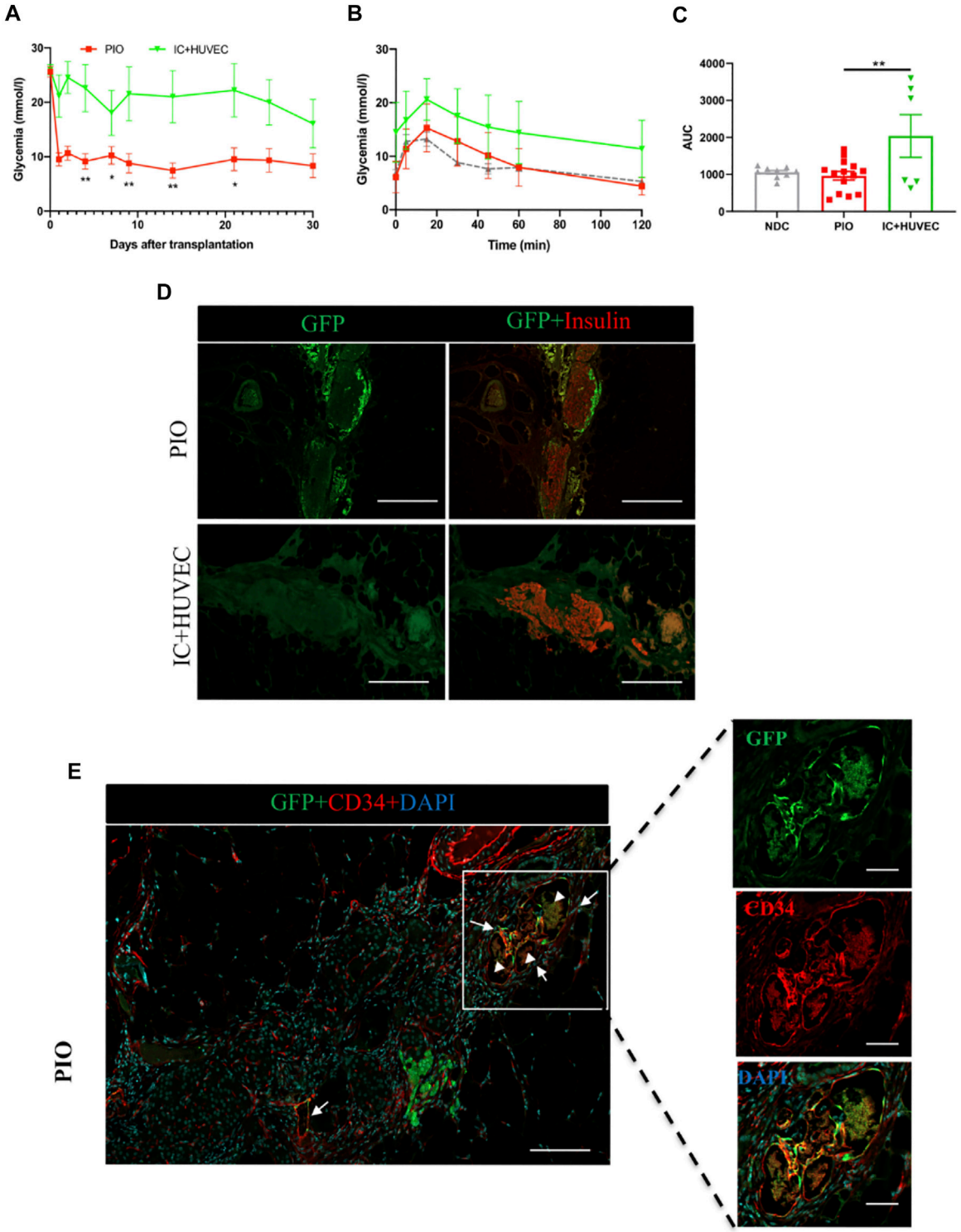


FIGURE 5 | *In vivo* function of IC + HUVEC spheroids, in immunodeficient, diabetic mice. **(A)** Mean glucose levels measured in NOD-*Rag1*^{null} mice transplanted with PIO (n = 14, red squares) and IC + HUVEC (n = 6, green inverted triangles). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days post-transplantation by a 2-tail unpaired Student t test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(B,C)** Intraperitoneal glucose tolerance test performed at 30 days post-transplantation and their corresponding AUC. Grey triangle represents the non-diabetic control (NDC) group (n = 9). Comparisons were made by a one-way ANOVA with Dunnett's multiple comparison test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(D)** Graft-bearing EFP recovered at 30 days post-transplantation and stained for GFP (green) and insulin (red). Scale bar = 100 µm. **(E)** Immunohistological staining for GFP (green), CD34 (red) and DAPI (blue). The yellow color represents the GFP-HUVECs with positive staining of anti-CD34. Arrows indicate chimeric blood vessels. Arrowheads indicate red blood cells. Scale bar for top panel = 100 µm and for the 3 bottom panels, 20 µm.

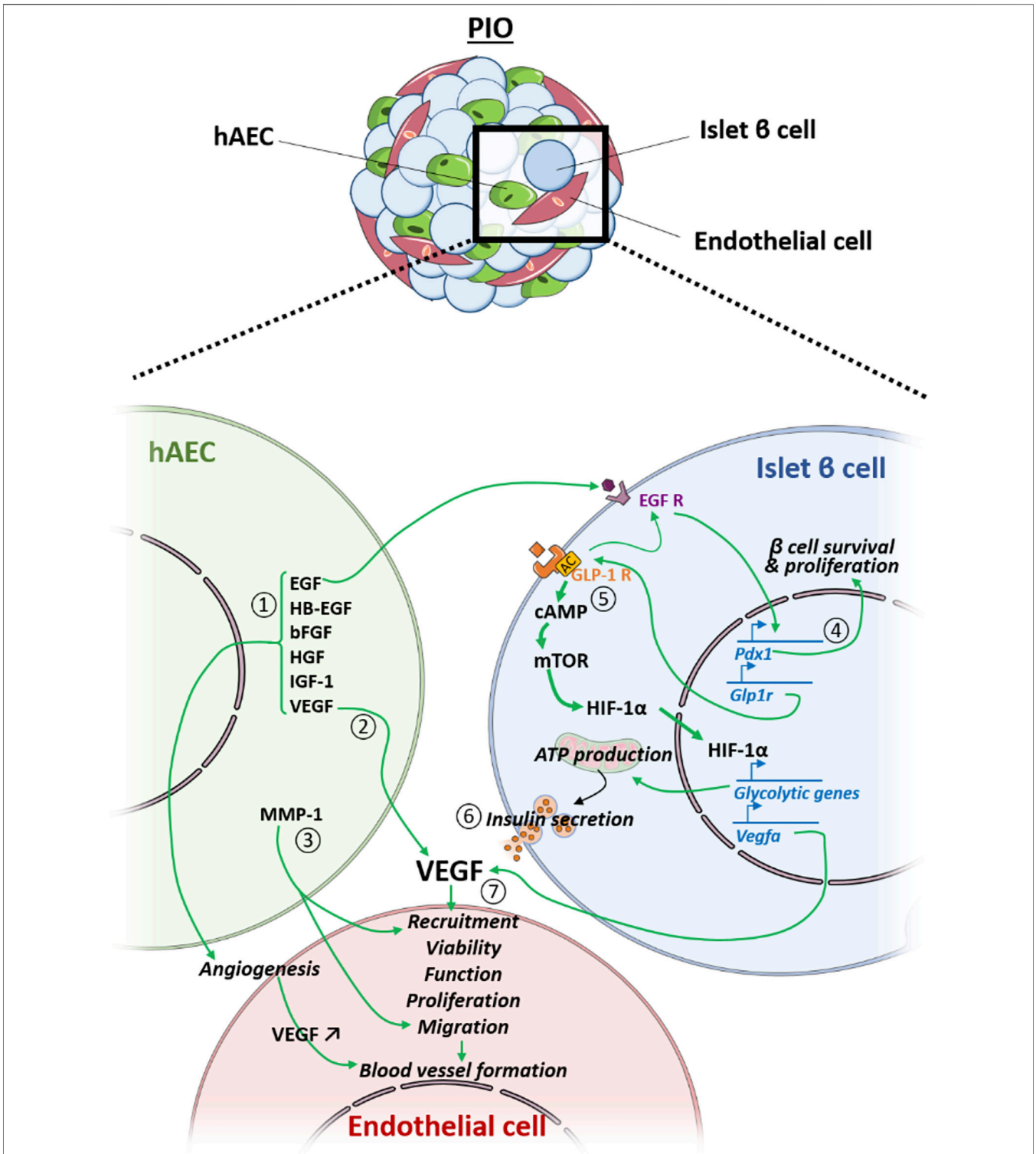


FIGURE 6 | Crosstalk between the hAEC, the endothelial cell (EC) and the islet β cell (IC) within the PIO. hAEC enhances revascularization of the PIO in a direct manner by secreting 1) angiogenic factors and 2) *vegf* that improve EC viability, function, proliferation and blood vessel formation, and 3) by producing ECM-degrading proteases (MMP-1) that facilitate EC migration and sprouting. Additionally, hAECs secrete EGF that 4) upregulates IC *pdx1* expression, leading to higher IC survival and proliferation, as well as 5) *glp1-r* expression, leading to an up-regulation of glycolytic genes and *vegf-a* through the mTOR/HIF-1a pathway, resulting in 6) an improved insulin secretion and 7) a better revascularization of the PIO.

incorporation of endothelial cells into islet-like constructs generated from embryonic stem cell-derived ß cells (30) or ß cell lines (31), and are mainly based on *in vitro* testing. In this study, we successfully generated functional pre-vascularized islet organoids using multiple cell types. The major finding of this study is that incorporation of hAECs and HUVECs into insulin-producing organoids hastens the rate of graft revascularization, and subsequently results in better engraftment of the β-cell mass.

HUVECs are the most commonly used, robust source of human endothelial cells in regenerative medicine and tissue engineering (33). However, limited proliferative potential of these cells hinders their clinical application. hAECs isolated from the amniotic membrane of discarded placenta is considered a non-controversial stem cell source (34). These cells demonstrated profound anti-fibrotic, anti-inflammatory, non-tumorigenic and low antigenic properties (35, 36). Furthermore, hAECs possess pluripotent stem cells characteristics, can be isolated in large quantities and are thus considered as an evolving therapeutic tool for the development of various clinical applications (35). Previously, we have shown that the generation of insulin-secreting organoids from primary IC in combination with hAECs improved islet engraftment and vascularization primarily by stimulating *VEGF-A* production from the graft *via* HIF1- α signaling pathway (17, 20). Therefore, in this study, we evaluated whether hAECs could accelerate the angiogenic potential of mature endothelial cells (HUVECs). Our results show that chimeric, prevascularized insulin secreting organoids are capable of establishing new vascular networks *in vitro* and *in vivo* when co-cultured with hAECs and HUVECs. The enhancement of the angiogenic potential of HUVECs by hAECs can be explained by three possible mechanisms: 1) *via* the secretion of ECM-degrading proteases facilitating EC migration and sprouting (37), 2) by up-regulating VEGF expression in endothelial and islet cells (38), and 3) by the reduction or suppression of inflammatory responses (39, 40). Our *in vivo* experiments have demonstrated the superiority of pre-vascularized islet organoids for insulin secretion and revascularization.

Another important finding is the existence of a cross-talk between the islet, endothelial and amniotic epithelial cells associated within one organoid (summarized in **Figure 6**), and that this communication can be successfully employed for improving outcomes of islet transplantation. In terms of revascularization, we observe that both blood vessel density and number of functional vessels were significantly higher in the grafts explanted from mice transplanted with PIO in comparison to control groups. *VEGF-A* is a proangiogenic factor that recruits endothelial cells and circulating endothelial progenitors (11). Our results demonstrated significant upregulation of *VEGF-A* gene expression in the grafts explanted from mice transplanted with pre-vascularized organoids. Immunohistochemical analysis of the explanted grafts confirmed that the major producers of *VEGF-A* were islet cells. This finding was in agreement with our previous studies, demonstrating that hAECs markedly increase production of *VEGF-A* in islet cells *via* paracrine signalling (17). In addition, hAECs themselves are known to secrete *VEGF-A* (41), which on the other hand could also enhance performance of HUVECs within the organoids. To verify this hypothesis, we used GFP-HUVECs and tracked transplanted cells inside the graft. We found GFP-HUVECs both inside and in the vicinity of the graft. At the same time, GFP-HUVECs were also detected to be integrated into the peri-islet functional blood vessels containing red blood cells. This indicates that the donor derived endothelial cells anastomosed with the murine vascular system and formed functionally perfused blood vessels. Interestingly, the same was not observed in mice transplanted with IC + HUVECs, in which no GFP-HUVECs were found in the recovered grafts. In addition, almost no blood circulation was observed inside the graft area. This indicates that hAECs contribute to the process of endothelial cell remodelling and stabilization finally leading to mature vessel formation. Our findings are in agreement with previously reported data, demonstrating that hAECs enhance EC viability, function, proliferation, migration and blood vessel formation *in vitro* and *in vivo* (41). Furthermore, amniotic cells secrete additional factors that are critical for angiogenesis, such as EGF, HB-EGF, bFGF, HGF, IGF-1 (42). Taken together, these data suggest that hAECs promote revascularization both directly by secreting angiogenic factors and indirectly by stimulating *VEGF-A* secretion by islet cells.

Accelerated revascularization can also provide important survival cues to the islet cells. Another important challenge to islet transplantation is to achieve stable, long-term insulin independence, preferably with single donor islet transplantation. In this study, improved revascularization was accompanied by prompt return of severely diabetic mice to a normoglycaemic state after transplantation of minimal mass of prevascularized islet organoids. Mice transplanted with PIO showed significantly improved insulin secretion and better glucose clearance compared to mice transplanted with PI, NI and IC + HUVECs. Investigations of underlying mechanisms showed that superior function of β-cells in PIOs was mediated by the GLP-1R signalling pathway. GLP-1R has been found to regulate homeostasis of β-cell mass by inducing β-cell proliferation and protecting against apoptosis. On the other hand, activation of the GLP-1R leads to the activation of multiple downstream pathways, including EGF receptor signalling (43), which in turn stimulates proliferation of β cells (44). EGF has been shown to enhance glucose-dependent insulin secretion and upregulate PDX1 expression (20). Although the precise mechanisms underlying this pattern of increased gene expression in the PIOs are not fully understood, we speculate that growth factor expression profile of hAECs, mainly EGF, could stimulate upregulation of the expression of genes involved in β-cell function (GLP-1R, PDX-1).

CONCLUSION

In this study, we demonstrate a novel approach to generate pre-vascularized islet organoids by combining primary ICs with two

additional supportive cell types, HUVECs and hAECs, and address some of the challenges of clinical islet transplantation such as donor supply scarcity, impaired islet engraftment and revascularization. Furthermore, our data demonstrate that hAECs not only promote cell viability and engraftment, but most importantly, play a primordial supporting role in the development of HUVEC-derived neo-vessels within the transplanted tissue.

However, to generate large numbers of uniform, size-controlled and functional prevascularized islet organoids, a scalable platform technology is a prerequisite to ensure standardization and reproducibility for new and innovative beta cell replacement strategies.

Addressing this challenge, recently, we showed that several spheroid generating methods are suitable to assemble uniform, size-controlled and functional islet-like clusters (45). The compared techniques included native islets as controls (IEQs), a self-aggregation technique, the hanging drop technique, the agarose 3D microwell technique and the Sphericalplate SP5D. We demonstrated that up to 9000 islet organoids can be easily generated per plate.

Moreover, the SP5D can be automatized, and robotic-mediated spheroid generation can further reduce variability and therefore improve standardization and reproducibility.

Taken together, these findings could be a basis for the design of novel extra-hepatic, extra-vascular islet transplantation sites.

CAPSULE SENTENCE SUMMARY

The pre-vascularized islet organoids were generated from dissociated islet cells, human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs). Our study demonstrates that pre-vascularized islet organoids exhibit enhanced *in vitro* function and most importantly, improved engraftment and accelerated vascularization *in vivo* in a murine model.

VANGUARD CONSORTIUM

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Commission Cantonale d'Ethique de la Recherche (CCER), in compliance with the Swiss Human Research Act (810.30). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Geneva.

AUTHOR CONTRIBUTIONS

C-HW: Performing experiments, data analysis and interpretation, manuscript writing. FL, DC-D, and KB: Performing experiments. MC, TB, CO, AF, and DB: Manuscript editing. LP: Technical support. AF, CO, VO-G and BT: Provision of study material. EB: Conception and design, supervision of project, financial support, administrative support, manuscript writing, final approval of manuscript. All other authors edited and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10214/full#supplementary-material

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GLOSSARY

AMCA Aminomethylcoumarin Acetate
AUC area under the curve
bFGF basic fibroblast growth factor
BSA Bovine Serum Albumine
CCER Commission Cantonale d'Ethique de la Recherche
CK-7 Cytokeratin 7
DAPI 4′,6-diamidino-2-phénylindole
DMEM Dulbecco's Modified Eagle Medium
EC endothelial cell
EFP epididymal fat pad
EGF epidermal growth factor
FBS fetal bovine serum
GFP green fluorescent protein
GLP-1R Glucoagon-like peptide 1 receptor
hAEC human amniotic epithelial cell
HB-EGF heparin binding epithelial growth factor
HBSS Hanks' balanced salt solution
HGF Hepatocyte growth factor
HIF1-a Hypoxia-inducible factor 1-alpha
HUVEC human umbilical vein endothelial cell
IC islet cell
IEQ islet equivalent
IGF-1 insulin-like growth factor-1
IPGTT intraperitoneal glucose tolerance test
LV lentiviral
MEM-NEAA Minimum essential medium non-essential amino acids
MOI multiplicity of infection
NI native islet
NDC non-diabetic control
PBS Dubbelco's Phosphate buffer saline
PCSK1 Proprotein Convertase Subtilisin/Kexin Type 1
PCSK2 Proprotein Convertase Subtilisin/Kexin Type 2
PDX-1 pancreatic and duodenal homeobox 1
PFA Paraformaldehyde
PI pseudo-islet
PIO prevascularized islet organoid
RPLP1 ribosomal protein lateral stalk subunit P1
RT-PCR reverse transcriptase polymerase chain reaction
SI stimulation index
STZ streptozotocin
VEC vascular endothelial cadherin
VEGF-A Vascular endothelial growth factor A

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REVIEW
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Genetic Engineering of Immune Evasive Stem Cell-Derived Islets

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Genome editing has the potential to revolutionize many investigative and therapeutic strategies in biology and medicine. In the field of regenerative medicine, one of the leading applications of genome engineering technology is the generation of immune evasive pluripotent stem cell-derived somatic cells for transplantation. In particular, as more functional and therapeutically relevant human pluripotent stem cell-derived islets (SCDI) are produced in many labs and studied in clinical trials, there is keen interest in studying the immunogenicity of these cells and modulating allogeneic and autoimmune immune responses for therapeutic benefit. Significant experimental work has already suggested that elimination of Human Leukocytes Antigen (HLA) expression and overexpression of immunomodulatory genes can impact survival of a variety of pluripotent stem cell-derived somatic cell types. Limited work published to date focuses on stem cell-derived islets and work in a number of labs is ongoing. Rapid progress is occurring in the genome editing of human pluripotent stem cells and their progeny focused on evading destruction by the immune system in transplantation models, and while much research is still needed, there is no doubt the combined technologies of genome editing and stem cell therapy will profoundly impact transplantation medicine in the future.

Keywords: regenerative medicine, type I diabetes, allograft rejection, CRISPR, HLA allobarrier

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INTRODUCTION

Diabetes mellitus is a complex metabolic disease which currently affects more than 30 million people in the United States and 463 million people worldwide with annual projections (1) indicated to continue to climb 2%–3% per year (2,3). Pancreatic islet endocrine cells are the major glucose and energy metabolism control mechanisms of the body. Despite continuing advances in insulin delivery technology and recombinant insulins, diabetes and its complications still claim the lives of millions of people as a result of ketoacidosis, hypoglycemic coma or chronic cardiovascular, eye, nerve and kidney damage (4). Existing beta cell replacement therapies, such as whole vascularized pancreas or islet transplantation, can achieve long-term normoglycemia and insulin independence in patients thereby forestalling end-organ complications. However, these therapies suffer from several key limitations. First, the shortage of organs make this option available to very few that fulfill the criterion, and second, the need for life-long immunosuppression to prevent allograft rejection. Severe complications related to immunosuppressive medication toxicities and chronic rejection continue to

plague these approaches limiting their long-term success (5). An ideal β cell replacement therapy strives towards both generating an abundant supply of functional β cells *and* identifying a means to downregulate immune responses to suppress rejection and/or autoimmunity that is not associated with immunosuppression-related toxicities while prolonging graft function.

Human pluripotent stem cells have the potential to provide an unlimited supply of insulin-producing β cells for treating patients with diabetes (T1D, T2D, MODY, monogeneic diabetes). Human embryonic stem cell (hESC) lines, and human induced pluripotent stem cells (hiPSCs), which are generated by genetically reprogramming terminally differentiated somatic cells into a pluripotent state, have entered clinical trials to treat a multitude of disease from heart failure to macular degeneration, spinal cord trauma and diabetes, among others. Human iPSCs hold the additional potential for patient-specific therapies, thereby theoretically removing the necessity for immunosuppression. To date there have been advances in directing human pluripotent stem cells (hPSC) through stepwise differentiation protocols into functionally mature glucose-responsive and potentially therapeutic stem cell-derived islets (SCDIs). Progress from multiple groups and companies have contributed to the development and review of these protocols and advancements (6-20) and has led to the recent initiation of clinical trials (10-25) (ClinicalTrials.gov identifiers NCT02239354, NCT03163511, NCT02939118 and NCT04786262). Recent peer reviewed publications, as well as company reports, of first clinical experiences highlight proof of concept (10,23).

As hPSC-derived islets move into initial clinical trials, a number of factors could impact immediate and long-term success of this very young field, including off-target cells and the complex role of immunogenicity, among others. In this review, we will focus on immunogenicity-related issues of SCDI therapies. We will discuss mechanisms of islet destruction, and genome engineering strategies designed to impede alloimmune destruction. Additionally, we will discuss new advances in humanized animal models designed for studying the effects of these genomic perturbations on human immune responses to stem cell progeny. Lastly, we will discuss current approaches for developing genetic screens for identifying additional immune-protective genes.

MECHANISMS OF ISLET DESTRUCTION

Understanding the mechanisms by which the immune system reacts to and can destroy transplanted islets will inform efforts to subvert these pathways and prevent rejection of transplanted stem cell-derived islet organoids. Innate and adaptive immunity as well as autoimmune memory responses are all potential barriers in T1D recipients. While human islet and pancreas transplantation is successful with greater than 80% of patients achieving short-term insulin independence, long-term success requires powerful, continuous immunosuppressive medications. Underscoring the clinical challenge, Human Leukocyte Antigen (HLA)-identical transplants may succumb to recurrent autoimmune destruction (26,27). Knowledge of the mechanisms of islet transplant rejection and autoimmunity largely derive from rodent studies; several excellent recent reviews update our knowledge in this area (28-30). Briefly, as autoimmunity in rodent models of type 1 diabetes requires both CD4 and CD8 T cells (31), autoantigen expression is required for graft infiltration by autoreactive CD8 T cells following syngeneic islet transplantation (32) and rejection of vascularized organs appears CD4 T cell-dependent (33) it is probable that both T cell subsets contribute to the combination of autoimmunity and alloimmunity that would occur following implantation of genetically-disparate or genetically engineered insulin-producing cells into an autoimmune recipient.

A potential opportunity for novel intervention relates to the innate immune instant blood mediated inflammatory reaction (IBMIR). IBMIR represents a key factor in the immediate loss of Islets transplanted into the liver and is currently managed with anti-coagulant and anti-inflammatory medications. A key molecular step in IBMIR is islet expression of tissue factor (TF) (34,35). TF expression is regulated by the pro-inflammatory transcription factor NF-kB following exposure to cytokines as well as by the activated inflammasome (36). Thus, engineering stem cells to be non-responsive to inflammasome activation, to be less sensitive to NF-κB activation, or to lack TF itself could be beneficial. However, to what extent SCDIs elicit IBMIR, express TF and/or are protected by cytokine inhibitors has not been studied despite ongoing clinical trials studying stem cell-derived islet transplantation into the liver. In addition, due to expression of ABO antigens on tissue cells, solid organ transplants and islet transplants must obey ABO compatibility, but whether this is true for SCDIs is unknown. Also, it is unknown whether SCDIs which generally contain immature and mature cell types, express high levels of ABO glycoprotein antigens.

Innate immune mechanisms include recruitment and activation of natural killer (NK) cells which offer further opportunities for enhancing islet resistance to immune attack. As NK cells are activated by ligands expressed on ischemically and mechanically damaged isolated islets, these ligands would represent promising candidates for gene editing SCDIs, but to what extent NK cells would be activated by SCDI grafts remains to be determined. Furthermore, editing NK ligands may promote NK cell-mediated attack as NK cells execute the "missing self" response, i.e., rejection of Major Histocompatibility Complex (MHC) Class I deficient or non-self MHC Class I expressing allografts. To overcome this problem some groups are engineering expression of non-classical HLA-E, HLA-G or CD47 into stem cells or rodent islets(37-39).

The cellular adaptive immune response is primarily mediated through alloreactive host T cells. Host T cells can be activated *via* multiple mechanisms including by 1) interaction of their T cell receptor with intact allogeneic MHC on donor cells (direct pathway), 2) donor peptides presented by self-MHC on recipient antigen presenting cells (APCs) (indirect pathway), or 3) through recognition of allogeneic MHC displayed on recipient APCs after their transfer *via* cell-cell contact or through extracellular vesicles (semi-direct pathway, MHC

cross-dressing) (40-43). Each of these T cell activation pathways requires specific steps that provide unique opportunities to engineer resistance into stem cells. Common steps in T cell activation include a requirement for co-stimulation and other reinforcing positive signals, as well as an absence of inhibitory signals, from antigen presenting cells. Studies in mice and humans show that co-stimulatory blockade with CTLA4Ig (abatacept), or analogs such as Belatacept, effectively inhibit cytotoxic T cell responses and prolong islet allograft survival but requires adjunctive immunotherapy (reviewed in (44)). Forced expression of cytotoxic T lymphocyte antigen 4 (CTLA4), or the programmed cell death (PD) molecules PD-1/PD-L1 in SCDIs could short circuit T cell activation and facilitate immunosuppression-free survival. However, there are many aspects to these processes that are not fully understood with regards to SCDIs. Whereas cadaver islets contain dendritic cells (DCs) acting as professional APCs potentially seeding direct alloresponses, SCDIs may not contain this population, or endothelial cells which can also express MHC following inflammatory signals, and therefore indirect or semi-direct responses may predominate influencing the choice of inhibitory molecules to be targeted in SCDIs.

New discoveries in mechanisms of immune homeostasis also provide new avenues for SCDI engineering. The ubiquitin editing enzyme A20, encoded by *TNFAIP3*, and FasL have been shown to play a dominant role in protecting islet allografts (45,46) together with a short course of rapamycin. A20 overexpression inhibits the expression of inflammatory mediators and raises inflammatory signaling thresholds which promotes the development of antigen specific Tregs supporting immune tolerance and islet survival (45). Another approach, FasL coating of islets or embedded in microgel with islets in conjunction with subtherapeutic rapamycin also promoted long-term allograft acceptance in rodents and non-human primates related to Treg induction (47,48). Stromal cell-derived factor 1-alpha, aka CXCL12, was also shown to promote islet allo and xenograft survival through multiple postulated immune regulatory mechanisms (49,50). Thus, these molecules could be tested in overexpression models of genome editing of SCDIs.

Though the major pathways of islet rejection are not fully understood, and may differ substantially between rodents and humans, the information we do have provides a rich source of opportunities for experimental interrogation of protecting SCDIs from cellular mechanisms of innate, adaptive and autoimmune mediated destruction.

IMMUNOGENICITY OF STEM CELL-DERIVED PANCREATIC LINEAGE CELLS

While undifferentiated stem cells maintain some level of immune privilege (51-53), they become recognized or visible to the immune system once differentiated. Therefore, development of strategies to avoid recognition of cells by the immune system and ultimate destruction will be critical to therapeutic effectiveness.

In mammalian systems every nucleated cell is adorned with cell surface antigens (54). In humans the genes responsible for these marker molecules are encoded by HLA genes. HLA genes are grouped into class I (HLA-A, -B and-C and less polymorphic -E, -F and -G), class II (HLA-DR, -DP, -DQ, -DM, -DN and -DO) and III (the complement cascade); HLA-A, -B, -DR, -DP, and –DQ are the most studied and important contributors to allorejection.

Studies have begun to interrogate the immunogenicity of SCDIs. While undifferentiated hPSC have low levels of MHC expression, leading to their evasion, as these cells differentiate the MHC signature is upregulated thereby increasing their vulnerability and exposure to the immune system (52,53). Similar to native human β cells, SCDIs express HLA Class I antigens which can be upregulated by cytokine exposure (55,56). However, while normal human β cells upregulate all MHC isotypes, gene expression profiling on the SCDIs revealed HLA-C to be predominantly expressed, a finding that may be due to the immaturity of the SCDIs (56). Interestingly, both stem cell-derived pancreatic progenitors and endocrine cells express complement inhibitory receptors, CD46, CD55 and CD59 (55). Additionally, it has been shown that human β cells upregulate PD-L1 when exposed to proinflammatory cytokines (57,58). Castro-Gutierrez et al. went on to show that while human primary β cells respond to inflammation by upregulating PD-L1, they found that their SCDIs did not (56); which is different from what Yoshihara et al. demonstrated (59).

Like human islets, SCDIs are vulnerable to alloreactive cytotoxic T lymphocyte (CTL) killing *in vitro* (55). In addition, preproinsulin (PPI)-specific CTLs recognize and kill SCDIs in the context of PPI peptides (55,56), similar to normal human β cells (60). SCDIs are similarly vulnerable to antibody dependent cellular cytotoxicity, but may be resistant to complement mediated cytotoxicity *in vitro* (55). Through genetic modification to introduce inducible PD-L1 expression, Castro-Gutierrez et al. showed that PD-L1 overexpression and HLA Class I knockout abrogated diabetogenic CD8 T cell activation (56). Collectively, these studies begin to define the immunogenicity of SCDIs.

METHODS OF GENETIC ENGINEERING

Precise and efficient genetic engineering leverages targeted DNA double strand breaks (DSB) to potentiate desired editing. CRISPR-Cas9 tools have shown wide utility and complement editing systems like ZFNs and TALENs to enable knockout (KO) and knock-in (KI) of transgene cassettes, tags, and patient risk variants (61). For example, gene editing has been used in hPSCs to show that a noncoding variant downstream of *GATA6* affects *GATA6* expression and pancreatic differentiation, suggesting that this minor allele variant acts as a genetic modifier of the neonatal diabetes phenotype in patients with *GATA6* heterozygous mutations (62). Similarly, we have applied CRISPR-Cas9-mediated gene editing to recreate patient-specific missense mutations in *GATA6* and *NGN3* or *NEUROG3* for

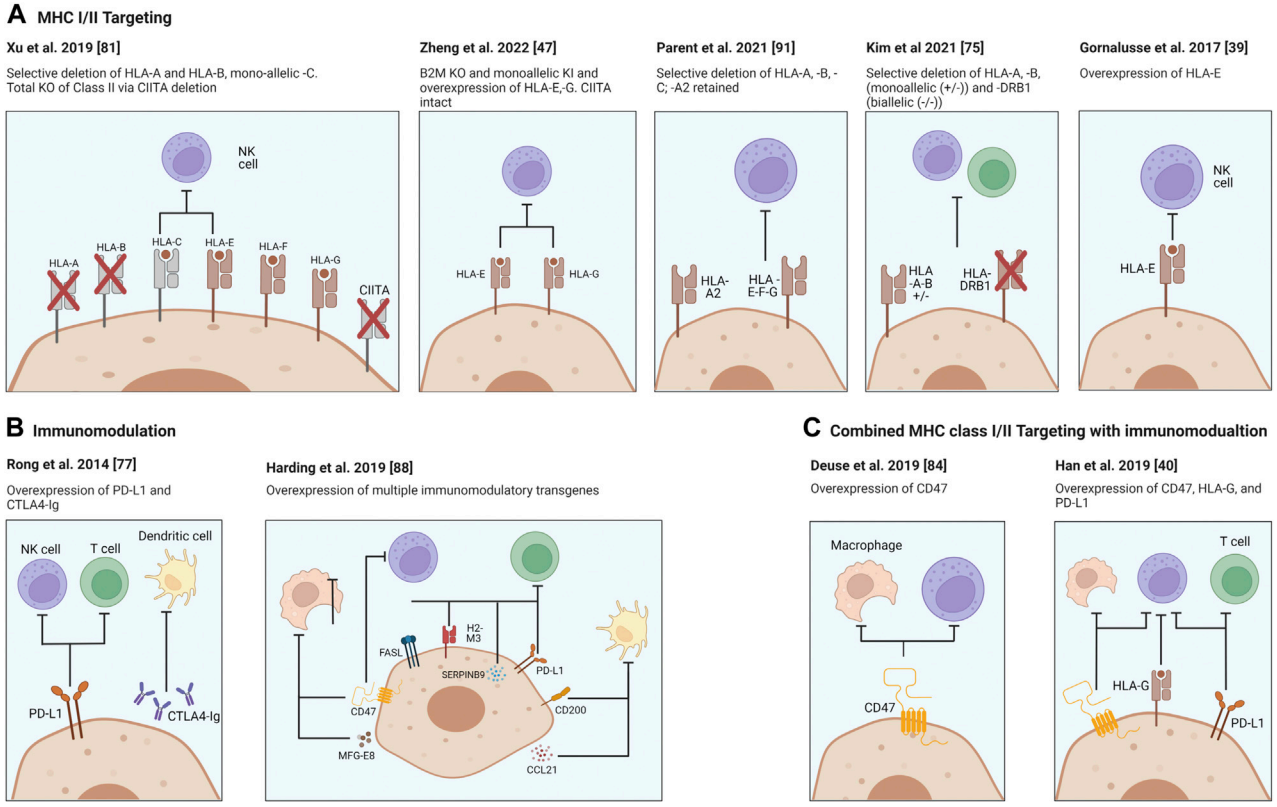

FIGURE 1 | Strategies for providing immune protection of SCDI. Genome engineering of immune check point molecules and/or *via* HLA Class I and II surface molecules. Targeting B2M in HLA-I causes the disruption of expression of all class I genes, major A-C and minor E-G. Surface presentation of HLA Class II molecules is disrupted *via* knocking out the transcription factor CIITA. Figure was produced using Biorender (biorender.com).

investigation of neonatal diabetes and pancreatic differentiation phenotypes (63,64).

KO and KI are keystone capabilities for engineering an immune privileged and safe beta cell therapy, but off-target editing effects and the proximity requirement to the DSB site for precise editing have limited the utility of early CRISPR-Cas9 systems. Many methods are being developed to overcome these limitations such as optimized Cas9 enzyme designs and new fusion constructs like those used in base editing (65). For instance, Cas9 nickases, variants of the Cas9 enzyme, were designed to cleave only one strand of the DNA to minimize off-target DSBs and subsequent undesired editing. Cleaving both DNA strands using a Cas9 nickase and two proximal gRNAs shows low off-target effects and allows efficient and complex editing in human iPSCs (66). An exciting recent expansion of this technology is prime editing, where, a Cas9 nickase is fused to a reverse transcriptase, and combined with a clever prime editing guide RNA design, allows precise nucleotide alterations that can be over 30 bp from the PAM site mitigating the proximity requirement (67,68).

The flexibility of CRISPR-Cas9 and new Cas9 variant-based editing tools can change the stem cell derived beta cell therapy landscape by supporting simple and robust manufacturing pipelines. Recent therapeutic efforts have largely taken allogeneic approaches that require only a single edited stem cell line to be produced and validated (e.g., ClinicalTrials.gov NCT05210530). Increased versatility and efficiency of genome editing technologies may enable allogeneic therapies with complex engineering that improves immunogenicity profiles through gene editing. In addition, a proof-of-principle study involving correction of an inherited mutation in the insulin locus also suggests feasibility of autologous therapies with personalized gene correction (69).

GENETIC MODIFICATIONS LEADING TO REDUCED ALLOIMMUNE DESTRUCTION AND INCREASED SURVIVAL OF STEM CELL-DIFFERENTIATED CELLS AND THEIR DERIVATIVES

For broad clinical use of stem cell-differentiated cells, it is imperative to reduce the alloimmune destruction after transplantation, if universal stem cell lines are to be more effectively utilized. Thus, a major active goal in the field is the development of compatible hypoimmunogenic cells which evade the immune system and reduce or eliminate the requirement for life-long immunosuppressive regimes while restoring tissue/

cellular function. These issues have been addressed in several recent reviews and several approaches depicted in **Figure 1** (70-74).

In order to utilize the power of stem cells the host immune response needs to be addressed. Disruption of β-2 microglobulin (B2M) interrupts surface presentation of MHC class I molecules encoded by HLA-A, -B, -C, -E, -F, and -G and prevents activation of $CD8^+$ cytotoxic T cells. On the other hand, disruption of Class II transactivator (CIITA), a master regulator responsible for expression of HLA Class II genes, reduces antigen presentation to host $CD4^+$ T cells. Each or both may be inactivated, and HLA class I and/or II knockouts may be coupled with the overexpression of immunomodulating transgenes. Two well characterized molecules, PD-L1 and CTLA4-Ig are two immune checkpoint proteins being employed and ectopically expressed to protect the cells from the host's T cells (75). Rong et al. have shown that these modifications prevented allogeneic rejection of teratomas in a humanized mouse model through disruption of T cell co-stimulatory and enhancing inhibitory pathways, both of which were necessary for allowing teratoma formation in their model (76). While promising this approach did not interrupt the MHC expression thereby potentially leaving engrafted cells vulnerable to allorecognition by the adaptive immune system.

However, complete removal of MHC Class I expression does not protect cells from NK cell attack and lysis but rather may activate them due to the "missing-self" response (77,78) and additionally may leave cells vulnerable to bacterial and viral infection due to inability to present these antigens to the host immune system. Addressing this concern, it has been demonstrated that it is possible to achieve alloimmune graft acceptance through genetic modifications, such as "knockins" and constitutive expression of immunomodulatory factors (**Figure 1**). To this end, Gornalusse et al. developed a B2M-HLA-E (a minimally polymorphic) fusion protein after complete B2M deletion in hPSCs (38) while Shi et al. similarly expressed a B2M-HLA-G fusion construct to stabilize the MHC and allow cell surface expression in B2M KO hPSC cells and showed hypoimmunogenicity and reduced NK-cell activation (38,79). This modification has the benefits of protecting stem cell derivatives from $CD8^+$ T cell targeting and from NK-mediated cell lysis. Importantly however, not all NK populations may be affected due to differences in membrane receptor presentation, such as NKG2A, KIR2DL4 and ILT2. Another example is that of Xu et al. who derived iPSCs with disruptions in HLA-A/B but retained HLA-C expression and could demonstrate $CD8^+$ T cell and NK cell evasion, although HLA-C presence may still allow presentation of bacterial and viral antigens (80). Other groups have also observed reduced NK cell activation upon non-classical MHC expression such as HLA-E and HLA-G. Zheng et al. found lentivirus overexpression of HLA-E and HLA-G in mesenchymal stem cells could prevent activation of the three major subtypes of NK cells (46). Lentiviral overexpression of a single-chain HLA-E was also used by Hoerster et al. to reduce allogeneic T cell proliferative and activation responses to *B2M* KO NK cells in co-culture assays (81). Taken together these approaches demonstrate methods to overcome NK cell "missing self" induced fratricide of KO somatic cell transplants.

Taking advantage of our knowledge regarding cancer cell survival pathways (82), another study looked to reduce NK cell activity through the overexpression of the transgene CD47, which is a ubiquitously expressed immunomodulatory suppressive gene (83,84). Deuse et al. demonstrated that CD47 was very effective at inhibiting NK cells and macrophages from killing MHC-deficient iPSCs in immunocompetent mice and report that these inhibitory signals are accomplished *via* an essential interaction with the signal-regulatory protein alpha (SIRPα). They further showed that blockade of the CD47 receptor renders the cells susceptible to NK cell killing.

Additionally in 2019 Han et al. sought to develop a strategy which addresses both adaptive and innate immune responses through genetic modifications to knockout the MHC class I and II expression followed by knock-ins (KI) to express the immunomodulatory factors PD-L1, CD47 and HLA-G (39). Of note, HLA-G is expressed during pregnancy at the maternal-fetal interface and is an NK cell inhibitory ligand (85,86). This study demonstrated that these modifications led to significant reduction in immune responses with respect to T cell, NK cell and macrophage-mediated killing *in vitro* assays.

While most studies focus on deletion of HLA-encoded MHC surface molecules, a study from Andras Nagy's group targeted the upregulation or over-expression of additional immunomodulatory factors, CCL21, PD-L1, FASL, Serpinb9, H2-M3, CD47, CD200 and MFGE8 in mouse embryonic stem cells (87). These factors individually target specific cell subsets of the immune system or act on different mechanisms, and therefore could act synergistically. For example, CCL21 encodes for a cytokine that recruits activated dendritic cells. PD-L1, FASL, Serpinb9, H2-M3 target T-cells and NK cells. CD47 and CD200 prevent phagocytosis and MFGE8 can push macrophages towards an anti-inflammatory state. Multiple clones were generated exhibiting different degrees of over expression of each protein and two optimal expressing closes were tested for survival after transplantation as undifferentiated cells in a variety of immunocompetent mouse strains. It was shown that the expression of these factors allowed transplanted cells to survive and form teratomas, without any intentional modifications of the MHC locus. While the aforementioned studies focused on achieving reduced alloimmune responses to non-islet hPSC-derived cell types, such as undifferentiated cells, cardiomyocytes, endothelial cells, hematopoietic cells and retinal pigment epithelial cells, in different studies, it remains to be confirmed whether such approaches will be as effective for SCDIs.

GENETIC MODIFICATIONS LEADING TO REDUCED ALLOIMMUNE DESTRUCTION AND INCREASED SURVIVAL OF STEM CELL-DIFFERENTIATED ISLET CELLS

Paving the way for the future possibility of allogeneic SCDI transplantation without immunosuppression, there has been significant progress towards improving immune evasion through genetic modifications (88). *B2M* knock-out aims to reduce T cell activation by preventing stable MHC class I

formation on the SCDI cell membrane. The role of MHC class I in the SCDI—T cell interaction was explored through a set of *in vitro* orthogonal approaches: a trans-well assay, antibody blocking of MHC class I, as well as genetic KO of *B2M* which resulted in decreased CD25 and CD69 expression in the responding $CD8^+$ T cell population (89). An alternative approach to improve immunocompatibility is PD-L1 overexpression, which was shown to dramatically improve SCDI functionality in a PBMC-SGM3 humanized mouse model, suggesting a measure of protection from alloimmune recognition (59). Notably, induction of endogenous PD-L1 through IFNγ pre-treatment of SCDIs also conferred protection upon transplantation to immune-competent mice implying a measure of protection against xenorejection. Although the transplanted cells were shown to regulate blood glucose out to 50 days post-transplantation, long-term time points were not included and could be of interest to characterize (59). In a separate study, PD-L1 overexpression in SCDI, achieved through an integrated inducible cassette, decreased IL2 secretion by diabetogenic TCR-expressing T cells (56). When further combined with a frameshift mutation in *B2M*, T Cell IL2 secretion was nearly abrogated, demonstrating the promise of multiplex editing involving MHC class I interference and PD-L1 overexpression (56).

MHC class I disruption is a major contributor to preventing T cell activation, but as mentioned in Section E, fully disrupting MHC class I surface expression may be associated with somatic cell graft lysis by NK cells (77,78). To address this concern, CRISPR-Cas9 was used in hPSCs to KO the polymorphic *HLA-A, HLA-B,* and *HLA-C* class I genes as well as MHC class II transactivator *CIITA* but retain the highly prevalent allelic variant *HLA-A2* and the other non-classical, less polymorphic *HLA-E, HLA-F,* and *HLA-G* genes that may protect cells from NK cell-mediated lysis (90). Co-culture of edited SCDIs with peripheral blood mononuclear cells (PBMCs) reduced CD107a (LAMP1) activated subset of NK cells and significantly improved survival following transplantation into immunodeficient mice which had been reconstituted with PBMCs from an $HLA\text{-}A2^+$ donor. The retained HLA-A2 is proposed as the factor that enables HLA-E expression upon IFNγ stimulation, as a failure of HLA-E expression in $HLA\text{-}ABC^{null}$ cells was restored upon introduction of HLA-A2-derived signal peptide. A complementary approach to combinatorial KI has been to discover and functionally characterize SCDI ligands that activate NK cells (91). Expression data suggested CD226 ligand PVR (CD155) and a co-stimulatory molecule of CD337 ligand B7-H6, B7-H3, to be promising NK activation candidates. While co-culture of *B2M* KO human SCDIs with human $CD16_{dim}$ NK cells caused ~80% of SCDI cells to become necrotic, co-culture of *B2M*, *CD155*, and *B7H3* triple KO SCDIs resulted in ~20% necrotic cells, indicating a measure of protection from NK lysis. Triple KO pancreatic progenitors were then subcutaneously transplanted to NSG mice. Within 72 h of human NK cell injection, luciferase signal from *B2M* KO cells was markedly reduced, but triple KO cells showed similar survival to WT and to *β2M* KO HLA-E overexpression pancreatic progenitors. Collectively, these studies highlight the value of investigating how immune cells subsets interact with transplanted cells and chart a path towards generating hypoimmunogenic and universal cell lines for allogeneic stem cell therapies.

Genetic engineering is a promising avenue for overcoming survival challenges post-transplantation, and looking forward, multiplex editing may advance SCDI therapies that do not require immunosuppression.

MODELING THE *IN VIVO* IMMUNE RESPONSE TO PSC THERAPIES

There is a critical need for assessing the *in vivo* immune response to PSC-based therapies prior to clinical trials. Human immune system (HIS) humanized mice offer a tractable pre-clinical *in vivo* model of the human immune response and have been used for a variety of transplantation immunology studies (92-95). There are a variety of HIS models available (96), but most useful for PSC transplant immunology studies are those models which incorporate both the infusion of human hematopoietic stem/progenitor cells (HSPCs) as well as thymic fragments into immune-deficient mouse strains to provide T cell developmental cues in the animals. The bone-marrow-liver-thymus (BLT) model (97) and NeoThy model (98) are two leading HIS iterations. Both harbor *de novo* generated human MHC-restricted T cells, and a complement of other adaptive and innate immune cell types useful for assessment of transplantation tolerance and rejection.

The BLT model has garnered concern over the immature nature of the fetal immune systems in the animals, in particular the naïve (99) and regulatory T cell subsets (100), spurring a search for higher-fidelity modeling of adult human immunity. We developed the NeoThy model using neonatal, instead of fetal, HSPCs and thymus in order to evaluate the impact of more developmentally mature tissue on the resultant immune cell repertoire function. Importantly in HIS models, not only does the humanizing tissue directly impact T cell development and function, but also the choice of immune-deficient mouse strain will impact the character and phenotype of accessory lymphoid and myeloid cells that develop, as will the method of myeloablation used for human HSPC engraftment (101).

Recently, immune-deficient mouse strains such as the NSG or NOG have been modified to improve human cell engraftment (102). Various groups have introduced mutations to these strains that obviate the need for irradiation-based myeloablation (103), for example, as well as adding human transgenes such as GM-CSF and IL3 that support a more-robust myeloid immune compartment (104), and therefore presentation of alloantigens.

Assessment of transplant rejection in HIS humanized mice can be determined by examining immune infiltration, activation and/or cytokine release post-transplant. To date, the humoral immune responses in these mice has been suboptimal, notably, with a lack of antibody class switching and T cell-dependent antigen responses to vaccination(105). Therefore, cellular immune responses are the primary focus until improved iterations of HIS mice can be developed.

Non-human primates (NHPs), such as rhesus macaques, currently play an important role in pre-clinical PSC studies (106,107). NHPs are useful for evaluation of human PSC-based therapies and associated immunosuppression requirements (108), as well as useful for gene editing studies (109). We recently developed a BLT-type "primatized" mouse model (110) for evaluation of the NHP immune response prior to conducting full-scale large animal studies. Experiments are ongoing to evaluate PSC cellular therapies in these primatized mice as a method to screen potential therapeutic and genetic modifications. Ethical considerations may prevent use of NHP primatized mice, as well as conventional HIS BLT mice using human fetal tissues.

A key consideration for the choice of *in vivo* model is the genetic composition of the immune system and humanized mice offer a unique opportunity to select humanization donors of particular genetic backgrounds. There are conflicting reports regarding the concept of autologous self-tolerance to iPSCs and/or their differentiated products (111,112,113) and it is possible to reconstitute a humanized (114) or primatized mouse model with an autologous immune system to test the hypothesis that an autologous graft will be tolerated as self. Importantly, the pathological target of a PSC therapy, will require careful consideration of the humanizing tissue source, especially in cases of autoimmunity e.g., T1D.

FUTURE PROSPECTS: GENETIC SCREENS

Genetic engineering tools also impact discovery efforts for stem cell derived β cell replacement. The progress of utilizing genome editing in hPSCs to create SCDI for transplantation without immunosuppression also points to the need to discover additional targets for gene editing to further improve engraftment and delay (or prevent) immune rejection. Genome-scale CRISPR screens have emerged as a powerful tool to address this need. In addition to CRISPR-Cas9 screens that we and others have performed to identify genes involved in the step-wise differentiation from hPSCs to insulin-secreting β cells)(115,116,117,118) recent studies have leveraged CRISPR screens to directly uncover immunomodulatory factors that mediate SCDI survival post-transplantation. For these experiments, a pool of cells is created where each cell has a different gene knocked out. Following an assay (ex. transplantation), the impact of knocking out every gene on a readout (ex. survival) is revealed. The first such screens were conducted in the mouse NIT-1 β cell line to uncover genes, which when mutated, would confer a survival advantage upon transplantation into a T1D mouse model(119). While most cells were destroyed upon transplantation, the authors collected the surviving cells and found that knockout of *RNLS*, a gene previously associated with autoimmune diabetes, protected cells from destruction through reduced stimulation of autoreactive $CD8^+$ T cells and increased resistance to ER stress. Furthermore, *RNLS* KO β cells differentiated from hPSCs had increased protection from ER stress, reproducing an important finding from the mutant mouse β cells. A limitation of conducting screens using the mouse system is that there are known differences between mouse and human β cells and immunological contexts(120). Addressing this limitation, a human SCDIs transplantation survival screen has also been conducted(121). Human SCDIs were transplanted into Hu-PBL-NSG-MHC^{null} mice that also received human PBMCs. SCDIs were harvested after 10 weeks and compared to mice that received SCDIs but did not receive human PBMCs. *CXCL10* knockout was discovered to confer a survival advantage, in addition to known genes like *HLA-A* and *B2M*. CXCL10 is an IFN-induced chemokine, and other members of the family (CXCL9 and CXCL5) were also screen hits, suggesting a common mechanism. *CXCL10* KO SCDIs were generated, transplanted into mice, and graft survival was assessed with or without PBMCs. While a majority of unedited SCDIs were destroyed when mice received PBMCs, *CXCL10* KO SCDI graft survival was significantly improved compared to mice that received unedited SCDIs but did not receive PBMCs. The state-of-the-art Hu-PBL-NSG-MHC^{null} mouse model enables superior PBMC engraftment by preventing human T-cell recognition of murine MHC and the concomitant acute GVHD, but there are also limitations as other aspects of the human immune system may yet prove relevant to understanding the totality of the SCDI-immune interaction(122). Going forward, we anticipate genetic screens to tap deeper into the vast coding as well as noncoding genome for improved survival and immunocompatibility of transplanted cells.

BRIEF CONCLUSION

The application of genome engineering to study and reduce the immunogenicity of SCDI is both an exciting area of inquiry and essential for widespread clinical application. Work is this space is at the vanguard and additional insights will undoubtedly be revealed by future investigations.

AUTHOR CONTRIBUTIONS

SDS, JO, and DH designed the review, and along with MEB, SJK, SM, ALB, and SG analyzed the literature and wrote the manuscript.

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CONFLICT OF INTEREST

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Strategies to Improve the Safety of iPSC-Derived β Cells for β Cell Replacement in Diabetes

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Allogeneic islet transplantation allows for the re-establishment of glycemic control with the possibility of insulin independence, but is severely limited by the scarcity of organ donors. However, a new source of insulin-producing cells could enable the widespread use of cell therapy for diabetes treatment. Recent breakthroughs in stem cell biology, particularly pluripotent stem cell (PSC) techniques, have highlighted the therapeutic potential of stem cells in regenerative medicine. An understanding of the stages that regulate β cell development has led to the establishment of protocols for PSC differentiation into β cells, and PSC-derived β cells are appearing in the first pioneering clinical trials. However, the safety of the final product prior to implantation remains crucial. Although PSC differentiate into functional β cells *in vitro*, not all cells complete differentiation, and a fraction remain undifferentiated and at risk of teratoma formation upon transplantation. A single case of stem cell-derived tumors may set the field back years. Thus, this review discusses four approaches to increase the safety of PSC-derived β cells: reprogramming of somatic cells into induced PSC, selection of pure differentiated pancreatic cells, depletion of contaminant PSC in the final cell product, and control or destruction of tumorigenic cells with engineered suicide genes.

Keywords: cell therapy, safety, type 1 diabetes mellitus, induced pluripotent stem cells, beta cells

INTRODUCTION

In patients with type 1 diabetes (T1D), glycemic control can be reestablished by allogeneic islet transplantation. However, this approach is severely limited by the scarcity of organ donors. A new source of insulin-producing cells would significantly increase the possibility of cell therapy becoming a broad and standard therapy for the treatment of all diabetic patients. Pluripotent stem cells (PSC), such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) derived from somatic cell reprogramming, can differentiate *in vitro* into insulin-producing cells with established protocols that recapitulate embryonic pancreas development. In the first clinical trials, PSC-derived β cells were transplanted into patients with type 1 diabetes (NCT03163511, NCT02239354, and NCT04786262). In this context, the safety of the final cellular product in developing PSC derivatives for transplantation prior to implantation is crucial (1,2). Indeed, not all PSC reach complete differentiation into functional β cells *in vitro*, and a fraction of the cells may remain undifferentiated, exposing recipients to the risk of teratoma formation post-transplantation.

The most common method for determining pluripotency is the teratoma formation model, which employs immunodeficient animal models, in which pluripotent cells develop into teratomas formed from all three germ layers. A direct comparison of the teratoma formation capacity between ESC and

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iPSC revealed that iPSC form teratomas more efficiently and quickly than ESC (3). It is also likely that the extended *in vitro* culture and manipulation of PSC facilitates accumulation of genetic lesions (4–6), as well as genetic and epigenetic abnormalities during reprogramming to pluripotency (1,7). Even a very small contaminant at the end of differentiation constitutes a risk. It has been found that as few as two ESC colonies implanted into immunodeficient mice can result in teratoma formation; when the clumps were trypsinized to single-cell suspensions before injection, 245 cells were sufficient to form teratomas after 10 weeks (8). Several groups have reported the formation of teratomatous tissue elements in grafts when not purified PSC-derived pancreatic endoderm cells were infused in mice (9–13). Although recent protocol refinements have reduced this risk and increased the percentage of mature β cells obtained, there remains a need to control contaminant pluripotency in β cells. Therefore, the therapeutic applications of PSC-differentiated derivatives require strategies for the control of innate tumorigenicity and the malignant transformation of inappropriately differentiated cells.

The foreseeable implementation of stem cell-based therapies for the treatment of thousands of patients requires extreme caution, as only a single case of stem cell-derived tumors can set the field back several years. The first published data on patients with T1D transplanted with PSC-derived pancreatic progenitors showed that the transplanted cells did not form tumors, but only a percentage of the implanted cells survived and secreted C-peptide (14,15). Therefore, whether a greater number of implanted and engrafted cells can give rise to teratomas remains unclear. In this review, approaches to increase the safety of PSC-derived β cells are discussed, which can be summarized in four different strategies:

(1) The generation of safe iPSC using advanced techniques for cell reprogramming that conjugate non-integrating delivery of Yamanaka's factors and high efficiency.
(2) The selection of pure differentiated cells based on specific β cell or pancreatic precursor markers, allowing for the selection of target cells only.
(3) The depletion of contaminant PSC in the final cell product, using chemical inhibitors or the selective killing of contaminant stem cells.
(4) The control of tumorigenic cells with suicide genes, in which stem cells are harbored with one or more suicide gene cassettes, resulting in cell death in the presence of specific prodrugs.

Herein, these approaches are discussed with the belief that the best results will most likely be obtained using a strategy that combines the choice of the safest PSC source, the selection of the cellular product, and protection via the use of safety switches.

GENERATION OF THE SAFEST INDUCED PLURIPOTENT STEM CELL

IPSC can be derived from any individual, with the advantages of possessing the same plasticity as ESC while avoiding the ethical problems arising from the use of human embryos. For these reasons, iPSC are considered valuable tools in regenerative medicine, disease modelling, and drug discovery. IPSC are generated through the genetic reprogramming of adult somatic cells; however, inserting reprogramming factors into adult cells raises safety issues. In fact, iPSC reprogramming was originally obtained by the overexpression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc), subsequently denoted as the "Yamanaka factors," with a retroviral delivery system in murine and human fibroblasts (16,17). The disadvantage of this original reprogramming method from a translational perspective is that reprogramming vectors are randomly integrated into the genome of transduced cells, leading to risks including teratomas and genomic instability (18,19). Several integration-free alternative methods have been developed and tested to overcome these safety issues. Without the intention of describing all the reported reprogramming techniques and how these have changed since the discovery of iPSC 15 years ago, this review focuses on the optimal reprogramming for the safe application of iPSC in the field of cell replacement therapies.

The most important factor that should be considered for the reprogramming of donor cells includes the "footprint" that a particular method deposits in the reprogrammed cell type. Within cellular replacement therapy, iPSC should have no footprint and no residual transgene sequences of the reprogramming vectors in the final iPSC product. This can be achieved using methods of transfection with episomal plasmids or minicircles, infection with non-integrating Sendai Virus (SeV) or adenovirus, transfection with synthetic mRNA/miRNA, or transposition with the piggyBac transposon, all of which leave no traces of the integration of the transgenes in the genome of reprogrammed iPSC (18). Alternatives include the use of lentiviruses and retroviruses that, with an additional step after reprogramming, allow for the excision of the transgene, such that only a small portion of the reprogramming vector remains integrated in the iPSC genome. Combining this characteristic of the "zero" footprint with an acceptable level of efficiency and the need for commercially available easy-to-use reagents that meet good manufacturing practice (GMP) standards, episomal plasmids and Sendai virus are currently the best choices for generating iPSC for projects with translational endpoints (20,21).

At present, the most commonly used strategy for reprogramming with SeV involves the delivery of Oct4, Sox2, Klf4, and L-Myc genes (22). Sendai virus is an enveloped virus with a single-chain RNA genome, and its two main characteristics make SeV the most attractive system for reprogramming. First, it can infect a wide range of cell types, infecting cells by attaching itself to the sialic acid present on the surface of multiple somatic cells, including PBMC, $CD34^+$ cells, and T cells. Second, SeV vectors are made of RNA and remain in the cytoplasm, ensuring that they do not integrate into the host genome or alter the genetic information of the host cell (22–24). Importantly, however, in the most recent version of SeV, the F gene, responsible for fusion protein expression, was deleted, and new temperature sensitivity mutations to the polymerase-

related genes were added to counteract the formation of non-transmissible virus-like particles. These modifications prevent transmission and limit the propagation of reprogramming vectors, helping to clear the virus faster after reprogramming and reducing cytotoxicity to cells (25).

SELECTION OF PURE DIFFERENTIATED CELLS

IPSC reprogramming using a safe method represents a step towards guaranteeing a safer cellular product. However, it does not protect completely against the risk of tumorigenesis. Indeed, although multistep differentiation protocols lead to the *in vitro* production of functional insulin-producing cells from PSC (26–30), the differentiated cultures can also contain undesirable proliferating cell types, such as residual pluripotent cells, which can jeopardize graft safety. The most intuitive and reasonable approach for the selection of β cells, capable of purifying the cell preparation to be transplanted while eliminating unwanted unsafe cells, is the positive selection of the target cells. This approach is mainly mediated by antibodies that bind to specific proteins expressed on the surface of pancreatic cells. Two main strategies have been developed: the selection of pancreatic endoderm (PE) progenitors and the selection of mature β cells. In both cases, it has been necessary to rely on transcriptomic and proteomic studies aimed at describing specific markers (31–35). Despite efforts to characterize insulin-producing cells and their precursors, there are currently no universally shared surface markers of these cell types. Finding endodermal-specific markers is not an easy task and requires the careful analysis of differentiating cells during embryogenesis. One elegant study mined microarray gene expression data from early murine embryos to identify two PE-specific cell-surface proteins (31,32), namely PDGFRα and Lrp2. However, the presence of RNA during development does not always correlate with the presence of the protein (33). Another study revealed that of all protein classes examined, cell-surface proteins in particular showed a poor correlation between protein and RNA abundance when comparing cell types (34). Therefore, RNA expression may be an unreliable predictor of specific surface protein expression; thus, proteomic approaches are needed to identify protein markers that can distinguish cell types in developing embryos. In a pioneering study, Rugg-Gunn et al. developed a direct proteomic approach to explore the cell-surface proteome for developmental lineages using affinity labelling and mass spectrometry. They identified molecules with potential importance in the separation and migration of endoderm, which allowed for the prospective isolation and characterization of viable PE directly from mouse blastocysts (35). The results obtained in the mouse model highlighted a strategy with which to find specific lineage markers for transfer into human cells.

An early work aimed at identifying β cell markers useful for the purification of cells during the last stages of differentiation from stem cells was published in 2011 by the group of scientists of Viacyte Inc., who proposed three proteins as specific to different stages (9). Using a flow cytometry-based screening of commercial antibodies, the researchers identified cell surface markers for the separation of pancreatic cell types derived from human ESC. In particular, CD200 and CD318 were used as markers of endocrine cells. However, when these sorted cells were implanted *in vivo*, they gave rise mainly to glucagon-positive cells. In contrast, CD142, also known as a tissue factor, was found to enrich PE cells, which give rise to all pancreatic lineages, including functional insulin-producing cells after transplantation into mice. In fact, the transplantation of CD142 sorted cell aggregates gave rise to functional, glucose-responsive, insulin-secreting cells *in vivo*, whereas the transplantation of unenriched material resulted in teratomatous graft rates of 45% (9). The main limitation of the use of CD142 as a selection marker for pancreatic differentiation is its low specificity. Several other cell types, including endothelial cells, monocytes, macrophages, and platelets, express CD142.

In the same year, a study reported CD24 as a new surface marker for pancreatic progenitors differentiated from human ESC (36). CD24 is a sialoglycoprotein normally expressed on mature granulocytes and B cells that modulates growth and differentiation signals in these cells. In this study, CD24 was identified as a positive marker of pancreatic progenitors by co-staining for PDX1 and a panel of cell surface antigens at the pancreatic progenitor stage of human ESC differentiation. $CD24^+$ cells co-expressed most of the key transcription factors of pancreatic progenitors, and the expression of important pancreatic genes was significantly enriched in $CD24^+$ cells compared with $CD24^-$ cells. Notably, $CD24^+$ cells could differentiate into insulin-producing cells, but $CD24^-$ negative cells could not. As in the case of CD200 and CD318, the use of CD24 did not include a follow-up to purify differentiated cells, and to date, CD24 plays a role mainly as a cancer stem cell marker for ductal adenocarcinoma (37).

A substantial new impetus to the surface marker-based selection approach came when three major papers on the GP2 protein were published in 2017. In the first study, the researchers performed microarray analysis to compare the gene expression pattern of $PDX1^+/NKX6.1^+$ pancreatic progenitors with that of $PDX1^+/NKX6.1^-$ cells and identified progenitor-specific cell surface markers (38). CD142 and CD200, two cell surface markers previously shown to enrich pancreatic endoderm cells and endocrine progenitors (9) were expressed in both cell populations. In addition, the researchers identified a cell surface maker, glycoprotein 2 (zymogen granule membrane GP2), which was enriched in the $PDX1^+/NKX6.1^+$ cell population obtained from PSC differentiation and fetal pancreas (38), which could potentially be used for the isolation of pancreatic progenitors. Furthermore, the researchers showed that the isolated $GP2^+$ progenitors efficiently differentiated into glucose-responsive insulin-producing cells *in vitro*. Another study reported that $GP2^+$ cells, obtained from the human pancreas at 7 weeks of development, purified and cultured *in vitro*, might give rise to acinar cells, in which GP2 is upregulated, as well as ductal and endocrine cells, in which GP2 is downregulated or silenced. In this study, human

fetal pancreatic differentiation was reconstructed using GP2 in combination with CD142 to mark pancreatic progenitors, which could give rise to $GP2^{hi}CD142^{+}$ acinar cells or enter the endocrine pathway and express NEUROG3 by turning off GP2 and CD142 (39). At the same time, Cogger et al., in Canada, used a proteomics approach to phenotypically characterize pancreatic progenitors derived from PSC and distinguish these cells from other populations during differentiation (40). In addition, GP2 has been identified as a specific cell surface marker for pancreatic progenitors (40). In the developing human pancreas, GP2 is co-expressed with the endocrine key transcription factors NKX6.1 and PTF1A. In addition, isolated PSC-derived $GP2^{+}$ cells were shown to generate β cells more efficiently than $GP2^{-}$ and unsorted populations, decreasing the percentage of unwanted PSC-derivatives, consequently increasing the safety of the final cell product. This last point was taken up and confirmed by a very recent study by the same group, wherein they showed that sorted GP2-expressing pancreatic progenitors give rise to all endocrine and exocrine cells *in vivo*, including functional β cells, without influencing the endocrine-to-acinar ratio within the graft, and that GP2 sorting prevents teratoma formation *in vivo*. These findings support GP2 as a candidate marker for cell selection with potential for clinical use (41).

Another surface marker for differentiating pancreatic cells, but at an earlier stage of differentiation, was recently reported: the CD177/NB1 glycoprotein. This glycoprotein was identified as a novel surface marker to isolate pancreatic progenitors from definitive endoderm cells derived from human PSC. Isolated $CD177^{+}$ definitive endoderm differentiated more homogeneously into pancreatic progenitors and into more functionally mature and glucose-responsive β cells than cells from unsorted differentiation cultures (42). Therefore, CD177 is a promising marker for cell selection during pancreatic differentiation to improve differentiation efficiency, but it is likely to be an early marker to purify progenitors for safety purposes. It is worth noting the work by Melton's team, whose research resulted in a differentiation protocol to produce β cells that are now being transplanted into patients in an ongoing clinical trial (NCT04786262). In a study published in Nature in 2019, Veres et al. used a strategy for endocrine cell enrichment based on single-cell dissociation followed by controlled re-aggregation (43,44). This technique was coupled to the selection of cells with a marker, CD49a/ITGA1, identified by single-cell transcriptomic analysis (45). Anti-CD49a staining and magnetic microbead labelling allowed for the efficient sorting of stem cell-derived β cells. This method produced clusters containing up to 80% β cells from embryonic and induced pluripotent stem cell lines. These highly purified β cells were responsive to glucose *in vitro* and had increased stimulation indices compared to unsorted, re-aggregated islets in both static and dynamic glucose-stimulated insulin secretion (GSIS) (45). It is reasonable to assume that this purification level reduces the risk of non-pancreatic contaminants in the final cellular product, thereby increasing its safety. In 2020, one study reported an antibody panel against cell surface antigens to enable the isolation of highly purified endocrine subsets from mouse islets, and CD71 was used as a specific marker of adult β cells. CD71 is a transferrin receptor that mediates the uptake of transferrin-bound iron whose expression is regulated in a glucose-dependent manner. β cells were also found to express high levels of several other genes implicated in iron metabolism, and iron deprivation significantly impaired β cell function (46). These findings have interesting implications on iron metabolism in β cell function, as well as for the discovery of CD71 as a novel surface marker of β cells, at least in mouse islets.

Another potential marker for the identification of adult β cells is CD81/TSPAN28. In a recent study, the researchers performed single-cell mRNA profiling of early postnatal mouse islets, re-analyzed several single-cell mRNA sequencing datasets from mouse and human islets, and complemented the findings by testing iPSC-derived endocrine cells, Min6 insulinoma, and human EndoC-βH1 β cell lines (47). They found that CD81 marks immature β cells in healthy islets and labels dedifferentiated β cells in metabolically stressed environments, such as during diabetes progression. Since it is possible that β cells derived from stem cells share some features of dedifferentiated or immature cells, CD81 could be a valuable tool for targeting β cells and purifying them from the bulk of progenitors and non-β cells present in the final cell product of differentiation. Since CD81 likely marks immature β cells, with reduced levels of expression associated with increased gene regulatory networks involved in maintaining β cell maturation, it could be used to select differentiating cells at the stage of immature β cells, when Nkx6.1 is upregulated, but cells do not yet secrete insulin (47).

Another possibility for the efficient purification of insulin-positive cells involves cell sorting based on the expression of insulin at the immature β-like stage. However, this purification method, successfully reported in some studies (30), requires the cell sorting of a genetically modified human ESC line in which a green fluorescent protein (GFP) reporter gene was inserted into the endogenous human insulin locus. One paper using a new approach was published this year, in which the researchers describe the generation of an array of monoclonal antibodies against cell surface markers that selectively label stem cell-derived islet cells (12). High-throughput screening identified promising candidates, including three clones that marked a high proportion of endocrine cells in differentiated cultures. These three antibodies, 4-2B2, 4-5C8, and 4-5G9, were used to magnetically sort PSC-derived islet cells, which led to the formation of islet-like clusters with improved GSIS and reduced growth upon transplantation. Thus, these antibodies selectively isolated islet cell populations from PSC differentiated *in vitro* using a scalable magnetic sorting approach, facilitating the large-scale production of safe and functional islets from stem cells (12).

DEPLETION OF CONTAMINANT PLURIPOTENT STEM CELLS IN THE FINAL CELL PRODUCT

Despite its efficiency, antibody-mediated cell sorting using surface markers to detect and select pancreatic cells does not

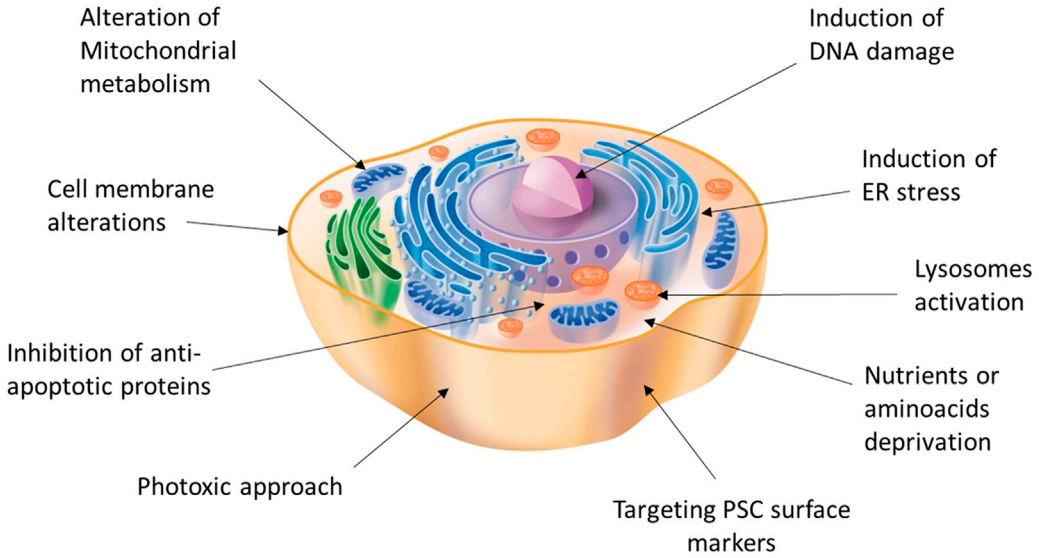

FIGURE 1 | Potential targets and strategies acting on different cell compartments for the induction of selective PSC death.

guarantee a lack of undifferentiated cells in the sorted group. Moreover, cell sorting is a technique that inherently exerts a strong mechanical stress, which can heavily affect cell viability. However, antibody-mediated strategies could still be combined with other positive selection solutions or even replaced with direct depletion of the contaminant pluripotent cells remaining after the differentiation process. In fact, the two main characteristics of PSC, namely pluripotency and active proliferation capacity, can be exploited for the development of highly selective strategies that facilitate their elimination (48). Therefore, a variety of approaches have been reported, including the use of drugs/phototoxic approaches linked to antibodies targeting PSC surface-specific antigens or small molecules for selective elimination (**Figure 1**).

Antibody-Mediated Selection

As previously described, cell sorting using antibodies against specific surface proteins has primarily been used to isolate desirable cell types after differentiation. Alternatively, undifferentiated PSC can be identified by exploiting specific surface marker expression profiles. Antibodies against tumor-related antigen (TRA)-1-60 and TRA-1-81 or stage-specific embryonic antigens (SSEAs), such as SSEA-3, SSEA-4 (49), and SSEA-5 (50) were used to negatively select PSC from a mixed cell population. However, when using magnetic-activated cell sorting (MACS), it was not possible to achieve complete separation, and thus the elimination of undifferentiated ESC, while using highly selective fluorescence-activated cell sorting (FACS), thereby compromising the viability of PSC derivative cells (49). Therefore, the use of an antibody capable of inducing cell death or separation based on a specific surface protein linked to a cytotoxic agent is a valid approach to reduce the potential for teratoma formation in heterogeneously differentiated cultures, as the specificity of antibodies can be exploited without using sorting techniques.

Choo et al. generated 10 monoclonal antibodies against the surface antigens of undifferentiated ESC, showing strong reactivity against undifferentiated, but not differentiated, cells. Among these antibodies, IgM mAb 84, which binds the antigen podocalyxin-like protein-1, was found to be cytotoxic to undifferentiated ESC in a concentration-dependent and complement-independent manner. Single-cell suspensions of undifferentiated ESC pre-treated *in vitro* with mAb 84 before transplantation into mice did not form tumors even 18 weeks after infusion (51). This strategy was later combined with MACS selection with an anti-SSEA-1 antibody for the selective removal of 99.1–100% of undifferentiated ESC (52). One of the main problems associated with this strategy is the large size of mAb 84, which can impede penetration into embryoid bodies (EB) or cell clusters. Consequently, four antibody fragment formats of mAb 84 were engineered and among these only one, scFv 84-HTH, a single chain variable fragment with a dimerizing helix–turn–helix motif, could recapitulate the cytotoxicity of mAb 84 on multiple hESC lines (53).

Another strategy that exploits hyperglycosylated podocalyxin expression is based on the recombinant lectin probe, rBC2LCN. Initially, this molecule was used for fluorescence-based imaging (54) and quantitative detection (55). However, it was later conjugated with a catalytic domain of *Pseudomonas aeruginosa* exotoxin A, which led to the formation of a recombinant lectin-toxin fusion protein, termed rBC2LCN-PE23. rBC2LCN-PE23 binds to human PSC, followed by its internalization, allowing for the intracellular delivery of the cytotoxic protein, which is sufficient to completely eliminate human PSC but not differentiated cells (56). Ben-David et al. also showed that a cytotoxin-conjugated antibody that selectively targets Claudin-

6-positive cells efficiently kills undifferentiated cells, thus eliminating the tumorigenic potential of human PSC cultures containing undifferentiated cells, as Claudin-6 is absent in adult tissues but highly expressed in undifferentiated cells (57).

In a recent study, desmoglein 2 (Dsg2), which is highly expressed in undifferentiated PSC versus somatic tissues, was targeted using the monoclonal antibody K6-1 linked to the chemotherapeutic agent doxorubicin (DOX). Dsg2-positive hPSC were selectively targeted by K6-1-DOX, which led to the pH-dependent endosomal release and nuclear localization of DOX, with subsequent cytotoxicity via an apoptotic caspase cascade. The drug is highly efficient in preventing teratoma formation upon iPSC transplantation (58); however, its effect on PSC-derived cells transplanted *in vivo* has not yet been investigated. Conversely, Sougawa et al. proposed a new clinical grade method to eliminate residual undifferentiated iPSC from differentiated cardiomyocyte cell culture using the anti-CD30 antibody-drug conjugate brentuximab vedotin, which selectively kills CD30-positive cells by inducing cell cycle arrest in the G2/M phase followed by apoptosis (59). The researchers demonstrated that undifferentiated iPSC express the surface marker CD30, a TNF receptor superfamily member, at high levels, and brentuximab vedotin treatment induces PSC apoptosis and prevents teratoma formation without affecting the differentiated cardiomyocytes (59). We recently applied this strategy in the field of diabetes, confirming that treatment with brentuximab vedotin efficiently induced cell death in human iPSC while sparing iPSC-derived β cell identity and function. The transplantation of non-treated human iPSC-derived β cells into NOD-SCID mice may result in teratoma formation within 4 weeks, whereas cells treated with brentuximab vedotin prior to transplantation did not result in the formation of teratomas. These findings suggest that targeting the CD30-positive iPSC residual fraction reduces the tumorigenicity of human iPSC-derived β cells, potentially enhancing the safety of iPSC-based β cell replacement therapy (60).

Another strategy for eliminating pluripotent cells is the phototoxic approach. Indeed, in 2003, a new method for selective cell targeting was described, based on the use of light-absorbing microparticles and nanoparticles heated by short laser pulses to create highly localized cell damage (61). This strategy was then applied for the ablation of hPSC from differentiating cell cultures using antibodies directed against the hPSC surface markers Tra-1-60 and Tra-1-81, which were targeted with nanogold particles. Subsequent laser exposure resulted in 98.9 ± 0.9% elimination of hPSC by photothermolysis, while co-treated differentiated cells maintained their normal proliferation and differentiation potential. Moreover, the *in vivo* transplantation of treated mixed hPSCs/differentiated cell cultures revealed that laser ablation can strongly reduce the risk of teratoma formation (62). Alternatively, the PSC-specific fluorescent probe CDy1 was found to induce the selective death of murine and human PSC. CDy1 is a fluorescent rhodamine compound that induces the generation of reactive oxygen species in PSC and determines selective PSC death by simple visible light irradiation, without affecting other differentiated cells. Notably, a single 1 minute exposure of CDy1-stained PSC to visible light confirmed the inhibition of teratoma formation in mice (63).

Small Molecules

The first report of a small molecule that induced the selective cell death of hESC dates back to 2004, when Bieberich et al. described that, in tumors formed after engraftment of differentiated neuronal cells into the mouse brain, Oct-4 expression co-localized with that of PAR-4, a protein that mediates ceramide-induced apoptosis during neural differentiation of ES cells. They then demonstrated that a ceramide analog, N-oleoyl serinol (S18), can eliminate human $Oct4^{+}/PAR4^{+}$ cells and increase the proportion of Nestin-positive neuroprogenitors, and that this enrichment prevents teratoma formation (64). However, this strategy exploits the characteristics of pluripotent cells committed to neuronal differentiation and is therefore not applicable for differentiation into other lineages, including β cells. Instead, a feature common to pluripotent stem cells, which distinguishes them from all somatic cells, is their high susceptibility to DNA damage (65), as PSC commit programmed cell death even under low genotoxic stress to ensure genomic stability (66). This rapid apoptosis process results from the high induction of mitochondria-dependent cell death mechanisms, which can be mediated through several processes, such as cytoplasmic p53, mitochondrial translocation of BAX, or through the inhibition of ESC-specific anti-apoptotic proteins, such as BIRC5 (Survivin) or BCL10 (67). This peculiarity has therefore been widely exploited in research on small molecules capable of inducing the selective death of PSC, since adult stem/progenitor cells express other pro-survival proteins. For instance, it was demonstrated that a single treatment of PSC-derived cells with chemical inhibitors of Survivin, such as the flavonoid quercetin (QC) or YM155, induced the selective and complete cell death of undifferentiated hPSC and prevented teratoma formation, while differentiated cell types derived from PSC survived and maintained their functionality (68,69). Recently, it was reported that another natural flavonoid, luteolin, is even more potent than QC in selectively inducing PSC death in a p53-dependent manner (70). However, the effect of this molecule has not been explored *in vivo*. Similarly, the sequential administration of the mitotic drug Taxol at very low doses followed by the CDK inhibitor purvalanol A has been shown to eliminate Survivin activity; this drug combination was able to induce apoptosis in ESC and teratomas (71), although tissue analysis was performed only 18 h after transplantation and a longer follow-up was not reported. However, the efficacy of purvalanol A for PSC-derived teratoma eradication (together with two CDK1 inhibitors, dinaciclib and Ro-3306) in another study showed that inhibiting CDK1 leads to the activation of the DNA damage response and negative regulation of the anti-apoptotic protein MCL1 in human and mouse ESC, but not in differentiated cells (72). Apoptotic susceptibility to DNA damage in PSC was also tested using the genotoxic anti-tumoral drug etoposide, which effectively purged the population of residual teratoma-forming cells within the progenitor population of cells upon *in vivo* transplantation, without causing genomic instability in the

surviving progeny (73). Furthermore, Brequinar, an inhibitor of dihydroorotate dehydrogenase (DHODH), a key enzyme in the *de novo* pyrimidine synthesis pathway, was shown to be effective in inducing cell cycle arrest, cell death, and stemness loss in mouse PSC (74). However, its effect has yet to be evaluated in PSC of human origin.

Using compound screening, the ER stress induction molecule JC011 was found to induce cell death in PSC; undifferentiated cells pre-treated with this compound failed to form teratomas in immunodeficient mice (75). Using a similar approach, the screening of a library of cytotoxic compounds identified methyl 27-deoxy-27-oxookadaate, a substrate for two ATP-binding cassette transporters (ABCB1 and ABCG2) whose expression is repressed in PSC, as a reagent that selectively induces the death of human pluripotent stem cells (76). Similarly, the high-throughput screening of over 50,000 small molecules identified 15 pluripotent cell-specific inhibitors (PluriSIns) (77). Among these, PluriSIn#2 induces PSC selective death by suppressing the expression of topoisomerase, an enzyme essential for maintaining DNA integrity. Notably, topoisomerase IIα (TOP2A) is uniquely expressed in undifferentiated cells and is downregulated during their differentiation. PluriSIn#2 does not directly inhibit TOP2A enzymatic activity, but rather selectively represses its transcription, thereby significantly reducing TOP2A protein levels (78). Doxorubicin, a proven chemotherapeutic agent, is another inhibitor of topoisomerase II that has been shown to increase cardiomyocyte purity by removing potential proliferative stem cells from terminally differentiated cells. Doxorubicin, however, does not discriminate between the two isoforms of topoisomerase II (α, PSC- and cancer-specific, and β, expressed in almost all cell types). Therefore, in this study, it was crucial to determine the optimal doxorubicin dosage that prevented cell proliferation of residual undifferentiated stem cells while being non-cardiotoxic towards more terminally differentiated cells (79). However, although effective, strategies that induce oxidative stress or DNA damage should be carefully evaluated and used with caution, as they may increase the risk of DNA damage in differentiated cell types.

Another possibility that would allow for the selective elimination of pluripotent cells involves taking advantage of the different pathways of PSC compared to differentiated cells. PSC produce most ATP via glycolysis, transitioning to oxidative phosphorylation (OXPHOS) for most ATP production during differentiation (67). Cardiomyocytes, for example, produce the most energy using glucose, fatty acids, and lactate by OXPHOS. It has been demonstrated that these differentiated cells can be purified from PSC using a medium lacking glucose and glutamine, but supplemented with lactate (80). However, many other differentiated cell types cannot uptake and metabolize lactate, making this strategy cell-specific. In particular, this strategy would not be suitable for β cells, as glucose is fundamental for insulin release and β cells lack the lactate transporter MCT13 and have reduced expression of lactate dehydrogenase (81). Similarly, even the use of an inhibitor of glucose transporter 1 (GLUT1) such as STF-31, which is able to selectively kill undifferentiated PSC (82,83), is not applicable to the β cell field, as GLUT1 is the main glucose transporter in human insulin-secreting β cells (84). The response to treatment with high concentrations or deprivation of specific amino acids is also different between undifferentiated PSC and differentiated cells, and these differences may be used for the selective elimination of PSC. For instance, a high concentration of l-alanine was able to selectively eliminate undifferentiated iPSC co-cultured with differentiated cells (85); however, this strategy would not be feasible for PSC-derived insulin-secreting cells, as prolonged l-alanine exposure induces changes in metabolism, Ca^{2+} handling, and desensitization of insulin secretion in pancreatic β cells (86). L-methionine-free media were also tested as a PSC-depleting agent in combination with cell culture at 42°C, demonstrating that this combination of culture conditions is capable of preventing tumor formation upon iPSC subcutaneous transplantation (87). In addition, in this case, the strategy does not seem applicable to β cells, as L-methionine has recently been shown to prevent β cell damage and modulate the β cell identity marker MafA (88).

The most selective compound for achieving PSC-specific selective killing among the PluriSIns identified is PluriSIn#1, an inhibitor of stearoyl-coA desaturase (SCD1), which catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids. Even if the expression level of SCD1 in PSC is comparable with that of other cell types, the biosynthesis of oleate by SCD1 is a vital process in PSC, which is highly sensitive to SCD1 inhibition. As a result, PluriSIn#1 activates a cascade of events that culminate in the death of these cells via apoptosis after the induction of ER stress, mitochondrial ROS, and mitochondrial DNA damage. The treatment of a mixed population of pluripotent and differentiated cells for 48 h with PluriSIn#1 was reported to prevent teratoma formation in mice (77). However, the researchers did not show the analysis of the grafts of the animals that did not develop teratomas and did not confirm that the differentiated cells were the only ones to have survived.

Another molecule capable of acting on mitochondrial metabolism is MitoBloCK-6, an inhibitor of the mitochondrial redox protein Erv1/ALR, which induces apoptotic cell death via the selective release of cytochrome C in PSC, but which has no effect on differentiated cells (89). However, it remains unclear how PSC are specifically sensitive to MitoBloCK-6. Similarly, the mechanism of action of metformin, which has been shown to be effective in preventing the occurrence or in decreasing the size of teratomas after transplantation of iPSC in an apoptosis-independent manner, has not yet been elucidated. The hypothesis is that metformin suppresses the expression of Oct4 and Survivin, two pivotal genes of malignant stem cells responsible for teratocarcinoma growth, circumventing the suppression of AMPK (AMP-activated protein kinase (AMPK), which allows iPSC to avoid anabolic inhibition, similar to cancer cells (90).

Molecules capable of targeting various other elements of the cell, such as lysosomes, proteins, and pumps present on the cell membrane, have also been described. Recently, Chakraborty et al. explored the use of WX8 and apilimod as inhibitors of PIKfyve phosphatidylinositol kinase, which is essential for lysosome

homeostasis, to selectively kill PSC under conditions where differentiated cells remain viable (91). PIKfyve inhibitors prevent lysosome fission, induce autophagosome accumulation, and reduce cell proliferation in both pluripotent and differentiated cells, but induce death specifically in pluripotent cells by non-canonical apoptosis (91). Recently, it has been shown that bee venom (BV) can specifically induce cell death in iPSC but not in iPSC-derived differentiated cells; however, the cause of this selectivity has yet to be clarified. BV was found to rapidly disrupt cell membrane integrity and focal adhesions, followed by the induction of apoptosis and necroptosis in iPSC, with BV exposure remarkably enhancing intracellular calcium levels, calpain activation, and reactive oxygen species generation (92).

In another study, the cytotoxic effects of the US Food and Drug Administration (FDA)-approved cardiac glycosides (CG), such as digoxin and lanatoside C, on ESC were investigated (93). CG is a specific inhibitor of the transmembrane sodium pump Na^+/K^+-ATPase, which leads to an increase in the intracellular concentrations of calcium ions. ESC expressed Na^+/K^+-ATPase more abundantly than adult stem cells. Thus, the viability of the ESC-derived cells was not affected by digoxin and lanatoside C treatments. Furthermore, *in vivo* experiments have demonstrated that digoxin and lanatoside C prevent teratoma formation (93).

In general, there are no single small molecules suitable for all types of differentiation, as these compounds often exploit the biological properties of pluripotent cells potentially shared by differentiated cells (i.e., JC011 and 27-deoxy are toxic for neurons, and MitoBloCK-6 is toxic for cardiac development). Notably, all the depletion approaches presented thus far have proven to be effective in selectively killing PSC without damaging the differentiated cells and preventing or limiting teratoma formation. However, despite their proven efficacy, almost none of these strategies have been tested in PSC-derived β cells for diabetes cell therapy. In addition, there is also a need to develop an alternative safe approach to selectively eliminate PSC *in vivo* after accidental transplantation into patients. To this end, genome editing strategies may be a solution to this critical problem.

A SAFETY SWITCH FOR A SAFER CELL THERAPY

One strategy to fully control the cellular product, even after transplantation, is to equip cells with a suicide gene that can eliminate cells that have gone astray, since mutations can occur anytime and differentiated cells can undergo malignant transformation *in vivo* (**Figure 2**) (94). Ideally, the insertion of a suicide gene, which can be stably expressed in both quiescent and replicating cells, should not impair the pluripotency, differentiation, or genomic stability of PSC (95). The choice of the gene editing approach is based on the type of target cells that will be edited. Since gene editing of hESC or iPSC has a lower efficiency rate compared to other cell lines due to lower resilience to DNA damage (96), protocols designed *ad hoc* for human PSC must be adopted. The crucial components to be evaluated for the efficient gene editing of PSC are the choice of the delivery vectors and of the suicide genes with their relative selection marker, as the selection of edited cells is fundamental to obtain a pure edited population. Selection methods include the addition of antibiotic-resistant cassettes or genome-edited cell sorting based on the induced expression of fluorescent reporters or surface antigens (90–92). These selection strategies can also be combined to obtain a purer population or to select a cell population edited with more than one construct.

Viral and Non-viral Vectors

The vector is essential for the delivery of gene constructs to PSC. Currently, both viral and non-viral gene delivery systems are used to this end.

Among the most common viral delivery systems, retrovirus (RV), lentivirus (LV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), and baculovirus (BV) have a higher transduction efficiency for PSC than adenovirus (AV) and adeno-associated virus (AVV) (97,98). In particular, RV (99) and LV (100) have the highest transduction efficiency; however, they permanently modify the host genome with the risk of causing insertional mutations when randomly incorporated (101). Conversely, EBV (102), HSV (103) and BV (104) are non-integrating viruses that mediate transient gene expression in dividing and non-dividing cells (97). Additionally, AV does not integrate into the host genome and allows long-term transgene expression, as AV persists as an episome in the nucleus. However, due to the active cell division or proliferation of PSC, the percentage of transduced cells decreases over time (105,106). Viral gene delivery systems are primarily based on DNA, RNA, and oncolytic vectors. The vectors based on DNA deliver a plasmid containing the gene construct (107), while the RNA-based vectors provide RNA-dependent RNA polymerase complexes coupled with negative-strand RNA templates (108). The oncolytic vectors, an emerging weapon in the cancer field, are able to specifically target and lyse tumor cells (109).

Non-viral gene systems allow for construct delivery via physical or chemical methods, including electroporation or liposomes, which show less toxicity and immunogenicity than viral vectors; however, their transfection efficiency is orders of magnitude lower than that of viral vectors (110). Among the non-viral gene delivery systems described thus far, the scaffold/matrix attachment regions (SMARs) are non-integrating vectors suitable for PSC engineering and can autonomously replicate without causing molecular or genetic damage. Moreover, SMARs provide sustained transgene expression during the reprogramming and differentiation of PSC and their progenies (111).

Regardless of the strategy used, if the chosen vector integrates the genetic material, the insertion site is of fundamental importance, as random gene insertion may lead to perturbation of endogenous gene activity and the inactivation of a random gene, leading to the death of the targeted cell or cancer promotion (112). Thus, the installation of the suicide switch into a genomic safe harbor is fundamental for the establishment of a safe and efficient system. Among the known safe harbors in the human genome, namely AAVS1, CCR5, and the human homolog of murine ROSA26 locus, AAVS1 is the most studied for PSC gene editing, as no gross abnormalities or differentiation deficits were observed in PSC

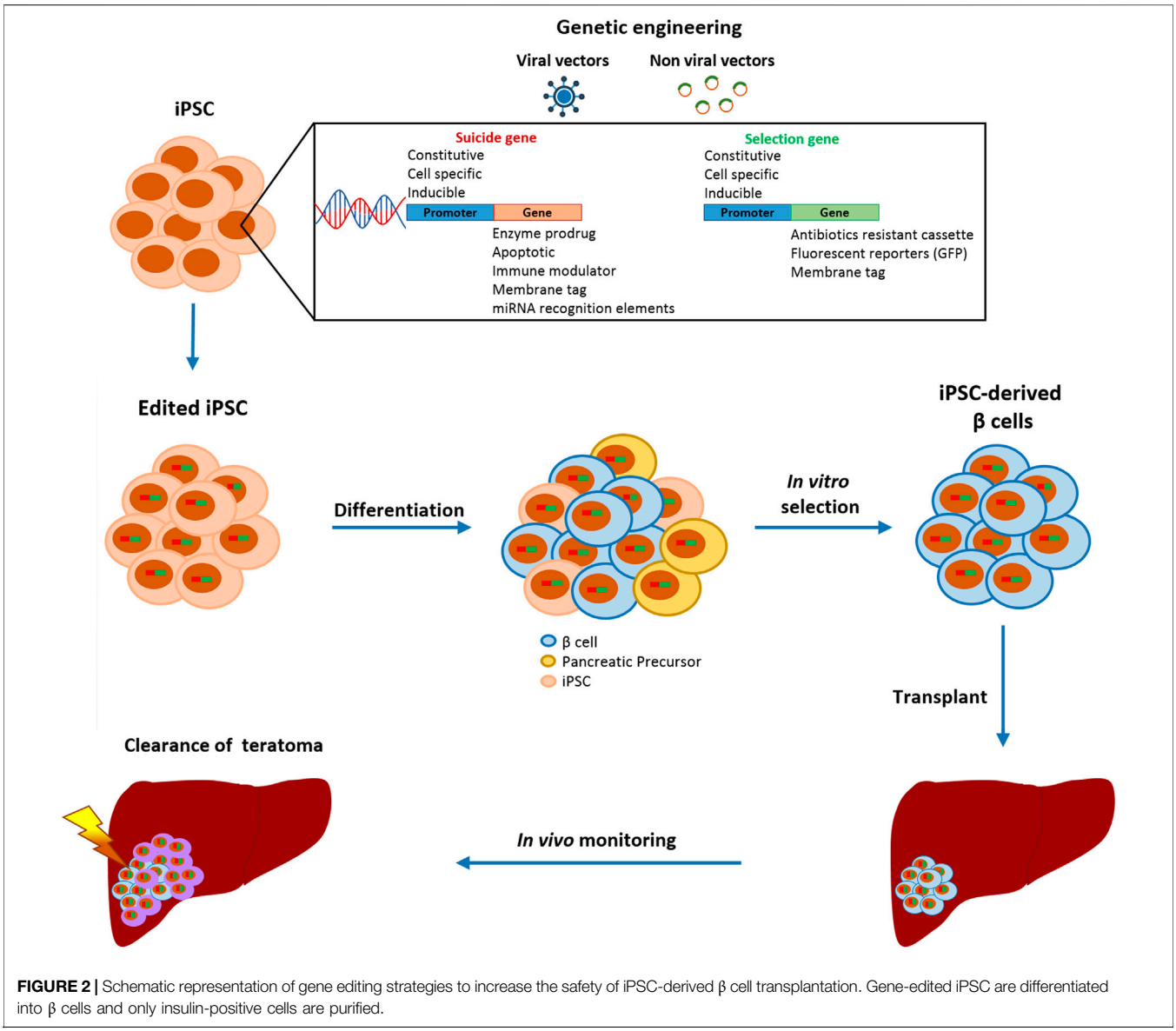

FIGURE 2 | Schematic representation of gene editing strategies to increase the safety of iPSC-derived β cell transplantation. Gene-edited iPSC are differentiated into β cells and only insulin-positive cells are purified.

harboring transgenes targeted in AAVS1 (112,113). Moreover, transgene expression at this locus is stable and consistent across different cell types (112,114).

To date, site-specific genome editing can be achieved by applying zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), or CRISPR/CAS9 systems (115). ZFN and TALEN are based on similar principles: they contain a FoxI endonuclease and exploit protein-DNA binding. Although both TALEN and ZFN have been applied for genome editing in human PSC (116,117), the ZFN system is the worst in terms of target specificity and off-target frequency (118). The more novel CRISPR system contains a Cas9 nuclease and has a binding principle based on RNA-DNA. Compared to ZFN and TALEN, the CRISPR/Cas9 system has the highest target specificity and lower off-target frequency (118,119). Therefore, CRISPR/Cas9 is becoming the most used system for the genetic manipulation of hPSC (120).

Suicide Genes

The choice of the gene and promoter to be used for its expression is of crucial importance for efficient gene editing. The most efficient and widely used suicide gene is herpes simplex virus thymidine kinase (HSV-TK), which induces apoptosis in edited cells upon treatment with ganciclovir (GCV) by inhibiting DNA synthesis (85). Schuldiner et al. were the first to demonstrate that using GCV enables the *in vivo* elimination of a teratoma originating from the injection of edited ESC into SCID mice using cells edited with a constitutive promoter, PGK, carrying the expression of the HSV-TK gene (121). However, this strategy is not applicable for selectively removing pluripotent undifferentiated cells from a heterogeneous cell preparation, as a constitutive promoter leads to the constitutive expression of the target gene in all undifferentiated and differentiated cells. Consequently, another possibility involves the selection of a

promoter that targets a gene specifically expressed by PSC, enabling the survival of differentiated progenitors. For example, adding a suicide gene under *TERT, OCT4*, *TERF1*, or *NANOG* promoters, which are highly expressed in a pluripotent state, can selectively remove undifferentiated cells (122–125).

The most commonly used suicide genes perform their function via enzymatic drug conversion activity, apoptotic potential, or the ability to direct the immune response against a cell by the addition of a tag (126). In particular, enzyme prodrugs can enzymatically convert an innocuous prodrug into a toxic compound that can kill the target cell (127). The toxic molecule generated can act only towards the edited cell or can have a broader action killing also the surrounding cells, called the "bystander effect," usually used to treat cancer (128). The most common prodrug enzymes used are HSV-TK, cytosine deaminase (CD) from *Escherichia coli* or yeast, and *E. coli*-associated nitroreductase (NTR), which make cells sensitive to the prodrugs GCV, 5-fluorocytosine (5-FC), and CB1954, respectively (127).

HSV-TK, CD, and NTR have been used as safety switches in the field of PSC (122,129,130), among which HSV-TK is one of the most studied and applied prodrug-activated enzymes (131). Interestingly, Rong et al. introduced the HSV-TK gene into the 3′-untranslated region of the endogenous NANOG gene in ESC and found that the safety switch allowed for the clearance of residual undifferentiated cells from differentiated neural populations *in vitro* and *in vivo* in an SCID mouse model (132). Another possibility involves introducing HSV-TK into human and murine ESC under the control of a cell division gene, such as *CDK1*, which is fundamental for the G2 to M phase transition. Specifically, Liang et al. introduced HSV-TK into the *Cdk1* 3′-untranslated region in homozygosity, which allowed for the maintenance of CDK1–TK expression without incurring a loss of gene function due to mutational events. Upon the transplantation of edited ESC-derived neural epithelial progenitors into mice, only the proliferative cells died after GCV administration, leaving the non-dividing differentiated cells intact (133). This approach may be interesting for application in PSC-derived β cells, as the final differentiated cell no longer has proliferation capacity compared to the PSC and progenitor cells. The miRNA regulatory system can also be used in suicide gene therapy strategies. For instance, the specific expression of the let7 miRNA family in differentiated cells, but not in pluripotent cells, has been exploited to construct an HSV-TK gene under the constitutive promoter human translation elongation factor 1A (EF1α) tagged to four tandem miRNA recognition elements (MRE) complementary to mature miRNAs of the let7 family. In this case, HSV-TK was specifically expressed in PSC that were selectively killed by GCV, whereas differentiated cells were fully protected (134).

Despite its effectiveness in killing target cells, some disadvantages of the HSV-TK system include immunogenicity, *in vivo* drug resistance, and the presence of inactivating mutations (94,134,135). Moreover, a recent study documented the acquisition of GCV resistance by iPSC expressing HSV-TK (87), underlining the need for the use of gene editing techniques that allow for insertion in genomically safe harbors that cannot be silenced.

Notably, a recent study on ESC-derived β cells applied a double fail-safe approach, capable of both killing residual PSC and selecting insulin-positive cells (130). Specifically, they used the HSV-TK cassette placed under the human telomerase reverse transcriptase (hTERT) promoter, which is highly expressed only by stem cells and tumor cells, to induce PSC-selective death when exposed to GCV. At the same time, nitroreductase (NTR) was used to select insulin-positive cells, as this construct is flanked by *loxP* sites and eliminated by Cre expression under the control of the human insulin promoter. Therefore, insulin-expressing cells are rendered insensitive to the prodrug CB1954. Using this method, only insulin-positive and non-proliferating cells survive selection, and cells that may de-differentiate after transplantation may still be selectively killed *in vivo* by GCV without affecting the rest of the graft (130).

Suicide genes, with apoptotic potential, are directly involved in triggering the apoptotic pathway. The most known are Fas ligand, Fas, FADD, caspase-3, caspase-8, caspase-9, p53, p33ING1, p73α, Bax, Apaf-1, IkappaBdN, Bcl-2, Bcl-x, and NBK (126), some of which have also been used to eliminate pluripotent cells. For instance, the inducible caspase-9 (iCASP9) suicide gene, under the control of the endogenous *OCT4* promoter, was applied to specifically kill undifferentiated PSC *in vitro* and *in vivo* (136). Similarly, the *SOX2* promoter has been exploited as a safeguard system for PSC-based therapies (137). However, *SOX2* is a less specific marker since it is also expressed in differentiated lineages, including ectoderm and endoderm (138,139). Thus, this strategy could not be applied to the β cell replacement field. Another study in iPSC used iCASP9 under the control of a constitutive promoter EF1α, which is able to eliminate pluripotent cells within 24 h of exposure to a chemical inducer of dimerization, AP20817 (140). Similarly, iCASP9, under the control of the synthetic promoter CAG, allowed for the killing and complete elimination of iPSC *in vitro* by inducible activation using AP1903, a lipid-dependent tacrolimus analog. In this case, a synthetic promoter was chosen to obtain higher expression levels (141).

Recently, a new drug-inducible safeguard combination has been adopted to eliminate *in vitro* and *in vivo* undifferentiated PSC. The construct NANOG-iCASP9, activated by the AP20187 molecule, has been used to induce PSC apoptosis and reduce the risk of teratoma formation prior to transplantation, while the construct ACTB-OiCASP9, activated by AP21967, killed all PSC-derived cell types to protect against *in vivo* adverse events. A third construct, ACTB-HSV-TK, activated by GCV, was used to kill all PSC-derived dividing cells *in vivo* (142). The iCASP9 suicide gene system is effective, safe, and less immunogenic owing to its human origin (143).

Another strategy to reduce the tumorigenic potential of ESC and iPSC involves exploiting the antitumor function of p53, which increases the gene copy number while retaining full pluripotency. Edited cells showed an improved response to anticancer drugs, which could aid in their elimination when tumors arise (144). Moreover, enzymatic activity already present in the cell, such as alkaline phosphatase (ALP), particularly overexpressed by iPSC, can be exploited to

selectively kill pluripotent cells. The peptide l-phosphopentapeptide, when dephosphorylated by ALP, forms intranuclear peptide assemblies that lead to cell death, but is innocuous to normal cells, which do not overexpress ALP (145).

Taking advantage of the recipient immune system represents another strategy to selectively kill a target cell. For instance, porcine xenoantigen α1,3-galactosyltransferase (GalT) was inserted under the control of the hTERT promoter in hESC. As in human serum, antibodies against the α-gal epitope and GalT expression are present only in edited PSC, and the immune system directly kills hESC upon transplantation, providing protection from *in vivo* cell dedifferentiation or *de novo* tumor formation that involves hTERT reactivation (146).

Marking cells with a distinctive tag expressed in the plasma membrane represents another method available in the field of suicide gene technology. The tag should preferably not be an immunogenic human sequence. This approach, which is mostly used in T cell transplantation, allows for the *in vivo* control of adverse events associated with the use of stem cell-derived differentiated cells. For instance, the overexpression of the CD20 tag has been assessed in combination with an anti-tag monoclonal antibody, which can be administered *in vivo*, for an antibody-dependent cytotoxic response (147). However, this strategy has yet to be applied to iPSC (148). One possible disadvantage of this method is that it incurs a toxic off-target effect if the antibody binds other cells that express the same receptor. Finally, a new frontier is the application of an engineered oncolytic virus to selectively replicate in and kill tumor cells. Mistui et al. developed a conditionally replicating adenovirus (CRA), and in particular, a variety of CRAs, such as Surv.m-CRA and Tert.m-CRA, that replicate only in undifferentiated cells as they are controlled by the Survivin and *TERT* promoters, which are more expressed in PSC (149).

In conclusion, gene editing represents a promising approach for the control PSC-derived cellular products, especially with regards to the elimination of cells with tumorigenic potential *in vitro* and intervening in time in case of tumor occurrence *in vivo*. Currently, there are many preclinical and clinical studies that confirm the validity of this approach. In general, for PSC gene editing, a vector capable of providing a stable and efficient insertion must be chosen because the stability of the insertion should be maintained in the pluripotent state and in the progeny, during cellular differentiation, and in the final differentiated stage. Moreover, accuracy is required to select the best promoter-gene construct expressed only in the target cell population, which does not undergo silencing, reduction of expression, or changes due to mutations. In addition, issues related to immunogenicity and toxicity of the inserted genes must also be considered.

DISCUSSION

A new source of insulin-producing cells would represent a significant step forward in cell therapy for the treatment of diabetes. Stem cells are strong candidates due to their infinite replication and differentiation capabilities, as well as their ability to be gene-edited. Among stem cells, iPSC are of particular interest because they can be derived from any individual, and there are numerous *in vitro* differentiation protocols capable of transforming them into β cells in an efficient and reproducible manner. Within the context of the use of iPSC-derived β cells in clinical applications, safety issues are an essential consideration. In this regard, we have identified and described four main steps to ensure the transplantation of safe cellular product in patients. First, iPSC must be reprogrammed with a non-integrating vector that is easily cleared from the cell, such as the latest generation of Sendai viruses. Second, the differentiated β cells must be purified as much as possible using the surface markers identified or in combination, such as GP2 at the precursor stage and CD49a at the β cell stage. However, if this selection is not 100% effective, treatments with molecules or antibodies that eliminate the residual stem component could be employed, for example adding PluriSIns or anti-CD30 monoclonal antibody to the iPSC derivatives. Finally, it is desirable to incorporate a suicide gene into iPSC, enabling the conversion of a non-toxic prodrug into an active cytotoxic compound that kills the cell itself. In this case, if tumor cells develop after transplantation, the graft can be eliminated by prodrug assumption. However, it must be taken into consideration that with the use of gene editing strategies, cell therapies will require further regulatory review steps to ensure patient safety.

AUTHOR CONTRIBUTIONS

SP, VZ, and VS designed the review, analyzed the existing literature and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Formation of Re-Aggregated Neonatal Porcine Islet Clusters Improves *In Vitro* Function and Transplantation Outcome

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Neonatal porcine islet-like cell clusters (NPICCs) are a promising source for islet cell transplantation. Excellent islet quality is important to achieve a cure for type 1 diabetes. We investigated formation of cell clusters from dispersed NPICCs on microwell cell culture plates, evaluated the composition of re-aggregated porcine islets (REPIs) and compared *in vivo* function by transplantation into diabetic NOD-SCID IL2rγ$^{-/-}$ (NSG) mice with native NPICCs. Dissociation of NPICCs into single cells and re-aggregation resulted in the formation of uniform REPI clusters. A higher prevalence of normoglycemia was observed in diabetic NSG mice after transplantation with a limited number (n = 1500) of REPIs (85.7%) versus NPICCs (n = 1500) (33.3%) ($p < 0.05$). Transplanted REPIs and NPICCs displayed a similar architecture of endocrine and endothelial cells. Intraperitoneal glucose tolerance tests revealed an improved beta cell function after transplantation of 1500 REPIs (AUC glucose 0–120 min 6260 ± 305.3) as compared to transplantation of 3000 native NPICCs (AUC glucose 0–120 min 8073 ± 536.2) ($p < 0.01$). Re-aggregation of single cells from dissociated NPICCs generates cell clusters with excellent functionality and improved *in vivo* function as compared to native NPICCs.

Keywords: islet transplantation, xenotransplantation, neonatal islet-like cell clusters, re-aggregated cell clusters, porcine islets, pseudo-islets

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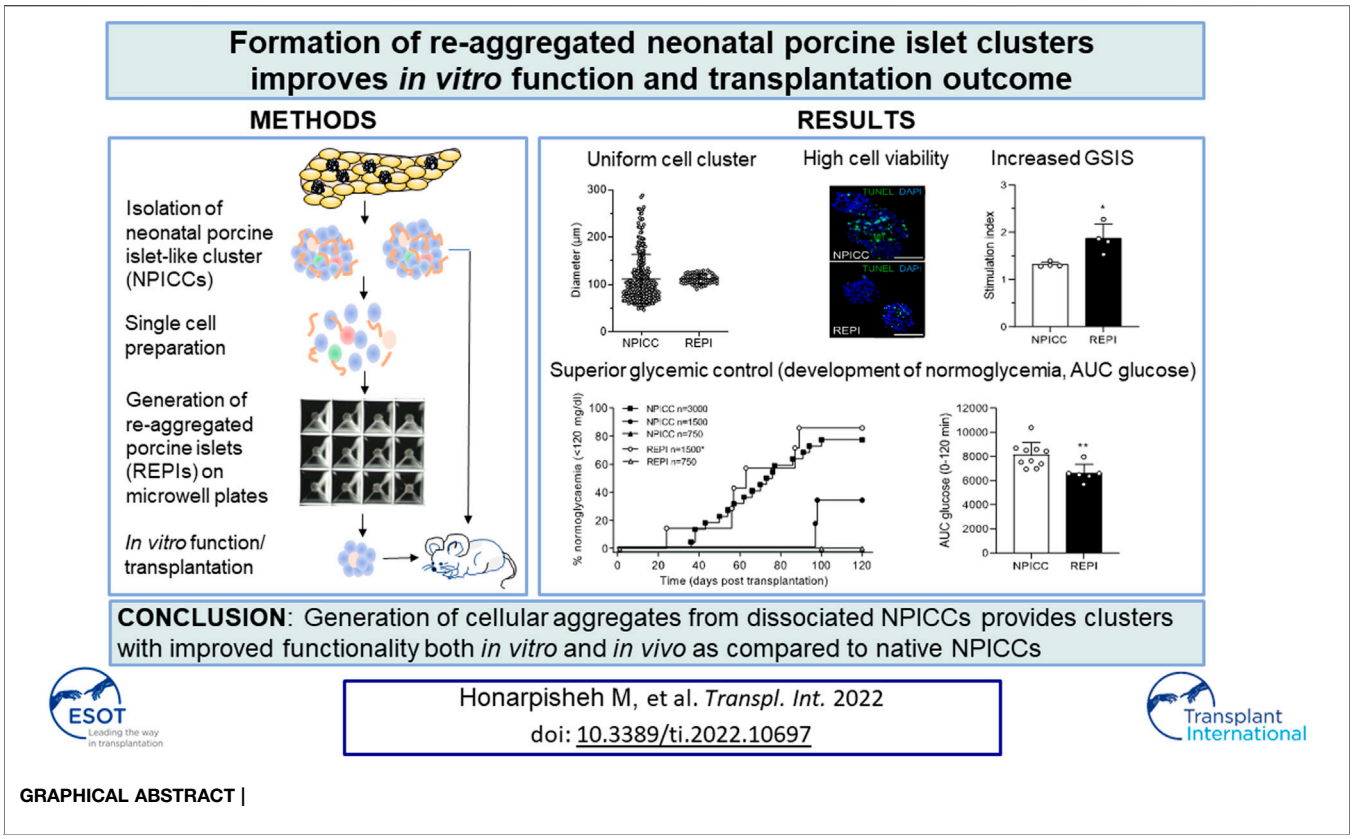
Abbreviations: AUC, area under the curve; IPGTT, intraperitoneal glucose tolerance test; NSG, NOD-SCID IL2rγ$^{-/-}$; NPICC, neonatal porcine islet-like cell cluster; REPI, re-aggregated neonatal porcine islet-like cluster.



GRAPHICAL ABSTRACT |

INTRODUCTION

Islet cell transplantation represents a promising therapy to achieve normoglycemia in patients with type 1 diabetes (1). Loss of islet mass during the isolation process and in the first days after transplantation is a major challenge for islet transplantation. This is mainly mediated by hypoxia due to the limitation of oxygen diffusion until sufficient revascularization has developed (2, 3). It has been shown that smaller islets display a better function *in vitro* and *in vivo* (4-6). Native mouse and human islets have been reported to spontaneously re-aggregate into cell clumps after being dissociated into single cells (7). Pre-defined uniform cluster size was achieved using the hanging drop culture or customized microwell devices (8-12).

Since the supply of high-quality human islets is limited by the paucity of organ donors, alternative cell sources such as porcine islet cells are demanded. Xenotransplantation of pig islets is very promising because supply of porcine islets is unlimited, techniques to isolate islets on a large scale are established (13), and recent progress on genetic modification of pigs has generated donor animals which provide islets which significantly decrease the severity of humoral and cellular immune responses (14). Successful long-term transplantation of neonatal and adult porcine islets into diabetic non-human primates (NHP) with insulin independence for a maximum of 965 days were described in several studies under systemic immunosuppression using potent co-stimulation inhibitors (15-17). However, translation of these studies to clinical trials is still limited because antibodies to block the CD40/CD154 costimulation pathway are not approved for application in human beings thus far (18).

Neonatal porcine islet like cell clusters (NPICCs) represent useful candidate cells for xenotransplantation (19) because they are easier to isolate as compared to adult islets and more robust against hypoxia and inflammation (20). The disadvantage is that NPICCs consist of immature cells and precursor cells which need several weeks for maturation after transplantation until glucose-dependent insulin secretion and normoglycemia have developed (13, 21). The requirement of a high number of islets to correct hyperglycemia after transplantation together with the lower yield from neonatal as compared to adult pig pancreas demands to increase either the isolation or the transplantation efficacy.

In the present study we investigated whether islet cell function can be improved by dissociation of NPICCs and re-aggregation into uniform cell clusters.

MATERIALS AND METHODS

Animals

German landrace hybrid piglets served as pancreas donors. NOD-SCID IL2rγ$^{-/-}$ (NSG) mice, which lack mature T cells, B cells and NK cells, were obtained from The Jackson Laboratory (strain 005557) and housed under standard SPF conditions. All animal

experiments were approved by the responsible authority and performed in agreement with the German Animal Welfare Act and Directive 2010/63/EU.

Islet Isolation and Generation of Dissociated Single Cells

NPICCs were isolated from pancreata (n = 12) of 2–5 days-old piglets by collagenase digestion as described previously (22, 23). Cell clusters were cultured in RPMI 1640 (PAN-Biotech, Aidenbach, Germany), 2% human serum albumin (Takeda, Konstanz, Germany), 10 mM nicotinamide, 20 ng/ml exendin-4 (Merck, Darmstadt, Germany) and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Germering, Germany) (basal islet culture [B-IC] medium). On day 4, NPICCs were harvested and islet equivalents (IEQ) were determined under a stereomicroscope. NPICCs were either re-cultivated in B-IC medium (control group) or washed with PBS and incubated in TrypLE Express solution (Thermo Fisher Scientific) for 8–12 min at 37°C with gentle mixing every 60 s until dispersion into single cells was observed under a stereomicroscope. Then, the cells were filtered through a 40-µm filter (Corning, Wiesbaden, Germany) to remove debris.

Formation of Re-aggregated Islet Cells

Dispersed islet cells were selected at random and seeded on Sphericalplate 5D (Kugelmeiers, Erlenbach, Switzerland), which contain 750 microcavities per well, in 2 ml B-IC medium to yield re-aggregated clusters composed of 750 cells. This cell number was chosen from previous studies reporting on an optimal islet aggregate size of about 100 µm (6, 10, 24, 25). Plates were centrifuged at 250xg for 3 min and incubated in B-IC medium for 3 days. Then, clusters were gently flushed out from the wells, washed with medium or buffer, and used for measurement of cell viability, glucose stimulated insulin secretion (GSIS), and transplantation.

Islet Cell Viability, Composition, and Recovery

Cell viability was detected by calcein AM (live cells) and propidium iodide (dead cells) dye staining according to the manufacturer´s instruction (Thermo Fisher Scientific). Samples were analyzed under a fluorescent microscope (n > 50 cluster) and by flow cytometry at day 7 after isolation. To determine the cluster architecture, NPICCs and REPIs were embedded in Epredia™ HistoGel™ Specimen Processing Gel (Thermo Fisher Scientific) and stained for insulin (guinea pig anti-insulin, 1:400, Agilent-Dako, Frankfurt, Germany) and glucagon (rabbit anti-glucagon, 1:100, Cell Signaling, Frankfurt, Germany) followed by incubation with FITC-labelled anti-rabbit IgG and Cy3-labeled anti-guinea pig IgG (Thermo Fisher Scientific). DAPI was used to counterstain cell nuclei. Recovery rate was determined by calculation of the ratio IEQ of REPIs to IEQ of native NPICCs. Apoptotic cells were analyzed on day 7 by TUNEL staining of NPICCs and REPIs using the DeadEnd Fluorometric TUNEL System assay according to the manufacturer´s instructions (Promega, Madison, WI, United States).

Static Glucose-Stimulated Insulin Secretion

On day 7 after isolation, 100–150 islet equivalent (IEQ) of NPICCs and REPIs were washed in glucose-free RPMI medium and Krebs-Ringer buffer (KRB) solution and pre-incubated in KRB containing 2.8 mmol/L glucose (low glucose) for 1 h at 37°C and 5% CO_2 followed by incubation in duplicates in KRB with low glucose or high glucose (20.0 mmol/L) for 1 h. Then, supernatants were collected and porcine insulin concentration was measured in duplicates by ELISA (Mercodia, Uppsala, Sweden). The stimulation index (SI) was calculated by dividing the insulin concentration in high glucose by insulin concentration in low glucose (23).

Transplantation

After induction of diabetes by intraperitoneal injection of 180 mg/kg body weight streptozotocin, diabetic NSG mice (blood glucose levels >350 mg/dl) received native NPICCs (3000, 1500, or 750 IEQs/mouse) or REPIs (750 or 1500 IEQs/mouse) under the left kidney capsule as described recently (22, 26). 3000 IEQs represent the standard dose of NPICCs for transplantation to achieve normoglycemia in about 80% of diabetic NSG mice. Blood glucose levels were monitored by FreeStyle Lite blood glucose test strips (Abbott, Wiesbaden, Germany). Diabetic mice with blood glucose levels >300 mg/dl were treated with insulin glargine (0.25–1 IE s.c. daily). Our primary endpoint was normoglycemia. The observation period was set to a maximum of 16 weeks. We used a stringent definition for normoglycemia which was specified as achievement of pretransplant glycemia (persistent random non-fasting blood glucose levels <120 mg/dl). This cut-off is based on the measurement of non-fasting blood glucose levels of untreated NSG mice (n = 107; 94.6 ± 15.2 mg/dl, range 62–128 mg/dl). In this control cohort there was no animal with non-fasting blood glucose levels above 120 mg/dl on two consecutive days. Because other studies often used non-fasting blood glucose levels <180 mg/dl to define normoglycemia after islet transplantation this threshold was also analyzed.

Characterization of Graft Function

Glucose tolerance in transplanted mice was determined by intraperitoneal glucose tolerance test (IPGTT) 10–14 days after development of normoglycemia using 2 g glucose/kg body weight. Blood samples were obtained from the tail vein at 0 and 10 min to measure porcine insulin in duplicates by ELISA (Mercodia) that had no cross-reactivity with mouse insulin. To provide evidence that normoglycemia was mediated by the grafted tissue and not by pancreatic islet regeneration, graft bearing kidneys were removed in three transplanted animals followed by daily blood glucose measurements for 3 days. When the post uninephrectomy blood glucose level was >400 mg/dl, the achieved normoglycemia was considered as graft-dependent.

Cellular and Morphological Characteristics Analyzed by Immunohistochemistry

Paraffin sections of the graft bearing kidney were stained with the following antibodies (Ab): guinea pig anti-insulin (1:400, Agilent-Dako), rabbit anti-glucagon (1:100, Cell Signaling), rabbit anti-somatostatin (1:50, Agilent-Dako), rabbit anti-pancreatic polypeptide (PP) (1:5000, Proteintech) and rabbit anti-CD31 (1:100, Cell Signaling). Secondary antibodies used were HRP- or alkaline phosphatase-conjugated anti-guinea pig IgG (Agilent-Dako) and anti-rabbit IgG (Vector Laboratories, California, United States). Fuchsin + substrate chromogen (Agilent-Dako) or 3,39-diaminobenzidine (Kem-En-Tec Nordic A/S, Uppsala, Sweden) were used as chromogen. To visualize glucagon and PP, the ImmPRESS® HRP horse anti-rabbit IgG polymer detection kit (Vector Laboratories) was used.

Cellular composition of NPICCs and REPIs were quantified by QuPath software (version 0.3.2) using pictures scanned by uScope MXII slide scanner (Microscope International, Dallas, United States). The numbers of cells that stained positive for glucagon, somatostatin or PP were expressed as number of positive cells per 100 insulin positive beta cells. To assess vascularization, the area of CD31 positive cells (endothelial cell marker) were detected and normalized to the islet area.

Flow Cytometry

Accuri C6 flow cytometer (BD Biosciences, Heidelberg, Germany) was used to determine the number of insulin-, glucagon-, and somatostatin-positive cells in NPICCs and REPIs. Single cells were prepared by digesting cell clusters with TrypLE solution (Thermo Fisher Scientific), washed with PBS +10% fetal calf serum (FCS), and filtered through a 30 µm pre-separation filter (Miltenyi, Bergisch-Gladbach, Germany). Then, cells were fixed/permeabilized with an intracellular staining buffer set (Thermo Fisher Scientific) and incubated with Fc-Block (anti-mouse CD16/CD32) for 10 min at room temperature. Thereafter, cells were stained with fluorochrome-labeled antibodies against insulin (anti-insulin-AF647, clone T56-706), glucagon (anti-glucagon-PE, clone U16-850), and somatostatin (anti-somatostatin-AF488, clone U24-354) (BD Biosciences). All antibodies were pretested for appropriate dilution and specificity using isotype control antibodies. Antibodies were incubated at 4°C for 30 min, washed two times with permeabilization buffer and analyzed on a flow cytometer with FlowJo software version 10.4 (TreeStar, Ashland, United States).

Statistical Analysis

Data are presented as means and standard deviations (SD). Statistical differences between two groups were analyzed with Shapiro-Wilk test for normality and F test for homogeneity of variance and examined with the Mann-Whitney *U* test. Time to normoglycemia was compared with log-rank test. The area under the curve (AUC) was calculated using trapezoidal rules. p values <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism (version 9.2, GraphPad, San Diego, United States).

RESULTS

Re-Aggregated Cells Show Uniform Size and Improved *In Vitro* Function

On day 3 after isolation, NIPPCs were dissociated by mild TypLE treatment resulting in single cells with a viability of 88.2 ± 5.6%. After 3–4 days of seeding in 3D-cluture plates, clusters composed of 750 re-aggregated cells showed a uniform size with a mean diameter of 113.3 ± 10.5 µm (range 94.8–130.4, median 113.4 µm) whereas the diameter of native NPICCs was much more heterogeneous (range 50.1–289.6 µm, median 95.2 µm) (**Figures 1A–C**). The size variation of native NPICCs was significantly higher as compared to REPIs ($p < 0.01$). As expected, there was cell loss during the cell dissociation and re-aggregation process. The recovery rate, defined as IEQ of REPIs compared to control NPICCs on day 7 after isolation was 63.5 ± 18.9% (**Figure 1D**).

Fluorescence microscopy revealed that insulin-positive cells were more uniformly dispersed and their relative abundance was increased in REPIs as compared to NPICCs (**Figure 1E**). This was confirmed by flow cytometry where slightly higher percentage of insulin- and glucagon-positive cells were detected. The proportion of somatostatin-positive cells was not altered. The percentage of endocrine cells (sum of alpha, beta and delta cells) was significantly increased in re-aggregated clusters (55.9 ± 2.2% vs. 50.0 ± 2.2% in NPICCs) ($p < 0.05$) (**Figure 1F**).

Glucose-dependent insulin secretion was assessed in four independent experiments with handpicked NPICCs and REPIs. REPIs exhibited a significantly higher GSIS (1.9-fold) as compared to native NPICCs (1.4-fold) ($p < 0.05$) (**Figure 1G**). Taken together, compared to conventional NPICCs, REPIs had a higher homogeneity in size, contained higher proportions of endocrine cells, and could serve as a suitable source for *in vivo* transplantation.

REPIs Display Improved Function After Transplantation

We next validated the *in vivo* function of REPIs in diabetic NSG mice by transplanting 750 and 1500 IEQ REPIs. We included groups transplanted with 750, 1500, and 3000 IEQ native NPICCs as controls. All animals remained diabetic after transplantation of 750 native NPICCs or 750 REPIs (**Figure 2A**). Prevalence of non-fasting blood glucose levels <120 mg/dl was 85.7% in mice transplanted with 1500 IEQ REPIs ($n = 6$ of 7) (median diabetes reversal time 63 days) as compared to around 33% ($n = 2$ of 6) in those transplanted with 1500 native NPICCs (median diabetes reversal time 98 days) ($p < 0.05$) (**Figure 2A**). Notably, the reversal rate and median diabetes reversal time of mice transplanted with 1500 REPIs (85.7% 63 days) were comparable to those of mice with 3000 native NPICCs (77.2%; 66 days), indicating a higher efficacy per IEQ unit for REPIs.

Similar results were obtained by using blood glucose levels <180 mg/dl as the threshold for normoglycemia. Percentage of mice below this cut-off was significantly higher

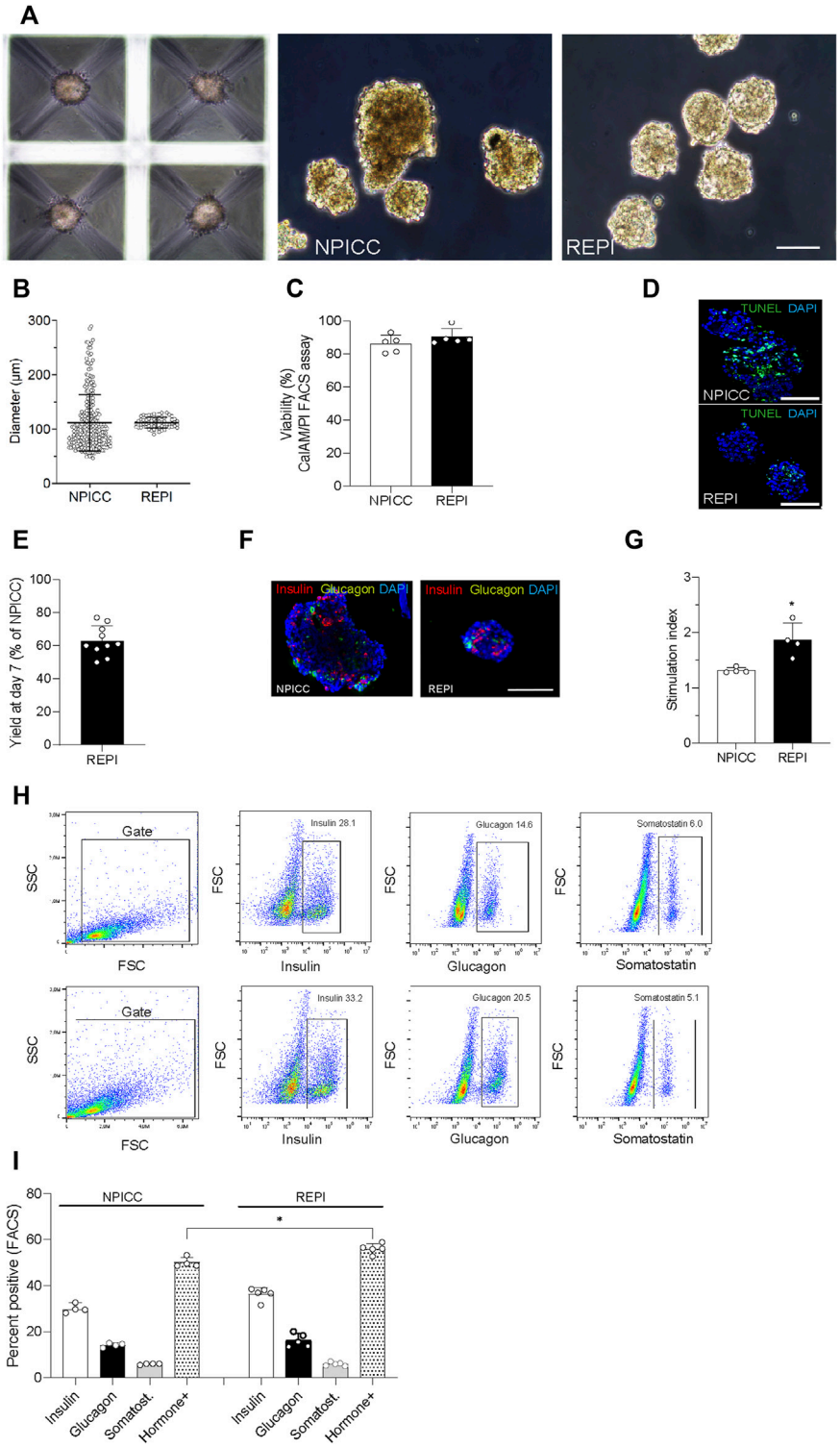


FIGURE 1 | Phenotype and architecture of native neonatal porcine islet-like clusters (NPICCs) and re-aggregated porcine islets (REPIs) on day 7 after isolation. **(A)** Phase contrast pictures of cells cultured in Sphericalplate 5D with 750 cells per microwell (left) and morphology of NPICCs (middle) and REPIs (right) after harvesting. Scale bars, 100 µm. **(B)** Size distribution of REPIs and NPICCs from $n = 5$ pancreata. Box plots with median diameter, 25th-75th percentile and minimum and maximum (whiskers). **(C)** Viability of cells analyzed by FACS (right) and fluorescence microscopy (left) using calcein AM (green, live cells) and propidium iodide (PI) (red, dead cells) dye staining ($n = 5$). **(D)** TUNEL assay revealed many TUNEL positive cells (green) in the inner core of large NPICCs. Scale bars, 100 µm **(E)** Recovery rate of REPIs as percentage of NPICCs (n = 10). **(F)** Immunofluorescence staining of insulin (red) and glucagon (green) in NPICCs and REPIs ($n = 4$). Scale bars, 100 µm. **(G)** Measurement of *in vitro* beta cell function assessed by glucose stimulated insulin secretion ($n = 4$). **(H)** Representative flow cytometric characterization of insulin, glucagon and somatostatin positive cells. **(I)** Analysis of the percentage of insulin (white bars), glucagon (black bars) and somatostatin (grey bars) positive cells and sum of hormone positive cells (dotted bars) ($n = 4$). Data are presented as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$ vs. NPICCs.

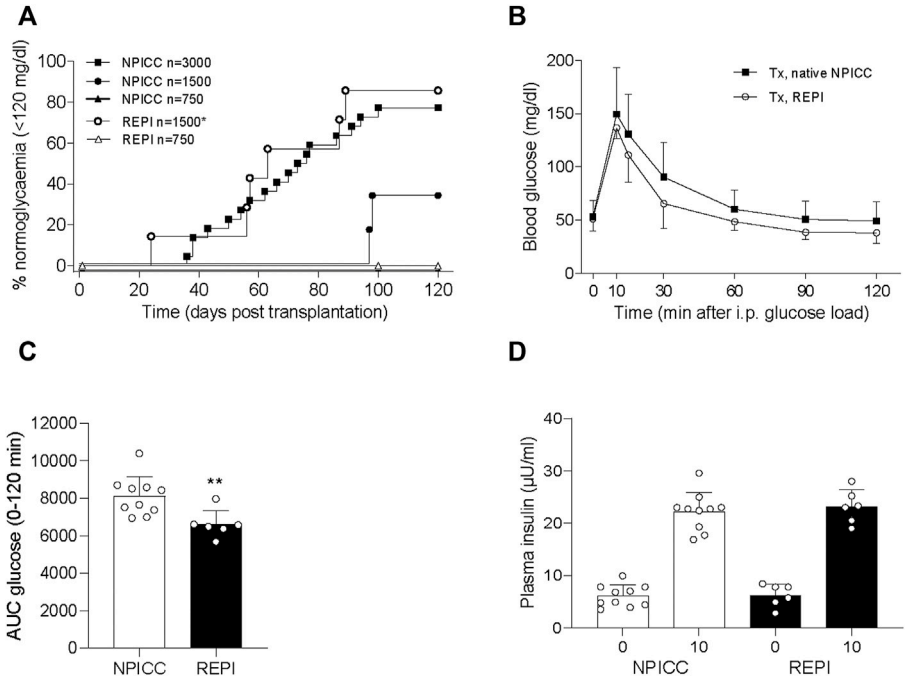


FIGURE 2 | Transplantation with REPIs improved reversal of diabetes. **(A)** Kaplan-Meier analysis of time to develop normoglycemia in diabetic NSG mice transplanted with 750 ($n = 4$) and 1500 REPIs ($n = 7$) or 750 ($n = 4$), 1500 ($n = 6$) and 3000 NPICCs ($n = 22$) on day 7 after isolation. After transplantation with 1500 REPIs significantly more animals developed normoglycemia defined by non-fasting blood glucose <120 mg/dl compared to the group transplanted with 1500 NPICCs (*$p < 0.05$). **(B–D)** Intraperitoneal glucose tolerance test (IPGTT) in diabetic NSG mice transplanted with 1500 REPIs ($n = 6$) or 3000 NPICCs ($n = 10$). **(B)** Glucose response curve during the IPGTT. **(C)** Glucose clearance during IPGTT assessed by calculating area under the curve (AUC) for glucose 0–120 min was improved in animals transplanted with REPIs ($n = 6$) **$p < 0.01$ vs. NPICC group. **(D)** Insulin secretion at 0 and 10 min after glucose challenge was similar in both transplantation groups. Data are presented as the mean ± SD.

in animals transplanted with 1500 IEQ REPIs (85.7%, median time 59 days) versus 1500 native NPICC (50%, median time 90 days) ($p < 0.05$) (**Supplementary Figure S1**).

We further performed IPGTT in mice developing normoglycemia. As shown in **Figure 2C**, the glucose clearance in the group that received 1500 REPIs (AUC 6260 ± 305.3) was significantly better than that in the group with 3000 NPICCs (AUC 8073 ± 536.2) ($p < 0.01$). Plasma insulin levels at 0 and 10 min during IPGTT were similar in all groups (**Figure 2D**). These data suggested that the re-aggregation of clusters from dispersed NPICCs resulted in a significant improvement of *in vivo* function.

Architecture of Re-Aggregated Clusters

To characterize the composition of endocrine cells and the density of vascularization following transplantation with NPICCs and REPIs, immunohistochemistry was performed at the end of the post-transplantation period. Three grafts per group were stained to detect endocrine cell components including insulin, glucagon, somatostatin and pancreatic polypeptide (PP) positive cells. Although there was a random rearrangement of endocrine cells in the first days after re-aggregation, this picture changed during *in vivo* maturation. As shown in **Figure 3**, the grafts derived from REPIs and from NPICCs consisted of a core of insulin-positive cells, surrounded by glucagon-, somatostatin- and PP-positive cells, which mainly located in the islet mantle (**Figures 3A,B**), suggesting the spatial reorganisation of endocrine cells *in vivo* during the post-transplant period.

There were small differences in the morphology between REPI and NPICC grafts. The relative proportion of insulin-positive cells in REPIs was slightly higher than in NPICC grafts (89.1 ± 3.4% vs. 82.6 ± 3.2%) . Conversely, glucagon- (10.9 ± 3.3% vs. 17.3 ± 3.2%) and somatostatin- (0.2 ± 0.1% vs. 2.3 ± 0.1%) positive cells were slightly decreased while the relative proportion of PP-positive cells was significantly reduced in REPI grafts (0.2 ± 0.1% vs. 1.2 ± 0.2%) as compared to NPICC grafts from the same pig donors ($p < 0.01$). (**Figure 3B**). Quantification of CD31 staining within the grafts revealed no difference in endothelial cell (EC) area in both groups (**Figures 3C,D**).

DISCUSSION

This study provides evidence that the *de novo* formation of uniform cell clusters from neonatal porcine islets in 3D-culture plates has the potential to generate pseudo-islets with a higher proportion of endocrine cells, an enhanced *in vitro*

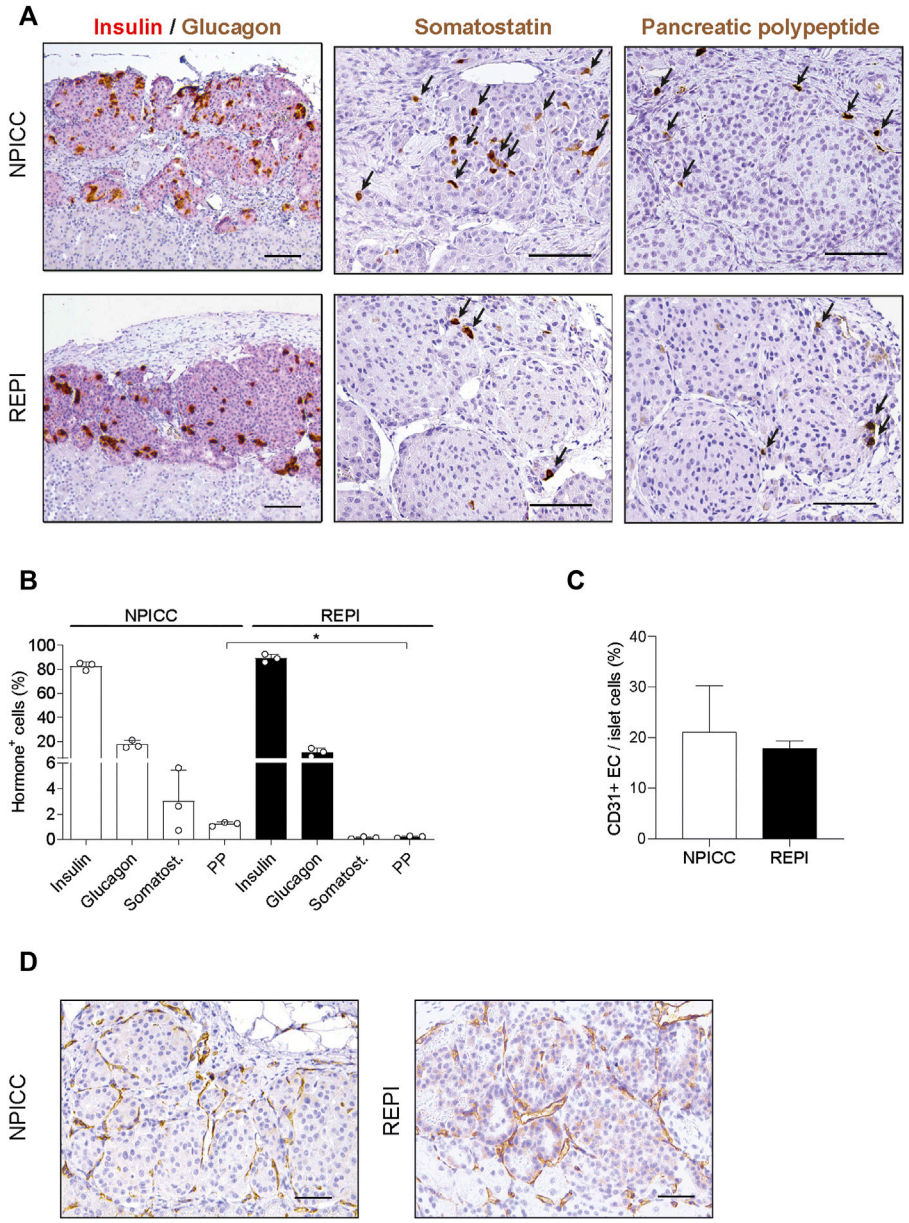


FIGURE 3 | Architecture and revascularization of transplanted grafts. **(A)** Representative immunohistochemical staining for insulin (red)/glucagon (brown), somatostatin (brown, arrows) and pancreatic polypeptide (PP) (brown, arrrows) in sections of grafted NPICCs and REPIs (1500 IEQ). **(B)** Quantification of endocrine cells within the grafts revealed a similar proportion of insulin, glucagon and somatostatin cells and a significant lower frequency of PP cells in grafted REPIs ($n = 3$ per group). $^{**}p < 0.01$. **(C)** Quantification of CD31 positive endothelial cells (EC) by immunohistochemistry ($n = 3$ per group). The results are expressed as CD31 positive area normalized to islet area. **(D)** Representative images of CD31 staining in grafts. Characteristic CD31 staining (brown) of blood vessels in the grafts. Data are represented as the mean ± SD. Scale bars, 100 µm.

function and an improved transplantation outcome than native NPICCs.

The study was initiated to evaluate the performance of REPIs in comparison to NPICCs originated from the same donor animals. For the first time we were able to show that neonatal porcine islets, which are mainly composed of immature endocrine cells and progenitor cells, benefit from single-cell preparation and re-aggregation in a similar way as reported from studies using pseudo-islets derived from dissociated adult human and rat islets. This includes an improved *in vitro* glucose responsiveness and a changed pattern of endocrine cell distribution of the gravity-mediated, newly formed cell clusters as compared to native islets (6, 11, 25, 27). In large NPICCs, we frequently observed a dark core with absent DAPI nuclear stain suggesting dead cells or components of extracellular matrix which increased by time of culture. By single cell preparation these

dead cells can be eliminated. After formation of weakly aggregated size-controlled clusters the diffusion of oxygen and essential nutrients may be accelerated (7, 25, 28). This improved supply may be one explanation why REPIs had a significantly higher percentage of endocrine cells and an increased *in vitro* insulin secretion capacity in response to high glucose.

The number of high-quality islets and the lag time to reach a full functioning of beta cells due to hormonal immaturity remains a major problem in NPICC transplantation. The most important question of the present study was whether the preparation of REPIs enables to improve development of normoglycemia after transplantation into diabetic mice. We used the marginal islet mass transplant model to test the ability of REPIs to reverse hyperglycemia. 1500 REPIs performed significantly better than 1500 native NPICCs and achieved diabetes reversal in a similar time frame as after transplantation of 3000 NPICCs. Improved outcome after transplantation of a limited number of re-aggregated rat and human islets as compared to control islets was described in several studies (6, 7, 11, 12). Thus, REPIs clearly provide an advantage over native NPICCs. In part, this may be due to a better hypoxia tolerance in the first hours after transplantation mediated by the increased oxygen tension due to a lower diffusion distance in comparison to the large NPICCs. Small native and re-aggregated human and rat islets have been shown to undergo a less sustained period of hypoxia and to develop a reduced necrotic core after transplantation underneath the kidney capsule or into subcutaneous tissue (4, 5, 7, 11). By immunohistochemistry a decreased number of PP cells as well as a trend towards a lower alpha and delta cell frequency was detected at the end of the observation period. Lower intraislet somatostatin and glucagon secretion may be another explanation for the significantly increased glucose clearance during IPGTT in mice transplanted with REPIs. Previous studies have shown that re-aggregated pseudo-islets develop a higher vessel density because they are more easily penetrated by newly forming capillaries (12). We detected similar volumes of CD31-positive endothelial cells in the grafts suggesting no significant differences in vascularization between both transplantation groups.

One important problem of the preparation of REPIs is the cell loss observed during single cell production and re-aggregation culture. To improve cell recovery from isolated NPICCs we performed gentle cell dissociation under visual controls to avoid over-digestion, rapid cell washing steps and centrifugation-forced cluster formation on 3D-culture plates. Nevertheless, the calculated recovery rate of REPIs in relation to NPICCs was only 63.5%. This cell yield seems to be low, but is in the range of the 50%–90% cell loss described in most studies using dissociated adult human and rat islets (4, 6, 7). The low cell yield may only partly explained by the cell dissociation process itself, because the cell viability was similar in REPIs and NPICCs. The elimination of damaged and irrelevant non-endocrine or inflammatory cells and the removal of extracellular matrix from the inner core of the islets may be one important factor for the observed reduction of REPI volume resulting in a decreased IEQ ratio. Considering not only the recovery ratio but also the equal transplantation outcome of 1500 REPIs and 3000 NPICCs, the use of REPIs results in an enhanced efficiency (1.2-fold per organ donor pancreas) for transplantation of diabetic mice with a curative islet dose. Thus, the better performance of REPIs fully compensates for the tissue loss during the dissociation and re-aggregation procedure.

In conclusion, we here demonstrate a simple and efficient method to easily produce re-aggregated clusters composed of neonatal porcine islet cells in a microwell format which has the potential for large scale generation of REPIs. The formation of REPIs has a significant impact on *in vitro* and *in vivo* beta cell function and the outcome of porcine islet transplantation. REPIs may facilitate future studies on NPICC cell manipulation to induce progenitor and beta cell proliferation and differentiation in order to increase pre- and post-transplant beta cell mass. The availability of size-defined clusters represent a substantial improvement for studies aiming to use encapsulated porcine islets. Moreover, it will become possible to fabricate clusters composed of different cell types including endothelial cells and immunomodulatory cells to further improve vascularization and to modulate graft rejection, thereby generating an improved self-protective islet graft. This will be the focus of our studies in the near future.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Regierung von Oberbayern.

AUTHOR CONTRIBUTIONS

MH, YZ, YL, LW-VB, and JS contributed to research design, conducted experiments, data analysis, and writing of the paper. EK, MK, AL, and EW performed animal breeding, explants of the pancreas and contributed to writing of the paper. All authors were involved in the critical discussion of the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10697/full#supplementary-material

Supplementary Figure S1 | Analysis of time to develop non-fasting blood glucose levels <180 mg/dl. After transplantation with 1500 REPIs (n = 7) significantly more animals developed glycemia below this cut off as compared to animals transplanted with 1500 NPICCs (n = 6) (*p < 0.05).

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ORIGINAL RESEARCH
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Preferred Islet Delivery Device Characteristics and Implantation Strategies of Patients With Type 1 Diabetes

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Islet delivery devices (IDDs) offer potential benefits for islet transplantation and stem cell-based replacement in type 1 diabetes. Little is known about patient preferences regarding islet delivery device characteristics and implantation strategies. Patient preferences for IDDs and implantation strategies remain understudied. We invited patients, parents and caregivers to fill in an online questionnaire regarding IDDs. An online survey gathered responses from 809 type 1 diabetes patients and 47 caregivers. We also assessed diabetes distress in a subgroup of 412 patients. A significant majority (97%) expressed willingness to receive an IDD. Preferred IDD attributes included a 3.5 cm diameter for 37.7% of respondents, while when provided with all options, 30.4% found dimensions unimportant. Respondents were open to approximately 4 implants, each with a 5 cm incision. Many favored a device functioning for 12 months (33.4%) or 24 months (24.8%). Younger participants (16–30) were more inclined to accept a 6 months functional duration ($p < 0.001$). Functional duration outweighed implant quantity and size ($p < 0.001$) in device importance. This emphasizes patients' willingness to accommodate burdens related to IDD features and implantation methods, crucial for designing future beta cell replacement strategies.

Keywords: islet transplantation, survey, type 1 diabetes, islet delivery device, patient preference

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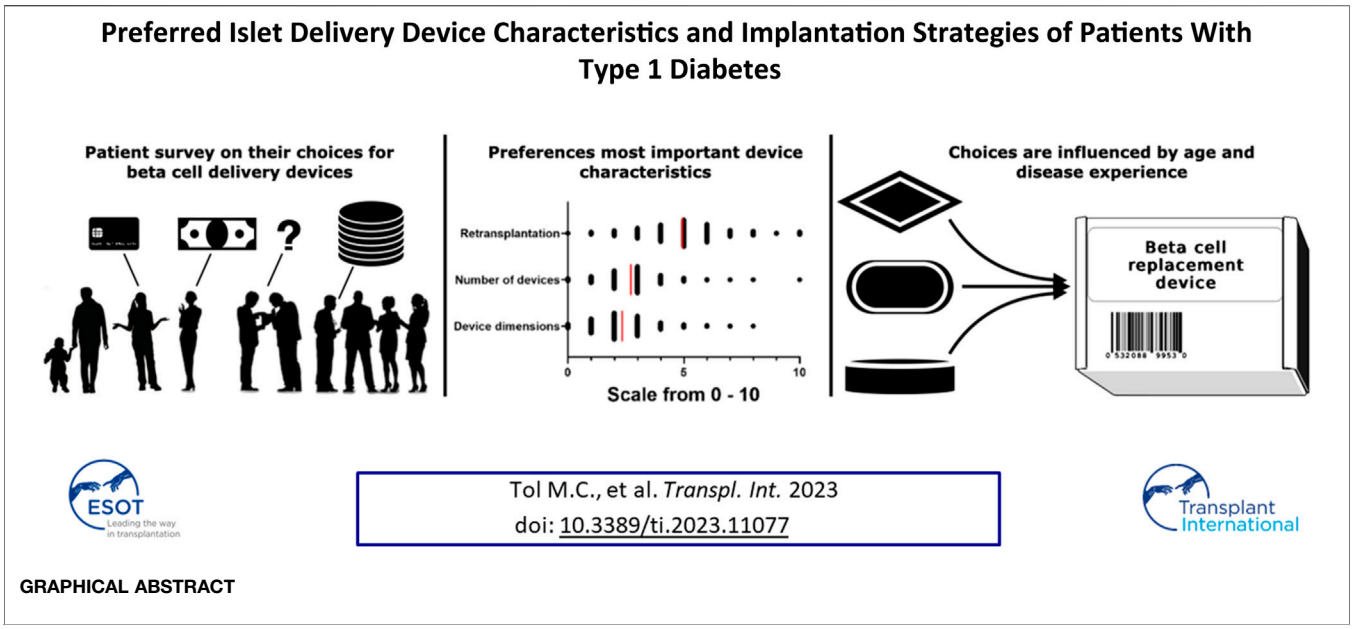
INTRODUCTION

In type 1 diabetes (T1D) insulin-producing beta cells are destroyed by the immune system and patients are dependent on life-long administration of exogenous insulin for glycemic control and survival [1]. Allogeneic islet transplantation (ITx) in the portal vein of the liver is performed in a small group of patients with T1D and severe problems with glycemic control and/or complications [2]. Usually due to an insufficient transplantable islet mass, instant blood mediated inflammatory reaction (IBMIR) and long-term islet attrition in the liver only a minority of patients will have long-lasting clinically relevant islet graft function [3, 4]. In the last 2 decades, researchers have tried to improve the efficacy of ITx with so-called islet delivery devices (IDDs) [5–7].

Islet delivery devices exist in many sizes, shapes, with or without different compartments and are made from different (bio)materials [7–9]. It has been proposed that IDDs could support ITx at an



GRAPHICAL ABSTRACT

extrahepatic site and potentially increase long-term functional capacity of transplanted islets [10]. There are two main types of islet delivery devices. Open devices support direct islet vascularization and efficient exchange of nutrients but require immunosuppressive medication [11]. Closed or immunoprotective devices are designed to prevent direct contact between the grafted cells and host immune cells thereby potentially preventing graft rejection. Recent developments in the generation of pluripotent stem cell-derived islets have focused more attention on the role of IDDs.

Despite the tremendous technical progress in the field of IDDs, there is a lack of information on user preferences. It is also unclear how diabetes distress and glycemic control affects preferences. In the current study, we evaluated preferences on IDD characteristics and implantation strategies in a cross-sectional study amongst a large group of Dutch patients with T1D.

MATERIAL AND METHODS

Subjects

Individuals aged 16 years and older with T1D were approached and invited to fill out a questionnaire about device preferences anonymously. One group of patients was approached by providing study information and a link to the questionnaire on various Dutch online platforms for patients with type 1 diabetes: Dutch Diabetes Research Foundation, Dutch Diabetes Association, Juvenile Diabetes Research Foundation (JDRF) Netherlands, Dutch Diabetes Meeting Point, diabetestype1.nl and Regenerative Medicine Crossing Borders (RegMedXB). Parents of children diagnosed with T1D younger than 16 years were also invited to participate. A second group of patients who had visited the diabetes outpatient clinic at the Leiden University Medical Center (LUMC) in the Netherlands during the past 2 years, were contacted by e-mail and invited to participate with a link to the online questionnaire.

Questionnaires

We developed a web-based questionnaire (Qualtrics, **Supplementary Appendix SA**) for self-reported background information (age, sex, time since diagnosis T1D, most recent time in range (TIR), most recent hemoglobin A1c (HbA1c), current treatment and current treatment center) and preferences regarding specific aspects of islet delivery devices and implantation strategies. These preferences comprised implant sites and device characteristics such as the number of devices, the dimensions and the minimal duration of function. Respondents were invited to add explanatory remarks to their answers. Explanatory remarks were coded into categories and validated by a second investigator. The second group of patients from the LUMC were also requested to complete the 20-item Problem Areas In Diabetes (PAID) questionnaire [12]. The anonymous data were collected from October 2021 until May 2022.

Data Handling and Analysis

Incomplete questionnaires were excluded from data analysis. A single respondent who indicated sex to be *other* was excluded from univariate and multivariate analysis by sex. Age categories were pre-specified in the questionnaire (16–30, 31–50, 51–70, >70 years, or parent/caregiver). PAID scores were categorized as low (0–16), moderate (17–39) or high (40–100) diabetes distress [13]. HbA1c levels were reported in mmol/mol Hb and if necessary converted from a percentage by the formula "mmol/mol Hb = (10.93 × %)–23.5". Data were analyzed in RStudio (version 2023.03.1) and GraphPad Prism (version 9.3.1) with $\alpha = 0.05$. Multivariate multinomial logistic regression was performed on variables with categorical

TABLE 1 | Patient characteristics.

	Overall (*N* = 856)
Sex	
Male	358 (41.8%)
Female	497 (58.1%)
Other	1 (0.1%)
Age (years)	
16–30	155 (18.1%)
31–50	311 (36.3%)
51–70	304 (35.5%)
>70	39 (4.6%)
Parent or caregiver	47 (5.5%)
Disease duration (years)	
<5	113 (13.2%)
5–15	196 (22.9%)
16–25	171 (20.0%)
>25	376 (43.9%)
Current treatment	
MDI[a]	403 (47.1%)
Pump therapy	440 (51.4%)
Other	13 (1.5%)
HbA1c, self-reported (mmol/mol Hb)	
Mean ± SD (N)	56.4 ± 12.4 (660)
Time in range, self-reported (%)	
Mean ± SD (N)	68.3 ± 17.3 (465)
PAID score	
Median (Q1–Q3, N)	25.0 (12.5–39.1, 412)
0–16	140 (34%)
17–39	169 (41%)
40–100	103 (25%)
Treatment center	
Local hospital	362 (42.3%)
University medical center	448 (52.3%)
Other	45 (5.3%)

[a]*MDI, multiple daily injections. PAID, problem areas in diabetes, indicating diabetes distress as low (0–16), moderate (17–39) or high (40–100). All units in N (%) unless otherwise indicated.*

TABLE 2 | Patient preferences for receiving a device and the preferred implant strategy.

	Overall (*N* = 856)
Willingness to receive a device	
No	26 (3.0%)
Yes, as soon as possible (for example, by taking part in safety studies	377 (44.0%)
Yes, after completion of all safety studies	380 (44.4%)
Yes, after the device has been in the clinic for several years	73 (8.5%)
Preferred strategy	
An implant with average functioning cells, requiring 1 surgical procedure	52 (6.1%)
An implant with cells functioning well, requiring two surgical procedures	523 (61.1%)
An implant with excellent functioning cells, requiring 1 surgical procedure and 10 min of daily care to add oxygen	176 (20.6%)
No preference	105 (12.3%)

All units in N (%).

outcomes, for all respondents with covariates age, sex, HbA1c and method of recruitment and just for the respondents of the diabetes outpatient clinic with age, sex, HbA1c and categorized PAID score. The most selected answers for the outcomes *preferred maximal size* and *minimal functional duration* were selected as reference. Comparison of continuous outcomes in multiple groups was done by repeated measures one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparison test. Univariate analyses of binary outcome were performed with chi-square test.

RESULTS

Respondent Characteristics

The online questionnaire was completed by 856 respondents (**Supplementary Figure S1**). The response rate was 43.1% amongst the approached patients of the LUMC diabetes outpatient clinic (412 respondents). Baseline characteristics of all respondents are presented in **Table 1**. The majority of respondents identified as female (58.1%) and were between the ages of 31 and 70 (71.8%). Forty-four percent of respondents had diabetes for more than 25 years. Mean self-reported HbA1c was 56.4 ± 12.4 mmol/mol Hb (N = 660) and mean self-reported TIR was 68.3% ± 17.3% (N = 465). The group of 412 patients also filled out the PAID questionnaire. The median PAID score was 25 (IQR: 12.5–39.1). Diabetes distress was determined to be low (PAID score 0–16) in 34%, moderate (PAID score 17–39) in 41% and high (PAID score 40–100) in 25% of respondents. Respondents recruited online were more often female, younger, had a shorter disease duration, and higher TIR than the patients contacted via the LUMC diabetes outpatient clinic (**Supplementary Table S1**).

Interest in Receiving an IDD

Nearly all (97%) respondents would like to receive an IDD (**Table 2**). Some respondents were willing to already take part in safety studies (44.0%), others would only accept the IDD after the completion of safety studies (44.4%).

Preferred Maximal Size

To explore the preferred maximal size of an implant, we surveyed 5 options (**Table 3**). We informed the respondents that a device would be flexible with a thickness of a credit card, and that it could be implanted, via a small incision, under the skin under local anesthesia at a location that would not be directly visible. Respondents were also informed that it could leave a scar. Most respondents (37.7%) preferred a maximal size corresponding to a FreeStyle Libre 2 sensor (diameter 3.5 cm), while 30.4% indicated that size was irrelevant. After correcting for sex, method of recruitment and HbA1c, respondents age >30 years compared to age 16–30 years were more likely to select a device with

TABLE 3 | Patient preferences for specific device characteristics and the expected improvements.

	Overall (*N* = 856)	Male (*N* = 358)	Female (*N* = 497)
Preferred maximal size			
2 Euro coin (diameter 2.5 cm)	95 (11.1%)	33 (9.2%)	62 (12.5%)
Freestyle Libre 2 sensor (diameter 3.5 cm)	323 (37.7%)	132 (36.9%)	191 (38.4%)
Credit card (8.5 cm × 5.5 cm)	166 (19.4%)	70 (19.6%)	95 (19.1%)
5 Euro banknote (12 cm × 6 cm)	12 (1.4%)	5 (1.4%)	7 (1.4%)
Size is irrelevant	260 (30.4%)	118 (33.0%)	142 (28.6%)
Maximal acceptable amount of implants			
Median (Q1–Q3)	4 (3–6.75)	4 (3–8)	4 (3–6)
Minimal expected functional duration			
3 months	113 (13.2%)	48 (13.4%)	65 (13.1%)
6 months	245 (28.6%)	108 (30.2%)	136 (27.4%)
12 months	286 (33.4%)	123 (34.4%)	163 (32.8%)
24 months	212 (24.8%)	79 (22.1%)	133 (26.8%)
Minimal expected improvement			
No more severe hyper- and hypoglycemia	104 (12.1%)	46 (12.8%)	58 (11.7%)
No more hyper- and hypoglycemia	301 (35.2%)	111 (31.0%)	190 (38.2%)
Less frequent insulin injections and monitoring	153 (17.9%)	73 (20.4%)	79 (15.9%)
Functional cure	298 (34.8%)	128 (35.8%)	170 (34.2%)

All units in N (%).

dimensions of a *2 Euro coin* (diameter 2.6 cm) rather than a *FreeStyle Libre 2 sensor* (p-values 0.007–0.048). Furthermore, parents/caregivers were less likely to opt for the choice "*size is irrelevant*" compared to the young reference group age 16–30 years (p = 0.043). Amongst the respondents from the outpatient clinic, having high diabetes distress increased the likelihood to select the option "*size is irrelevant*" over "*FreeStyle Libre 2 sensor*" compared to low diabetes distress (p = 0.003). Of the 602 respondents who left a comment at this question, 36.7% indicated that their choice for maximal size was motivated primarily by comfort: the device should not be visible nor hinder daily activities.

Maximal Number of Implants

As it is likely that several implants would need to be implanted for maximal efficacy, we asked how many devices a respondent would simultaneously accept to be cured of type 1 diabetes given that an incision of 5 cm would be needed per implant. Respondents could choose between 0 and 10 devices. Respondents indicated a median of 4 (IQR 3–6.75) implants to be acceptable (**Figure 1**). The option for 10 implants was selected by 186 (21.7%) people, of whom 110 (59.1%) commented that the number of devices is not relevant if it ensures a cure. Of 479 respondents that left a comment, 10.9% indicated scar formation, 11.5% recovery after surgery and 22.3% a balance between cure and daily discomfort to be considerations in selecting a maximal number of devices.

Minimal Expected Functional Duration

It is conceivable that the first generation of IDDs is functional for only a limited time. Therefore, respondents were informed that the cells in the implant would probably not be functional indefinitely, and that a replacement would be necessary under local anesthesia. We queried respondents what the minimal functional duration of an IDD should be before replacement (**Table 3**). A minimal functional duration of 3 months is the least desired option (13.2%). Replacement of an IDD of at most twice a year (minimal functional duration 6 months) was acceptable for 28.6%. A third (33.4%) of respondents would like the device to function for at least a year and 24.8% for at least 2 years. After correcting for method of recruitment, HbA1c and sex, respondents age 16–30 years were more likely to accept a minimal functional duration of 3–12 months when compared to those age 31–70 years ($p < 0.001$) and more likely to accept 6–12 months when compared to all other age categories (p-values 0.002–0.03).

Amongst respondents from the diabetes outpatient clinic, women were more likely than men to select a minimal functional duration of 24 months over 12 months (p = 0.01). Additionally, respondents age 31–50, and 51–70 years were more likely than those age 16–30 years to accept a minimal functional duration of 12 months compared to 6 months (p = 0.021 and p = 0.015, respectively). Of the 436 respondents who left a remark, 29.1% indicated that their choice revolved around minimizing emotional impact and impact on daily life due to hospital visits. Time to recovery and potential complications were important for 20%.

Most Important Device Characteristic

To gain more insights into which device characteristic was considered most important, the respondents distributed 10 points between device characteristics *size*, *quantity* and *functional duration*. Respondents preferred *functional duration* over *quantity* and *size* (4.9 ± 1.8 vs. 2.7 ± 1.2 vs. 2.3 ± 1.3 points, respectively, $p < 0.001$, **Figure 2**).

Respondents' Preferences on Implantation of Islet Delivery Devices

To evaluate what implant sites were acceptable, we asked what body parts were most preferable. The three most acceptable sites were the abdomen (65.7%), upper leg (63.7%), and upper arm

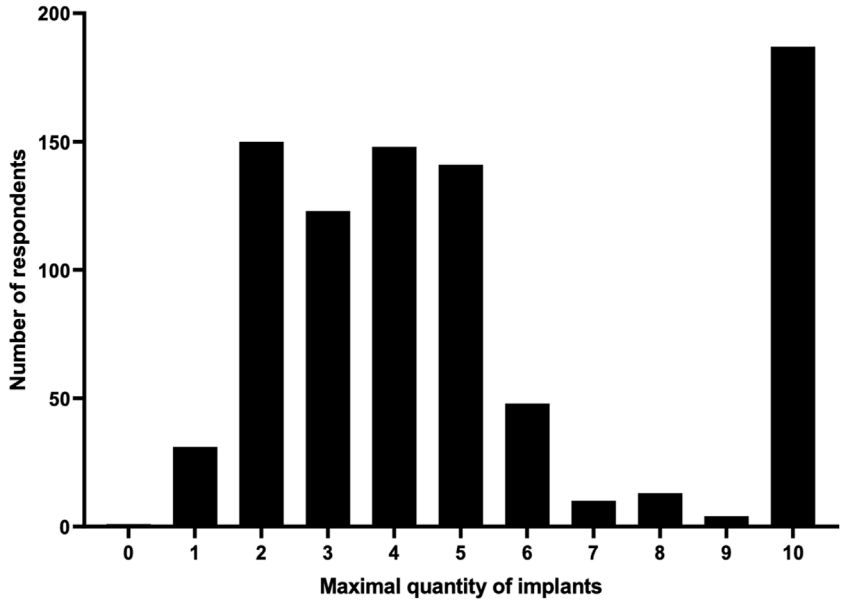


FIGURE 1 | Maximal number of implants.

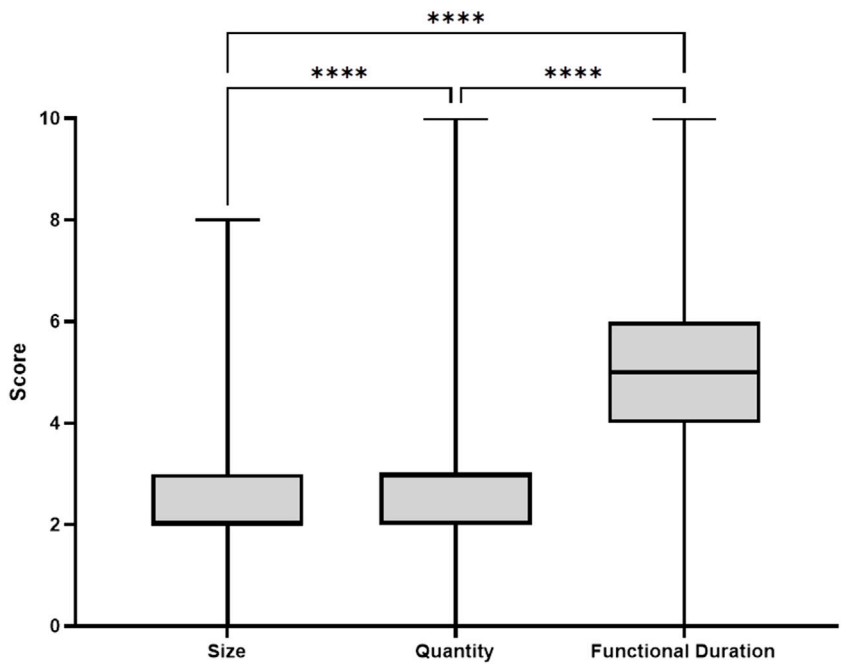


FIGURE 2 | Scores indicating the most important device characteristic. Box indicated Q1–Q3.

(54.8%), whereas the forearm (15.7%), chest (15.2%) and lower leg (14%) were the three least accepted implant sites (**Table 4**). The only difference in sex was that men were more positive about the chest as implant site than women (20.1% vs. 11.7%, $p < 0.001$).

Minimal Expected Improvement

Islet delivery devices can potentially improve glycemic control and ideally lead to insulin independence. Respondents indicated in 34.8% of cases that they would only accept IDDs if it would cure them from diabetes (**Table 3**). In all other cases, various

TABLE 4 | Patient preferences for specific implantation sites.

	Total (*N* = 856)	Male (*N* = 358)	Female (*N* = 497)	*p*-value
Abdomen				
Yes	562 (65.7%)	247 (69.0%)	314 (63.2%)	0.09
No	294 (34.3%)	111 (31.0%)	183 (36.8%)	
Upper leg				
Yes	545 (63.7%)	217 (60.6%)	327 (65.8%)	0.14
No	311 (36.3%)	141 (39.4%)	170 (34.2%)	
Upper arm				
Yes	469 (54.8%)	185 (51.7%)	283 (56.9%)	0.15
No	387 (45.2%)	173 (48.3%)	214 (43.1%)	
Butt				
Yes	362 (42.3%)	147 (41.1%)	215 (43.3%)	0.57
No	494 (57.7%)	211 (58.9%)	282 (56.7%)	
Hip				
Yes	321 (37.5%)	132 (36.9%)	189 (38.0%)	0.78
No	535 (62.5%)	226 (63.1%)	308 (62.0%)	
Back				
Yes	305 (35.6%)	115 (32.1%)	190 (38.2%)	0.077
No	551 (64.4%)	243 (67.9%)	307 (61.8%)	
Forearm				
Yes	134 (15.7%)	47 (13.1%)	87 (17.5%)	0.1
No	722 (84.3%)	311 (86.9%)	410 (82.5%)	
Chest				
Yes	130 (15.2%)	72 (20.1%)	58 (11.7%)	<0.001
No	726 (84.8%)	286 (79.9%)	439 (88.3%)	
Lower leg				
Yes	120 (14.0%)	55 (15.4%)	64 (12.9%)	0.35
No	736 (86.0%)	303 (84.6%)	433 (87.1%)	

The number and proportion of participants are shown for all variables.

forms of improvement would also be acceptable. No longer suffering from hyperglycemia and hypoglycemia was the most selected non-curative improvement (35.2%).

Preferred Device Strategy

Multiple device application strategies regarding islet delivery devices are currently considered. We surveyed the preference for three hypothetical scenarios (**Table 2**). Most respondents (61.1%) preferred a scenario with well-functioning cells that requires two surgical procedures over excellent-functioning cells requiring one surgical procedure and 10 min of daily care to supply oxygen (20.6%). A minority (6.1%) opted for moderately functioning cells after 1 surgical procedure. No preference was indicated by 12.3%.

DISCUSSION

The main outcome of our cross-sectional study in a large group of Dutch patients with type 1 diabetes is that patients with T1D are willing to accept a considerable burden of islet delivery device characteristics and implantation if this leads to a functional cure or clinically relevant improvement in hyper- and hypoglycemic events. Islet delivery devices could play an important future role in islet replacement strategies using insulin-producing cells from alternative cell sources such as pluripotent stem cells. Generating insights in the preferences of future recipients may support a smooth transition from IDD development to acceptance in the clinic.

In a previous report, Mohammadi et al. were the first to describe patient perspectives on implants for treatment of diabetes [14]. The results from their study indicated that patients with T1D prefer a device to be as small as possible and that a majority of the patients favored subcutaneous implantation. To gain more insight into what locations were preferred by patients we investigated which locations would be more preferable. Although not one location had a near total acceptance rate, most respondents accepted the abdomen, upper leg and upper arm which are sites that are often used for insulin injections and/or sensor placements.

The acceptance rate of IDDs was very high. Nearly all respondents indicated they would accept an IDD within the context of participation in a safety trial. T1D has a high disease burden [15] and diabetes distress partially mediates the relationship between depression and glycemic control [16]. It may not be surprising that device characteristics that may generate more discomfort are acceptable as long as it leads to a functional improvement or cure.

A morphomics framework was developed by McDermott et al. in which the body composition of 642 participants was evaluated using computed tomography images to analyze the maximal device dimensions [17]. In their model, maximal device dimensions were significantly larger in males, adults and dependent on BMI. The ideal device would be elliptical and could have an average surface area of 156 cm^2 in males. This equals the size of two banknotes, which according to our study results is only acceptable by one-third of the respondents.

The limitations of our study were the use of a non-validated questionnaire to assess the preferences for device characteristics and the self-reported glycemic control. Self-report bias is an important limitation in studies using questionnaire. We accepted a putative difference between reported and actual HbA1c and TIR as these measures were used as an indicative marker rather than a prognostic or etiological factor. Selection bias may also have played a role as it is possible that non-interested patients with T1D did not start or complete the survey, and were therefore not considered or registered for data analysis. The response rate of 43.1% from the diabetes outpatient clinic similar to that of a different survey study amongst patients with diabetes [18]. The outcomes of our survey will allow researchers to incorporate device preferences of potential recipients at an early stage during device design and development [14].

CONCLUSION

The vast majority of patients with type 1 diabetes would accept islet delivery devices when they become available. Respondents indicate that the minimal functional duration of an IDD

is the most important characteristic. Implanting multiple IDDs is an acceptable strategy, although the potential discomfort while performing daily activities should be considered. The outcomes of this survey should not only serve as a recommendation for designing IDDs, but may also aid clinicians and researchers in setting up the appropriate clinical protocol for beta cell replacement strategies using cell delivery devices.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The Medical Ethics Committees of Maastricht (METC azM/UM), and of Leiden Den Haag Delft (METC LDD) considered the Medical Research Involving Human Subjects Act (Dutch: WMO) not to be applicable to this study protocol. The Board of Directors of the LUMC approved this study to be executed in the hospital.

AUTHOR CONTRIBUTIONS

MT and DdB co-wrote this manuscript, analyzed the data, and designed the study in an equal manner. WB advised on the patient survey and writing of the article. EdK contributed to the writing, and AvA supervised the study and contributed to the writing. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

AvA is founder and shareholder of Lighthouse Biomedical BV a start-up company aiming to commercialize beta cell delivery devices in the future.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11077/full#supplementary-material

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POINT OF VIEW
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The Relevance of Advanced Therapy Medicinal Products in the Field of Transplantation and the Need for Academic Research Access: Overcoming Bottlenecks and Claiming a New Time

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Point of View

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The field of transplantation has witnessed the emergence of Advanced Therapy Medicinal Products (ATMPs) as highly promising solutions to address the challenges associated with organ and tissue transplantation. ATMPs encompass gene therapy, cell therapy, and tissue-engineered products, hold immense potential for breakthroughs in overcoming the obstacles of rejection and the limited availability of donor organs. However, the development and academic research access to ATMPs face significant bottlenecks that hinder progress. This opinion paper emphasizes the importance of addressing bottlenecks in the development and academic research access to ATMPs by implementing several key strategies. These include the establishment of streamlined regulatory processes, securing increased funding for ATMP research, fostering collaborations and partnerships, setting up centralized ATMP facilities, and actively engaging with patient groups. Advocacy at the policy level is essential to provide support for the development and accessibility of ATMPs, thereby driving advancements in transplantation and enhancing patient outcomes. By adopting these strategies, the field of transplantation can pave the way for the introduction of innovative and efficacious ATMP therapies, while simultaneously fostering a nurturing environment for academic research.

Keywords: advanced therapy medicinal products (ATMPs), regulatory processes, clinical trials, rare diseases, transplantation

Advanced Therapy Medicinal Products (ATMPs) defined in Regulation (EC) No 1394/2007 in the European Union, are medicinal products for human use including gene therapy medicinal products (GTMP), somatic cell therapy medicinal products (sCTMP), tissue-engineered products (TEP), or combinations of these [1]. The European Medicines Agency (EMA) regulates ATMPs through the Committee for Advanced Therapies (CAT), which provides scientific advice and evaluates marketing authorization applications for ATMPs based on quality, safety, and efficacy. The CAT's opinion forms the basis for marketing authorization by the European Commission. As of the most recent published report (**Figure 1**, quarterly highlights and approved ATMPs, 2009-January 2023), the CAT provided 597 scientific recommendations on ATMPs, 559 scientific advice to companies, and reviewed 116 applications for Priority Medicines designation, granting 50 of them. The first ATMP to receive authorization in the EU was ChondroCelect®, a tissue-engineered product used for treating cartilage defects in 2009, followed by Glybera®, the first gene therapy, in 2012, and PROVENGE®, the first somatic cell therapy, in 2013.

The field of ATMPs is still relatively new, and the number of Marketing Authorization Applications (MAAs) submitted to CAT remains low [3]. Despite the high level of presubmission activity, the CAT only received 36 MAAs for ATMPs and authorized 25 of them (**Table 1**). This is partly due to the complex and challenging nature of developing ATMPs, which often requires a significant investment of time and resources to achieve regulatory approval [4, 5]. One reason for the slow take/emergence of ATMPs is the limited patient populations for rare diseases, which makes it challenging to conduct clinical trials and demonstrate safety and efficacy [6]. Another factor is the evolving regulatory landscape, which requires the establishment of clear guidelines and standards for the development and approval of these therapies. Additionally, ATMPs are often developed for diseases with limited treatment options, which can pose additional challenges for clinical trial design and regulatory approval (i.e., small patient populations, disease heterogeneity, lack of established endpoints, limited comparative data, long-term follow-up requirements, regulatory complexities, and specialized manufacturing).

Regarding the authorization of MAAs, it is worth noting that the CAT in the European Union maintains stringent requirements for evidence of quality, safety, and efficacy before granting marketing authorization for ATMPs [7]. This rigorous approval process implies that not all MAAs submitted will be authorized, and certain products may necessitate additional data or further development to meet the necessary criteria. Furthermore, continuous monitoring and surveillance of authorized ATMPs are conducted, and the CAT may request supplementary data or undertake regulatory measures if concerns arise regarding safety or efficacy. This ongoing monitoring ensures that ATMPs continue to meet the required standards of quality, safety, and efficacy after being authorized for marketing. European Union has implemented various measures to tackle the challenges associated with the approval of ATMPs. One such measure involves the establishment of incentives to support the development of orphan drugs, including ATMPs. Orphan drugs are specifically designed to treat rare diseases that affect a relative small number of patients. Companies engaged in the development of orphan drugs, including ATMPs, can be encouraged by several

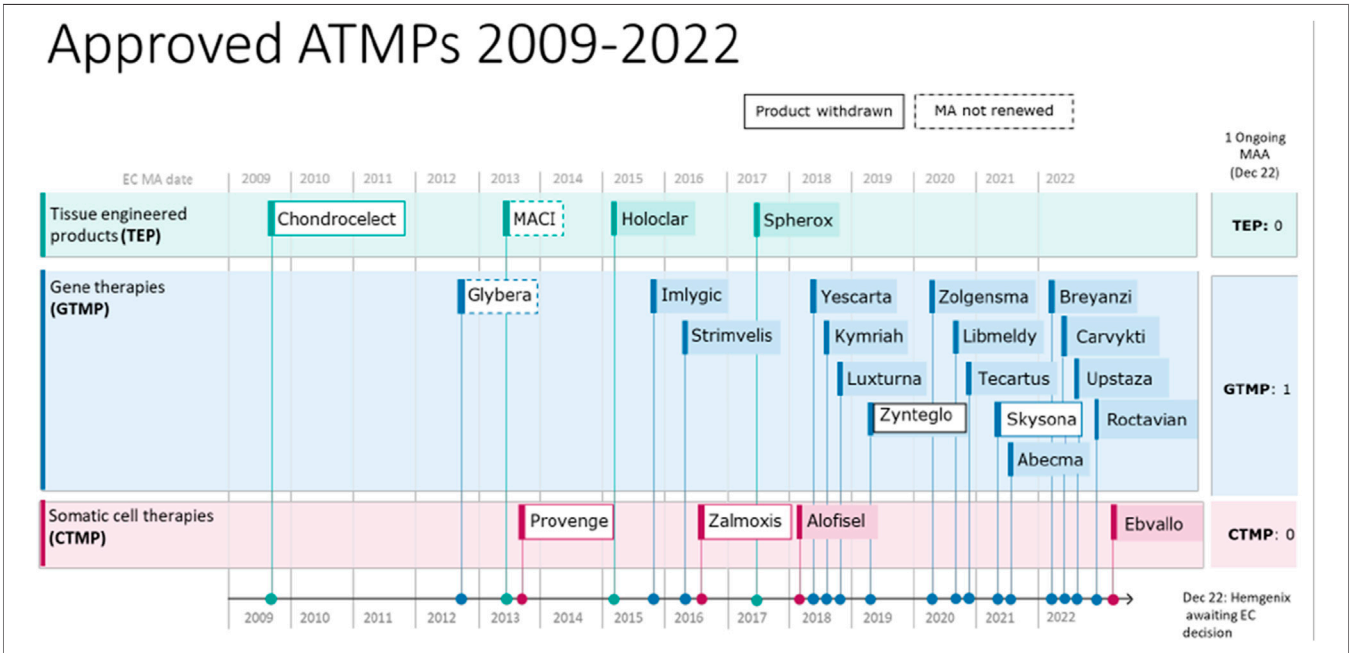

FIGURE 1 | Summary of authorized Advanced Therapy Medicinal Products (ATMPs) between 2009 and 2022, encompassing both withdrawn or non-renewed ones. Source: [2].

TABLE 1 | List of authorised ATMPs by EMA.

NAME	INN	Active substance	Type of ATMP	Indication	Company	Authorisation Date	Orphan	PRIME	MA
Chondrocelect		Characterized viable autologous cartilage cells expanded *ex vivo* expressing specific marker proteins	TEP	Repair of single symptomatic cartilage defects of the femoral condyle of the knee in adults	TiGenix N.V.	5/10/2009	No	No	withdrawn July 2016
Glybera	alipogene tiparvovec	human lipoprotein lipase (LPL) gene variant LPLS447X in a vector. The vector comprises a protein shell derived from adeno-associated virus serotype 1, the Cytomegalovirus promoter, a woodchuck hepatitis virus posttranscriptional regulatory element and AAV2 derived inverted terminal repeats	GTMP	Familial lipoprotein lipase deficiency (LPLD)	uniQure biopharma B.V.	25/10/2012	Yes	No	not renewed; ended Oct. 2017
MACI		Autologous cultured chondrocytes	TEP, combined ATMP	Repair of symptomatic cartilage defects of the knee	Vericel Denmark ApS	27/06/2013	No	No	not renewed; ended June 2018
Provenge	Sipuleucel-T	Autologous peripheral-blood mononuclear cells including a minimum of 50 million autologous $CD54^+$ cells activated with prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor	CTMP	Treatment of asymptomatic or minimally symptomatic metastatic (non-visceral) castrate-resistant prostate cancer in male adults in whom chemotherapy is not yet clinically indicated	Dendreon UK Ltd	6/09/2013	No	No	withdrawn May 2015
Holoclar		*Ex vivo* expanded autologous human corneal epithelial cells containing stem cells	TEP	Treatment of adult patients with moderate to severe limbal stem cell deficiency, unilateral or bilateral, due to physical or chemical ocular burns	Holostem Terapie Avanzate s.r.l	17/02/2015	Yes	No	
Imlygic	talimogene laherparepvec	Attenuated herpes simplex virus type-1 (HSV-1) derived by functional deletion of 2 genes (ICP34.5 and ICP47) and insertion of coding sequence for human granulocyte macrophage colony-stimulating factor (GM-CSF)	GTMP	Unresectable melanoma that is regionally or distantly metastatic	Amgen Europe B.V.	16/12/2015	No	No	
Strimvelis		Autologous $CD34^+$ enriched cell fraction that contains $CD34^+$ cells transduced with retroviral vector that encodes for the human adenosine deaminase (ADA) cDNA sequence from human haematopoietic stem/progenitor ($CD34^+$) cells	GTMP	Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID)	Orchard Therapeutics (Netherlands) BV	26/05/2016	Yes	No	
Zalmoxis		Allogeneic T cells genetically modified with a retroviral vector encoding for a truncated form of the human low affinity nerve growth factor receptor and the herpes simplex I virus thymidine kinase	CTMP	Adjunctive treatment in haploidentical haematopoietic stem cell transplantation (HSCT) of adult patients with high-risk haematological malignancies	MolMed SpA	18/08/2016	Yes	No	withdrawn Oct. 2019

(Continued on following page)

TABLE 1 | (*Continued*) List of authorised ATMPs by EMA.

NAME	INN	Active substance	Type of ATMP	Indication	Company	Authorisation Date	Orphan	PRIME	MA
Spherox		Spheroids of human autologous matrix-associated chondrocytes	TEP	Repair of symptomatic articular cartilage defects of the femoral condyle and the patella of the knee	CO.DON Gmbh	10/07/2017	No	No	
Alofisel	Darvadstrocel	Expanded human allogeneic mesenchymal adult stem cells extracted from adipose tissue	CTMP	Treatment of complex perianal fistulas in adult patients with non-active/mildly active luminal Crohn's disease, when fistulas have shown an inadequate response to at least one conventional or biologic therapy	Takeda Pharma A/S	23/03/2018	Yes	No	
Yescarta	Axicabtagene ciloleucel	Autologous T cells transduced *ex vivo* using a retroviral vector expressing an anti-CD19 chimeric antigen receptor (CAR) comprising a murine anti-CD19 single chain variable fragment linked to CD28 co-stimulatory domain and CD3-zeta signalling domain	GTMP	Diffuse large B cell lymphoma (DLBCL) and high-grade B-cell lymphoma (HGBL)	Kite Pharma EU B.V.	23/08/2018	Yes	Yes	
Kymriah	Tisagenlecleucel	Autologous T cells genetically modified *ex vivo* using a lentiviral vector encoding an anti-CD19 chimeric antigen receptor (CAR)	GTMP	B cell acute lymphoblastic leukaemia (ALL); diffuse large B cell lymphoma (DLBCL); follicular lymphoma (FL)	Novartis Europharm Limited	23/08/2018	Yes	Yes	
Luxturna	voretigene neparvovec	Gene transfer vector that employs an adeno-associated viral vector serotype 2 capsid as a delivery vehicle for the human retinal pigment epithelium 65 kDa protein (hRPE65) cDNA to the retina	GTMP	Inherited retinal dystrophy caused by confirmed bi-allelic RPE65 mutations	Novartis Europharm Limited	22/11/2018	Yes	No	
Zynteglo	Betibeglogene autotemcel	Genetically modified autologous $CD34^+$ cell enriched population that contains haematopoietic stem cells transduced with lentiviral vector encoding the βA-T87Q-globin gene	GTMP	Transfusion-dependent β thalassaemia	Bluebird bio (Netherlands) B.V.	29/05/2019	Yes	Yes	withdrawn March 2022
Zolgensma	Onasemnogene abeparvovec	Non-replicating recombinant adeno-associated virus serotype 9 based vector containing the cDNA of the human SMN gene under the control of the cytomegalovirus enhancer/chicken-β-actin-hybrid promoter	GTMP	Spinal muscular atrophy (SMA)	Novartis Europharm Limited	18/05/2020	Yes	Yes	
Libmeldy	Atidarsagene autotemcel	Genetically modified autologous $CD34^+$ cells enriched population that contains haematopoietic stem and progenitor cells (HSPC) transduced *ex vivo* using a lentiviral vector expressing the human arylsulfatase A (ARSA) gene	GTMP	Metachromatic leukodystrophy	Orchard Therapeutics (Netherlands) BV	17/12/2020	Yes	No	

(Continued on following page)

TABLE 1 | (*Continued*) List of authorised ATMPs by EMA.

NAME	INN	Active substance	Type of ATMP	Indication	Company	Authorisation Date	Orphan	PRIME	MA
Tecartus	Brexucabtagene autoleucel	Autologous T cells transduced *ex vivo* using a retroviral vector expressing an anti-CD19 chimeric antigen receptor (CAR) comprising a murine anti-CD19 single chain variable fragment linked to CD28 co-stimulatory domain and CD3-zeta signalling domain	GTMP	Mantle cell lymphoma (MCL)	Kite Pharma EU B.V.	14/12/2020	Yes	Yes	
Skysona	Elivaldogene autotemcel	Autologous $CD34^+$ cell-enriched population that contains haematopoietic stem cells transduced *ex vivo* with lentiviral vector encoding ABCD1 cDNA for human adrenoleukodystrophy protein	GTMP	Cerebral adrenoleukodystrophy	Bluebird bio (Netherlands) B.V.	16/07/2021	Yes	Yes	withdrawn Nov. 2021
Abecma	idecabtagene vicleucel	Autologous T cells transduced with lentiviral vector encoding a chimeric antigen receptor (CAR) that recognises B-cell maturation antigen	GTMP	Multiple myeloma	Bristol-Myers Squibb Pharma EEIG	18/08/2021	Yes	Yes	
Breyanzi	Lisocabtagene maraleucel	Autologous purified $CD8^+$ and $CD4^+$ T cells, in a defined composition, that have been separately transduced *ex vivo* using a replication incompetent lentiviral vector expressing an anti-CD19 chimeric antigen receptor (CAR) comprising a single chain variable fragment binding domain derived from a murine CD19-specific monoclonal antibody (mAb; FMC63) and a portion of the 4-1BB co-stimulatory endodomain and CD3 zeta chain signalling domains and a nonfunctional truncated epidermal growth factor receptor	GTMP	Diffuse large B-cell lymphoma (DLBCL), primary mediastinal large B-cell lymphoma (PMBCL) and follicular lymphoma grade 3B (FL3B)	Bristol-Myers Squibb Pharma EEIG	4/04/2022	No	Yes	
Carvykti	ciltacabtagene autoleucel	Autologous T cells transduced *ex vivo* using a replication incompetent lentiviral vector encoding an anti-B cell maturation antigen chimeric antigen receptor (CAR), comprising two single domain antibodies linked to a 4-1BB costimulatory domain and a CD3-zeta signaling domain	GTMP	Multiple myeloma	Janssen-Cilag International NV	25/05/2022	Yes	Yes	
Upstaza	Eladocagene exuparvovec	Non-replicating recombinant adeno-associated virus serotype 2 based vector containing the cDNA of the human dopa decarboxylase gene under the control of the cytomegalovirus immediate-early promoter	GTMP	Aromatic L amino acid decarboxylase (AADC) deficiency with a severe phenotype	PTC Therapeutics International Limited	18/07/2022	Yes	No	

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TABLE 1 | (*Continued*) List of authorised ATMPs by EMA.

NAME	INN	Active substance	Type of ATMP	Indication	Company	Authorisation Date	Orphan	PRIME	MA
Roctavian	valoctocogene roxaparvovec	Non-replicating recombinant adeno-associated virus serotype 5 based vector containing the cDNA of the B-domain deleted SQ form of human coagulation factor VIII gene under the control of a liver-specific promoter	GTMP	severe haemophilia A (congenital factor VIII deficiency)	BioMarin International Limited	24/08/2022	Yes	No	
Ebvallo	Tabelecleucel	Allogeneic Epstein-Barr virus-specific T-cell	GTMP	Epstein-Barr virus positive post-transplant lymphoproliferative disease (EBV+ PTLD)	Pierre Fabre Medicament	16/12/2022	Yes	Yes	
Hemgenix	Etranacogene dezaparvovec	Non-replicating, recombinant adeno-associated virus serotype 5 based vector containing a codon-optimised cDNA of the human coagulation Factor IX variant R338L (FIX-Padua) gene under the control of a liver-specific promoter (LP1)	GTMP	Severe and moderately severe Haemophilia B (congenital Factor IX deficiency)	CSL Behring GmbH	Opinion Dec. 2022	Yes	Yes	Commision pending

Abbreviations: ATMP, advanced therapy medicinal product; GTMP, gene therapy medicinal product; CTMP, cell therapy medicinal product; TEP, tissue engineered product; MA, Marketing authorization.

incentives, such as market exclusivity, reduced fees, protocol assistance, and eligibility for EU research funding. Additionally, the EU has introduced a regulatory pathway known as "conditional marketing authorization." This pathway enables expedited access to medicines that address unmet medical needs. Conditional approval can be granted to ATMPs and other drugs based on promising initial data. This regulation assures that the medicine meets strict EU standards for safety, quality and efficacy, and that suppporting data is still generated post-approval to complete its safety profile. The most recent example which allowed fast track authorization is the COVID-19 vaccines which were released in the EU to support the mass vaccination campaign against the corona virus. Companies that receive conditional approval are required to provide further evidence to substantiate the benefits of the medicine, thus allowing patients to access potentially life-saving treatments at an earlier stage. These measures are aimed at promoting the development of ATMPs and improving access to innovative therapies for patients affected by rare diseases or unmet medical needs. However, despite these encouragments, they may not be sufficiently effective to facilitate widespread approval and dissemination of ATMPs.

The sluggish approval process for ATMPs has various implications that warrant concern [8]. Foremost among these is the potential to leave patients with rare and complex diseases without access to life-saving treatments [9]. The delay in approvals can prolong their suffering and even lead to unnecessary fatalities, while the social economic effects of their disease lead to increased healthcare costs and less access to the labor market. Furthermore, the restricted adoption of ATMPs may impede scientific advancement in regenerative medicine and cell therapy, as they offer a new and promising means of treating diseases, as more positive examples could lead to increased interest from the scientific community to explore these alternatives. A limited number of approved ATMPs could discourage investors and researchers from pursuing this area of research, leading to a lack of progress in creating innovative cell therapies. Moreover, the high costs associated with developing ATMPs may cause companies to be wary of investing in them without a comprehensive understanding of regulatory requirements and commercial viability. The tardiness in approvals could intensify this hesitancy, leading to a decline in investment in ATMPs and a deceleration in the development of new treatments.

The challenges that ATMPs face in overcoming the "economic valley of death" after obtaining marketing authorization have raised even greater concerns [10]. In fact, out of the 25 authorized ATMPs, seven had their marketing authorization withdrawn or not renewed. It is evident that the financial commitment for earlier trials, even in academic settings, is considerably high and the cost of upgrading an ATMP manufacturing process to obtain GMP certification is substantial, often exceeding that of similar clinical-grade cell products by 2–3 times. To make the process economically sustainable, academic scientists have established collaborations with small or large companies or founded biotechnology start-ups [11]. Nonetheless, the investments needed to take an ATMP to marketing authorization are very

high, not only due to the costs of running clinical trials, but also the manufacturing costs of viral vectors and cellular products, as well as the stringent standards imposed by regulatory agencies to ensure the safety and quality of these products. Furthermore, the patient population that would benefit from these therapies is often very small, ranging from several thousand for less rare diseases to a few dozen for very rare diseases. The high costs of research and development and production have resulted in companies demanding very high prices for these therapies, ranging from several hundred thousand to a few million euros per patient. This can cause lengthy negotiations or even rejection by National Health Systems unwilling to cover the costs, even for a few patients. As a result, some efficacious and approved products, such as Skysona for adrenoleukodystrophy and Zynteglo for beta-thalassemia, have been withdrawn from the European market, leaving patients who might have benefited from these life-saving therapies without access to them. Secondly, companies may decide to drop an approved ATMP, even when it is approved and when a reimbursement policy has been negotiated, simply due to insufficient economic return [12]. This is particularly true for rare diseases where the very low number of patients poses significant challenges. For instance, Glybera, an approved gene therapy product for Type I hyperlipoproteinemia, withdrew after treating only one patient. Similarly, Strimvelis®, the first *ex vivo* gene therapy product approved in Europe, was passed on to Orchard Therapeutics after being created by GSK and the San Raffaele Telethon Institute of Gene Therapy in Milan. While the therapy proved effective in treating some patients, Orchard Therapeutics recently pulled it from the market due to commercial considerations. Finally, Valline Holding Srl made the decision to cease financial backing to Holostem, the company responsible for creating Holoclar®, the first stem cell-derived ATMP approved in Europe.

Developing ATMPs for rare diseases is crucial to the advancement of medical innovation and technology transfer [13]. The development of these therapies requires significant investments in research, development, infrastructure, and regulatory frameworks, which can be leveraged to develop treatments for more common diseases. This approach encourages collaborations between academic institutions, industry partners, and regulatory agencies, stimulating innovation and accelerating the translation of research into clinical applications. In addition, the development of ATMPs for rare diseases can create an ecosystem that supports innovation and technology transfer. Researchers and developers can use these rare diseases as a platform to refine cutting-edge technologies, such as gene editing and stem cell therapies, which can then be applied to other disease areas with similar genetic and cellular foundations, such as cancer or neurodegenerative diseases. Moreover, developing ATMPs for rare diseases can spur investment in related fields, including manufacturing and supply chain logistics, helping to lower the production costs and improve the scalability of ATMPs, ultimately making them more accessible to patients worldwide.

ATMPs are extremely relevant to the field of transplantation as they offer a promising way to address many of the challenges associated with organ and tissue transplantation [14, 15]. One of the main challenges of transplantation is rejection, which occurs when the recipient's immune system recognizes the transplanted organ or tissue as foreign and attacks it. Immunosuppressive therapy is currently used to prevent rejection, but this can have significant side effects and long-term complications, including increased susceptibility to infections and cancer. ATMPs offer a potential solution to this problem by modifying the recipient's immune system to accept the transplanted organ or tissue as "self." For example, chimeric antigen receptor (CAR) T cell therapy involves genetically engineering the patient's own immune cells to target and destroy cancer cells [16]. This approach has shown promise in treating post-transplant lymphoproliferative disorders, which are a common complication of solid organ transplantation. Another ATMP approach is the use of regulatory T cells (Tregs), which are a subset of immune cells that play a key role in immune tolerance [17]. Treg therapy is being developed to induce immune tolerance and reduce the need for immunosuppressive therapy, which could improve patient outcomes and reduce the risk of complications. ATMPs are also being used to address other challenges associated with transplantation, such as the limited availability of donor organs and tissues. Tissue engineering is one approach that involves using biodegradable scaffolds and cells to create functional replacements for damaged or diseased tissues (cartilage, bone, skin, vessels, islet, etc.). Xenotransplantation is another approach that involves transplanting organs or tissues from one species to another. ATMPs such as gene editing and immune cell therapies are being developed to overcome the immunological barriers associated with xenotransplantation and make it a viable option for treating organ failure.

The field of transplantation is at a critical juncture, as there is an urgent need to address the challenges associated with organ and tissue transplantation. ATMPs offer a promising way to achieve this goal, but their development and access to academic research must be sustained and expanded to fully realize their potential [18]. The transplant community has a critical role to play in sustaining the ATMP field, as they are uniquely positioned to identify the unmet needs and opportunities for innovation in transplantation. This includes advocating for increased funding for ATMP research and development, as well as promoting collaborations between academic researchers, industry partners, and regulatory agencies to accelerate the translation of promising ATMP therapies to the clinic. In addition, the transplant community can support the development and adoption of innovative approaches to transplant surgery, such as *ex vivo* organ perfusion, which has been shown to improve the quality of donor organs and increase the number of viable organs available for transplantation. By embracing new technologies and approaches to transplantation, the transplant community can create a more supportive environment for the development and adoption of ATMPs. Moreover, it is essential that the transplant community engage in ongoing education and training on the latest advances in ATMPs, including their potential clinical applications, regulatory considerations, and ethical implications. This will ensure that transplant clinicians and researchers are equipped with the knowledge and skills needed to effectively translate and apply ATMPs in the clinical setting.

Ultimately, the success of the ATMP field in transplantation will depend on the sustained commitment and collaboration of the transplant community. By working together to overcome the regulatory and funding challenges associated with ATMP development and access, the transplant community can help to ensure that patients in need of organ and tissue transplantation have access to the most innovative and effective therapies available. The timely and invaluable action of launching a task force by the European Society of Organ Transplantation (ESOT) to address ATM field issues in Europe demonstrates a recognition of the pressing challenges faced in the academic institution. It highlights a strong commitment to finding effective solutions. By assembling experts and stakeholders, the task force can capitalize on their collective knowledge and expertise to address crucial issues, fostering innovation, efficiency, and safety in European member states.

More specifically, to overcome the bottleneck in the development and access to ATMPs for academic research in the field of transplantation, several strategies can be implemented [19, 20]:

i) Streamlining regulatory processes. One of the major barriers to the development and access of ATMPs is the complex and lengthy regulatory approval process. To overcome this bottleneck, regulatory agencies can work to streamline their processes and reduce the time and cost of approval, while still ensuring the safety and efficacy of these therapies [21]. The EMA/CAT definition of ATMPS warrants reconsideration. Is it appropriate to regulate minimally modified cell therapy products, such as Stromal Vascular Fraction (SVF) differently based on non-homologous (ATMP) versus homologous therapy (simple cell therapy)? For instance, comparing SVF for plastic surgery (simple cell therapy) and SVF for scleroderma (ATMP), should not the classification be determined by the manufacturing process rather than the clinical end use? Could production facilities conduct risk assessments as evidence of manufacturing process quality?
ii) Increasing funding for ATMP research. The development of ATMPs requires significant investment in research and development. A challenge arises from the fact that public funding typically does not support these endeavors. National science funding primarily prioritizes the creation of new knowledge, focusing on academic research rather than the establishment of clinical trials or conducting safety studies, activities classified as TRL4 and higher in the EU. Consequently, scaling up an ATMP becomes unfeasible due to insufficient funding. To support this, funding agencies can increase their investment in ATMP research, with a focus on academic research and development.
iii) Promoting collaborations and partnerships. Collaboration between academic researchers, industry partners, and regulatory agencies is critical to the development and translation of ATMPs. To facilitate this, there is a need for increased support for partnerships and collaborations, including funding, infrastructure, and regulatory support.
iv) Establishing/Supporting Pre-ATMP Facilities. The development of pre-ATMP facilities is crucial for improving the efficiency and success rate of ATMP projects. It provides researchers with a valuable platform to thoroughly test their products, ensuring compatibility, safety, and efficacy before committing significant resources to full-scale GMP production. By avoiding potential pitfalls related to raw material and starting material selection early on, researchers can streamline the translation of ATMPs into clinical trials, fostering a more effective and efficient development pathway.
v) Establishing centralized ATMP facilities. The successful development and production of ATMPs rely on specialized facilities and expertise. To address the limited availability of these resources, the establishment of centralized ATMP facilities can provide academic researchers with accessible infrastructure and regulatory affairs expertise. This point is crucial, as it emphasizes the importance of not only having such facilities but also ensuring their affordability and having the necessary regulatory affairs expertise readily available. Having a cleanroom facility alone is insufficient for achieving clinical translation. The progress towards this goal can greatly benefit from experts who possess knowledge not only about regulatory hurdles but also about establishing effective quality management systems, training personnel in Good Manufacturing Practices (GMP), defining release criteria, and navigating other approval requirements. Typically, one option is to rent a cleanroom facility and seek the assistance of consultants for regulatory affairs. Additionally, safety studies are often outsourced to third-party organizations with ISO certification. However, this approach tends to be expensive, and obtaining research funding for these activities can be extremely challenging. This is because they extend beyond the scope of academic work and require substantial financial investments. Consequently, a gap is created presenting significant obstacles in terms of both financial resources and expertise for research groups interested in pursuing ATMP development. It would be beneficial if centralized facilities could offer a cost-effective combination of services specifically designed for academic researchers.
vi) Enhancing the efficiency and accessibility of the "Hospital Exemption" (HE) approval pathway. The HE pathway, a regulatory framework outlined in European Regulation No 1394/2007, provides a means for manufacturing and utilizing ATMPs outside the standard centralized marketing authorization pathway, subject to specific conditions. In February 2021, ARI-0001 (CART19-BE-01), an ATMP designed to target $CD19^+$ B-cell malignancies, achieved a significant milestone [22]. The Spanish Agency of Medicines and Medical Devices (AEMPS) authorized its use under the HE pathway for treating adult patients over 25 years old with relapsed/

refractory acute lymphoblastic leukemia. This achievement is remarkable as ARI-0001 becomes the first CAR-T therapy to receive approval from a governmental drug agency outside the central marketing authorization pathway. However, it is important to acknowledge the emergence of divergent interpretations and variations in HE implementation across countries and within industrial or academic organizations. These differences [8, 23–26] underline the need for greater harmonization of HE rules. While it is widely agreed that the HE pathway should not be exploited to bypass established procedures for marketing authorization and clinical trials in Europe, different viewpoints exist regarding the reasons for utilizing this pathway. Academic organizations emphasize the need to ensure uninterrupted patient treatment during clinical development, reduce costs, provide therapeutic options for individuals ineligible for clinical studies, accommodate early stages of product development with rapid manufacturing and advancements, and enable access to ATMPs with limited commercial viability that may not progress towards marketing authorization. Conversely, industrial organizations primarily raise concerns about the potential risks associated with establishing a dual-tier system with varying regulatory standards. Numerous challenges are associated with the HE pathway that necessitate attention: a) the lack of harmonization of HE rules among EU Member States, b) the necessity to enhance flexibility and efficiency in the regulatory process for HE-ATMPs, where even minor product modifications are regarded as "new products," c) the substantial requirement of human, logistic, and financial resources, which pose barriers for both public facilities and private investors, particularly small and medium enterprises, d) ensuring access to HE-ATMPs for patients treated in hospitals other than the one involved in product development, and e) facilitating technology transfer and knowledge sharing to promote access to these therapies in hospitals within or beyond the Member State.

vii) Engaging with patients and patient advocacy groups. Patients and patient advocacy groups should have an important role in the clinical development and translational process of ATMPs. Early exploration and engagement of patient perspectives are essential to understand and address the barriers and facilitating factors that may affect the uptake and impact of ATMPS on patient communities. Empirical research on patient perspectives is therefore important in order to ensure responsible clinical translation of ATMPs.

Finally, accessibility implies not only availability, but also affordability [27]. Given the expected high prices of ATMPs, there are concerns about equitable distribution of ATMPs. However, especially in countries in which ATMP facilities and trained staff are lacking, these treatments may not become accessible to patients who may need them most. To provide equitable access to ATMPs across different regions and communities, investments must be made in robust supply chains and knowledge sharing. Even in less resource-constrained settings, strategies for fair pricing will be required, as well as adequate reimbursement policies and the provision of support programs to alleviate financial burdens on individual patients. Particular attention should be paid to individuals with rare diseases, as they often face significant challenges in accessing effective treatments. Achieving fair distribution of ATMPs entails addressing patients' needs, including by raising awareness, improving early diagnosis, and establishing support networks. By doing so, we can ensure that patients with unmet medical needs have equitable opportunities to benefit from ATMPs. Equity in ATMPs extends to the realm of clinical trials as well. It is essential to ensure the inclusion of diverse populations, including historically underrepresented groups, in research studies. This inclusive approach enables a comprehensive understanding of the benefits and risks of ATMPs across various patient populations, thereby avoiding potential biases and ensuring equitable access to the benefits of research. Lastly, global disparities must be addressed to achieve equity in access to ATMPs. Efforts should be made to bridge gaps between different countries and regions, allowing individuals worldwide to benefit from these therapies. This can be accomplished through international collaboration, regulatory harmonization, and the transfer of knowledge and technology. By placing fair distribution of ATMPs at the center of our ethical considerations, we can work collectively to establish a healthcare landscape where all individuals, regardless of their socio-economic status, disease rarity, geographic location, or background, have equitable access to the transformative potential of ATMPs.

To claim for a new time, it is important to advocate for changes at the policy level that support the development and access to ATMPs for academic research [28]. This includes advocating for increased funding, streamlined regulatory processes, engagement of patients and patients advocacies and collaboration between academic researchers, industry partners, and regulatory agencies [29]. By working together to overcome the bottleneck in the development and access of ATMPs, we can create a more supportive environment for innovation in the field of transplantation and help to improve patient outcomes.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: [2].

AUTHOR CONTRIBUTIONS

LP, PK, and EB contributed to conception and design of the study. LP wrote the first draft of the manuscript. HS, DJ, JK-C, and AA wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

Authors MM, KP-K, and PK were employed by Kugelmeiers Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Clinical Translation of Bio-Artificial Pancreas Therapies: Ethical, Legal and Psychosocial Interdisciplinary Considerations and Key Recommendations

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The field of regenerative medicine offers potential therapies for Type 1 Diabetes, whereby metabolically active cellular components are combined with synthetic medical devices. These therapies are sometimes referred to as "bioartificial pancreases." For these emerging and rapidly developing therapies to be clinically translated to patients, researchers must overcome not just scientific hurdles, but also navigate complex legal, ethical and psychosocial issues. In this article, we first provide an introductory overview of the key legal, ethical and psychosocial considerations identified in the existing literature and identify areas where research is currently lacking. We then highlight two principal areas of concern in which these discrete disciplines significantly overlap: 1) individual autonomy and 2) access and equality. Using the example of beta-cell provenance, we demonstrate how, by harnessing an interdisciplinary approach we can address these key areas of concern. Moreover, we provide practical recommendations to researchers, clinicians, and policymakers which will help to facilitate the clinical translation of this cutting-edge technology for Type 1 Diabetes patients. Finally, we emphasize the importance of exploring patient perspectives to ensure their responsible and acceptable translation from bench to body.

Keywords: transplantation, regenerative medicine, tissue engineering, type 1 diabetes, informed consent

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INTRODUCTION

The current mainstay of treatment for Type 1 Diabetes uses exogenous insulin administered either as intermittent injections multiple times a day, or through a continuous infusion pump. Unfortunately, these techniques cannot precisely mimic the function of the native pancreas and the regulation and administration of exogenous insulin can be a stressful and burdensome self-management task for patients [1]. For instance, they must continuously monitor dietary intake, physical activity, and resulting blood sugar levels, and to adjust insulin dosage when necessary [2]. Transplantation of either the whole pancreas or islet cells offers the potential to return the recipient to a more stable state of euglycemia without the need to administer insulin and prevent or delay the onset of diabetic

complications [3]. However, both treatments are associated with significant risks such as complications of the procedure itself [4], increased propensity for infections and neoplasms as a result of immune suppression [5], and, in due course, graft failure [6, 7].

In addition, transplantation is not available for all patients with diabetes [8]. In low- and middle-income countries, transplant programs are limited by available health infrastructure. Even when islet and pancreas transplantation programs are established, the persistent global shortage of high-quality donor organs means that the availability of this therapy must be restricted [9]. Transplantation is reserved for a limited subgroup of patients with severe complications of diabetes, such as hypoglycaemic unawareness, suboptimal glycaemic control despite maximal medical input, and kidney failure [10]. Therefore, it is clearly imperative to develop alternative therapies, which help patients return to euglycemia without the limitations of existing transplantation options.

Regenerative medicine offers the most compelling prospects for developing such therapies. Regenerative medicine uses advanced biotechnologies, including tissue engineering and gene editing, to "replace or regenerate human cells, tissues or organs, to restore or establish normal function [11]." Research groups seeking to harness this technology to establish a novel treatment for diabetes have used a variety of biological and synthetic components. Insulin secreting cells from either deceased donors, xenogeneic cells or renewable induced allogenic stem cells are combined with other biological or synthetic (non-biological) devices such as scaffolds to hold or encapsulate the cells [12–23]. These products with both biological and device-based components can be seen as "hybrid" beta-cell replacement therapies and are sometimes referred to as "bioartificial pancreases."

The prospect of a hybrid beta-cell replacement therapy for Type 1 Diabetes patients that is safe and effective is tantalizing. However, for such a therapy to be a successful alternative it must also overcome the limitations of existing therapies. Specifically, the ideal hybrid beta-cell replacement therapy for Type 1 Diabetes it would be:

- Personalised to reduce or remove the need for post-transplant immunosuppression
- Widely accessible and available for patients

If realized, this hybrid beta-cell replacement therapy could have a revolutionary effect on the management of Type 1 Diabetes world-wide. Even so, they are also uniquely complicated, not just from a scientific perspective but also from ethical, legal, and psychosocial perspective. While many of these issues in isolation are not novel, in combination they present a new level of complexity that is unique and challenging for researchers, clinicians and policymakers.

PART 1—ETHICAL, LEGAL, AND PSYCHOSOCIAL OVERVIEW

Ethics

A recently published systematic review highlighted the ethical challenges of conducting early phase clinical trials of bioartificial organs [24]. Of most relevance to the clinical translation of this therapy for Type 1 Diabetes patients are 1) the source of the various cells used 2) recipient selection 3) informed consent and 4) access and justice considerations.

First, where hybrid products combine components made from cells and tissues (biomaterial) from different sources, each source (allogenic stem cells, deceased donor human islet cells and xenogeneic cells) will come with its own set of ethical considerations [24]. We explore this in more detail in Part 2 of this paper. Second, in the early phases of clinical translation, like with many novel therapies, not all Type 1 Diabetes patients may be eligible for hybrid beta-cell replacement therapy. This therapy requires an invasive and potentially irreversible surgical procedure, and the therapy may interact and integrate with the body, with unknown potential harms and complications [24]. Yet this is not immediately lifesaving. So, the balance of risks and benefits may not be favorable for Type 1 Diabetes patients who are relatively healthy. The first patients undergoing the therapy would likely be those who do not succeed in achieving adequate control of their glucose levels, suffer from the complications thereof, and have exhausted standard treatment options. Third, patient desperation for a cure can pose challenges to obtaining informed consent. Their desperation may lead to misunderstanding regarding the potential risks associated with participating in a clinical trial [24]. Uncertainties about complications arising from the novel nature of the therapy can hinder the provision of accurate information about the risk-benefit ratio of the intervention, this will also be explored in more detail in part 2 of this paper. Last, concerns regarding the accessibility of treatments for Type 1 Diabetes are not new nor specific to hybrid beta-cell replacement therapies, but these existing inequities may be amplified by this new technology. For example, currently some patients in developed countries cannot use the best available device-based treatments due to restrictive national reimbursement policies [25–27]. As with other regenerative medicine technologies, the costs of research and development of hybrid beta-cell replacement therapies will invariably be high. Nevertheless, access to these therapies should be equitable [8]. Ideally, they should be provided first and foremost to patients who stand to benefit the most.

Law

As with the ethical issues, each new cell type or component included in the therapy brings with it legal and regulatory requirements, so that even within the European Union, this may result in a complex web of national and international regulatory instruments.

To demonstrate—when using human deceased donor pancreases as a beta-cell source, firstly national laws regarding organ donation must be adhered to. Organs which are donated are then subject to EU Directive 2010/45/EU on standards of quality and safety of human organs intended for transplantation [28]. Secondly, if beta-cells are extracted from the very same pancreas, but are cultured or manipulated, they may then be subject to directives on tissues and cells [29, 30] and/or genetically modified organisms [31, 32]. Thirdly, stem cells that have been gene-edited or induced may be subject to regulations on genetically modified organisms [31, 32]. Fourthly, supporting

matrixes or scaffolds could be subject regulations governing tissues and cells [30] or medical devices depending on if they contain viable cells [33]. Fifthly, in the European Union a product such as this, which combines cells with devices, is likely to fall under the definition of an advanced therapy medicinal product (ATMP), which have additional specific regulatory requirements to adhere to (EU Regulation No 1394/2007) [34]. Thus, even with the advantages of harmonisation across the EU, there may still be multiple regulations or directives which are relevant to these products or their components throughout development and clinical translation.

What is more, there is not a global classification and many jurisdictions have taken alternative approaches, which has further increased the complexity of regulations and safeguards that developers must satisfy. Regulatory complexity and heterogeneity have been cited by legal academics and developers as a barrier to innovation even within single jurisdictions [35]. In response to such concerns, the World Health Organisation have issued a consultation urging for "Regulatory Convergence of Cell and Gene Therapy Products" to encourage research and enable broader access to such therapies [8].

In addition to these regulatory requirements, the areas of law which govern the everyday practice of clinical medicine, such as those relating to consent to medical therapy, confidentiality and equality of access must be considered. Taking the example of consent for medical treatment, this is protected at the highest level of European Law. The European Court of Human Rights (ECHR) has confirmed that Article 8 of the European Convention on Human Rights (ECHR)- the right to a private and family life- offers broad protection for individual autonomy [36, 37]. This includes the right to consent to, or refuse, medical treatment provided the person "is in a position to make up his own mind [38]." For consent to be considered valid, three principles are usually used—that it should be informed, given freely, and without coercion, and that the person should have the legal capacity to do so.

As hybrid therapies transition from investigational therapies to clinical practice informed consent could become a key factor in two ways. Firstly, the ability of potential recipients to understand the necessary information for such complex hybrid products has already been highlighted by researchers as a concern and is likely to be similarly problematic in clinical settings [24, 39]. Secondly, in contrast to research which is likely to only involve subjects able to consent, if a successful device moves into routine clinical care, it should also be available to those who have impaired capacity to consent, either by virtue of their age (minors) or due to impaired cognitive faculties. In each of these cases—determining the amount of information required for consent to be considered "valid" and the processes required to determine medical care in persons unable to give consent—the legal framework is decided at a national level [36] with differing practices, potentially resulting in differing availability across jurisdictions.

Psychosocial

Whether new hybrid therapies succeed in improving the health and wellbeing of Type 1 Diabetes patients also depends societal context in which they are developed [40, 41]. Understanding of patients' perspectives on hybrid therapies is important in order to ensure their responsible and acceptable translation from bench to body [42]. Yet there is a paucity of empirical research in this area [24]. It will be essential to explore the perceived advantages and disadvantages of hybrid therapies relative to treatment options currently available [42] as this is likely to impact uptake and adoption. For instance, while advanced device-based treatment options improve glycaemic control [43, 44] and make disease management easier [45], it remains challenging for patients to successfully learn how to handle these devices. For instance, several participants in a closed-loop system trial reported that they spent more time thinking about their diabetes while using this system than while undergoing standard treatment [1].

Another reported disadvantage of current treatments is the visibility of these devices due to having to wear a component on the body, such as a sensor on the arm. Some patients refrain from wearing pumps in public, to hide their disease from others, to preserve their self-image and to prevent stigmatization associated with having a disease [45]. An online survey study investigated the willingness of Type 1 Diabetes patients in the US to receive a personalized beta-cell replacement therapy as well as their preferences regarding the size, shape, visibility and transplantation site of the therapy. Findings suggested that the aesthetics are of importance to the majority of the patients surveyed [46]. There is also the implicit relationship between the human donors and recipients to be considered. For instance, some patients may have moral objections to having cells from a deceased person incorporated into the treatment [47], this will be further explored in part 2.

Key Interdisciplinary Issues

In order for hybrid beta-cell replacement therapies to be clinically translated, these key ethical, legal, and psychosocial issues need addressing. However, these issues are not the domain of discrete disciplines, but are interwoven and must be addressed in conjunction to find successful solutions and this novel area of medicine to flourish (see **Figure 1**).

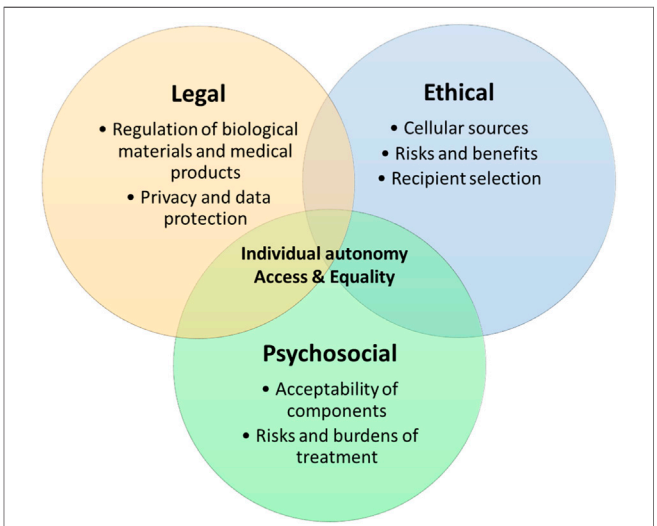

FIGURE 1 | Overlapping ethical, legal and psychosocial issues.

We set out two principal areas where these disciplines converge:

- Individual autonomy
- Access and equality

PART 2—EXAMINING CELL SOURCE AND PROVENANCE USING AN INTERDISCIPLINARY LENS

Issues related to the sourcing of the cells used to generate complex tissue-engineered products, such as bio-artificial organ, are the most frequently discussed aspects in the scientific literature [24]. To generate a hybrid beta-cell replacement therapy, a reliable and ideally renewable source (e.g., allogenic stem cells) of insulin secreting beta cells (see **Table 1**) must be identified. Each of these cell types will have scientific and practical advantages or disadvantages, but they also have distinctive ethical, legal and psychosocial features.

Here, we examine the impact of cell source and provenance on the exercise of individual autonomy and on achieving equitable access to this novel therapy using an interdisciplinary lens.

Individual Autonomy

The exercise of individual autonomy plays a significant role at multiple stages through the process of creating a therapy which utilises cells and tissues- in the act of donating biomaterials, the preferences and acceptability of cell sources to potential recipients and ultimately in gaining informed consent for the procedure.

Living and Deceased (Stem) Cell Donors

As outlined in the legal summary in the European Union, the acquisition, storage and use of human blood, cells, tissues, and organs is closely regulated. Most of these regulations are focused on the necessary conditions for procurement and testing of cells. Their purpose is to protect donors from exploitation and protecting recipients from risks such as transmission of infections or malignancies. However, the regulation preamble also hints at a philosophical purpose. It states:

> "As a matter of principle, tissue and cell application programmes should be founded on the philosophy of voluntary and unpaid donation, anonymity of both donor and recipient, altruism of the donor and solidarity between donor and recipient [48]."

So, these regulations serve to protect both donors and recipients of allogenic cells, but also to promote a certain culture of altruistic and voluntary donation—an exercise of personal autonomy for the good of the community.

However, handling donor cells is not without ethical and psychosocial considerations. The collection and use of allogenic (stem) cells for clinical applications could raise concerns regarding the confidentiality and privacy of the donor, and on ownership and commodification of donated cells [47, 49–51]. Explicit informed consent from the cell donor or their family is required. As a prerequisite for donor consent, donors should be comprehensively informed on current and future cell usage, financial rights, policies regarding the return of findings and the option to withdraw [52]. Safeguarding donor privacy is essential, for example, achieved through anonymizing samples. However, achieving absolute anonymity of donor cells in the field of regenerative medicine is questionable for three reasons: 1) due to advancements in big data and genomics; 2) it is may not preferable as it hinders the return of results to donors and 3) unfavourable since donors lose control and the ability to manage their samples, including the option to withdraw. Recent empirical studies on tissue donation for organoid biobanking [53, 54] highlight that tissue donors desire information, control, and the ability to withdraw. Donors seek knowledge of research outcomes among recipients and the impact of the treatment, to ensure their contribution is meaningful. Their motivation to donate is most often rooted in the idea of beneficence to these unseen and unknown recipients. In addition, the question of ownership of collected cells and engineered tissues, involving the donor, the recipient and the producing parties, remains debated. When altruistically donated cells are turned into profitable products without financial compensation to cell donors, there is potential to violate human dignity and lead to exploitation.

TABLE 1 | Possible insulin secreting beta cell sources.

Allogenic stem cells
Deceased donor islet cells
Xenogeneic cells

From the perspective of recipients there could be moral and religious objections for the use of deceased donors or (decellularized) donor cells, which form psychological or social barriers to treatment [51]. For instance, some patients may argue that they do not want parts of another person to merge with their own cells. Recipients may create an image of, or develop a perceived bond with, the donor who provides the cells for treatment [47]. They may (or may not) struggle to accept that the cells that their treatment is dependent on are cells from a deceased person [47]. Or they may not wish to accept a therapy that contains genetically modified cells, because of the uncertainty of potential tumorigenicity or unwanted side effects. In addition, using modified cells in therapies for patients may portray the human body as malleable [51] and raise questions as to whether human cells should be subjected to engineering.

Xenogeneic Cells

Cells derived from genetically modified animal (namely, porcine) sources have been suggested as alternatives. However, the use of xenogeneic cells raises even more challenging potential psychosocial and ethical barriers leading to varying legal approaches. These include moral concerns for animal rights and welfare, religious beliefs [51, 55] and the risks for wider society, particularly that of zoonosis [51, 56]. The EU has issued guidelines for an approach to the medicinal use of animal cells [57]. However, due to the culturally sensitive nature of this topic the overall permissibility of xenotransplantation is a matter devolved to individual member states. Despite centralized recommendations, markedly different approaches have been

adopted. For example, in Germany groups such as the Deutsches Primaten zentrum (German Primate Centers or DPZ) have been working extensively on xenotransplantation for over 20 years and are leading centers in porcine to non-human primate transplantation research. In contrast, the Netherlands have had a complete ban on xenotransplantation in place since 2002 [58]. Should a therapy containing animal cells come to market when it is not clear yet if it would be legally permissible in all European jurisdictions nor if it would be acceptable to a broad range of Type 1 Diabetes patients?

Informed Consent of Recipients

As asserted in our opening analysis of legal and ethical issues there is an obligation and challenge to obtain informed consent of recipients, but this is also morally essential. Recipients have the right to respect their autonomy and to have the opportunity to reject the treatment—and choose another—based on moral, religious, or any other system of beliefs. Ultimately the success (or failure) of hybrid beta-cell replacement therapies will hinge on if recipients find the product acceptable and will consent to its use.

However, the transition from medical research to clinical practice, also results in a change in the legally proscribed content of consent. In Europe, for medical research, the process and required information for consent is laid down explicitly and in detail in the Clinical Trials Regulations [59]. This means that all information must be "kept comprehensive, concise, clear, relevant, and understandable to a layperson [59]." However, no such consensus or legal standards have been agreed upon in the case of medical treatment.

Determining the content of consent may be challenging owing to the complexity of these hybrid therapies. Considerable uncertainties may also exist in some areas. For instance, long-term monitoring will be necessary to assess potential health risks of the use of highly manipulated and/or (genetically) modified xenogeneic or allogenic cells, such as transmission of zoonotic infections, epigenetic or genetic instability of the graft, or immunological or tumorigenic reactions in the recipient [52]. Long-term monitoring requires, at a minimum, a practical commitment for recipients, but from a psychological perspective, it suggests that there may be safety risks, which may be perceived as threatening. Furthermore, recipients will be required to relinquish some of the learned control they have developed over years of self-management regimes [60], which may cause relief but also anxiety at the idea that if something goes wrong with the hybrid product, they may not know or be able to influence this process. For example, systems with non-user-modifiable algorithms, which take full control of blood glucose, can be experienced by patients as resulting in a loss of autonomy [61]. The initial recipients are likely to be patients with poorly regulated diabetes and extensive secondary complications. These are patients with a perceived lack of alternative treatment options hoping for a cure, which could influence their decision making. If this influence amounts to coercion or interference with the exercise of their autonomy, it will be dependent upon individual patient circumstances.

While it is often assumed that a complete understanding of the technical and biological details of the product is required for informed consent, it is not clear whether "incomplete" understanding renders patients' decisions to undergo treatment (or not) less autonomous. One study exploring the views of tissue engineers on relevant issues and goals of clinical trials with human tissue-engineered products suggests that participants may not always want to be informed in technical information about the composition of the product, but want to be informed mainly about issues that could directly affect their health status and quality of life [62]. However, we do not know, due to the lack of research in this area, what the needs and preferences are of Type 1 Diabetes patients with regards to consenting to receive a hybrid beta-cell replacement therapy.

Initially, there will be many uncertainties, and recipients will need to consider their own moral boundaries in balancing risks and potential benefits, and in envision their level of acceptance and psychological response to having a therapy implanted in their bodies, which is comprised of various cell sources. From an ethical and psychological perspective, clinicians are likely to have to go beyond what might be considered the minimum requirements of information to ensure that prospective recipients are appropriately informed and counselled to make their decisions.

Access and Availability

Global accessibly and availability incorporate various areas that require attention including access among disadvantaged populations, recipient prioritisation and allocation of scarce resources.

Access Globally

Ethical, legal and psychosocial concerns arise regarding the potential limitations in accessibility due to the anticipated high costs associated with the clinical translation of regenerative therapies. High costs to develop the therapy will limit accessibility. If only those with financial means can benefit from the therapy, it may increase socioeconomic disparities at both local and global levels. However, over time, as the therapy becomes more established, costs are expected to decrease, potentially leading to costs-effectiveness [39]. For healthcare systems in the global South, where Type 1 Diabetes patients face already poor health outcomes and the production costs of insulin and insulin pumps are high [63, 64], it is expected that limited financial resources, laboratory facilities and specialized personnel required will hinder the manufacturing and administration of cell-based replacement therapies [8]. In addition, eventually these therapies for Type 1 Diabetes patients generated from donated cells could be patented. While patenting may promote innovation, quality control, and prevent misuse, it may also hinder open science and research, as well as equitable patient access to the therapy to patients [51].

Scarcity of Resources

In order to circumvent resource scarcity, reliable and ideally renewable source (e.g., allogenic stem cells) of insulin secreting beta-cells should be used to generate a hybrid beta-cell replacement therapy. Therefore, there are grounds to argue that funds should be channelled towards regenerative medicine solutions. However, there may be a concern that this allocation may divert resources away from other promising healthcare (technical) solutions for Type 1 Diabetes patients (e.g., hybrid-loop devices).

PART 3—PRACTICAL RECOMMENDATIONS TO FACILITATE TRANSLATION

Optimising Informed Consent

One practical solution to some of the challenges outlined regarding cell sourcing is to optimize the informed consent process. To guide this process and promote optimal informed consent, we propose the following recommendations:

First, language is important. There are different terms in circulation, ranging from bioartificial pancreas to cell-based products, some of which call into mind the *transplantation* of (bioartificial) *organs*, while others refer rather to medical devices (bio-*artificial*) or to advanced therapeutic medicinal products (*cell-based* therapy). Researchers, manufacturers and clinicians should be aware that terminology may affect patients' perspectives on (the risks and potential benefits of) and understanding of hybrid beta-cell replacement therapy. Standardize nomenclature would help promote understanding for all parties involved.

Second, it may be difficult to understand the precise composition of a complex hybrid beta-cell replacement therapy and the implications of accepting it. Given this complexity and individual differences in the ability to understand information needs, strategies are needed to make information accessible and tailored. To support accessibility various modes of delivery can be used in addition to written, for example, diagrams, pictograms or visual timelines [65]. Scientific jargon should be avoided [66] and the required reading level should be no higher than high-school level. Tailoring of information can be achieved through a stepped approach, whereby a minimum of information is agreed upon with optional add-ons for those with greater information needs.

Third, as part of this minimum set of facts, patients should be informed as part of the informed consent process that removal of the bio-engineered pancreas (in its entirety) may not be possible [67].

Last, researchers and/or manufacturers should recognise that their responsibilities in relation to information provision go beyond that of obtaining informed consent from donors and recipients, and consider how they may effectively engage patient communities, donors and donor families and society at large, in discussions on cell-based replacement therapies for Type 1 Diabetes.

Conduct Qualitative Research

A better understanding of patients' perspectives on hybrid beta-cell replacement therapies will be crucial for the development of adequate informed consent processes. Qualitative research would be of added value to gain understanding of how patients needs and preferences can be met and under which conditions they would undergo treatment with a hybrid beta-cell replacement product. Insights from research on patients' perspectives regarding hybrid beta-cell replacement therapies would help facilitate the clinical translation process. Patient representation through qualitative research will be necessary to ensure acceptance, uptake and adoption of such treatments.

Public Policy

Effort should be dedicated to enhancing accessibility to ensure equitable distribution of the therapy. Moreover, to guarantee equal access to novel therapies, reimbursement policies will be necessary. These reimbursement decisions should not solely be based on clinical benefits, but also on patient's preferences compared to alternative treatment options. Finally, regulations for cellular and gene therapies should be more globally harmonized.

CONCLUSION

By utilizing an interdisciplinary approach to the analysis of the legal, ethical and psychosocial matters surrounding the translation of hybrid beta-cell replacement therapies, our group has not only identified unifying themes linking each discipline, but also revealed important next steps in resolving key barriers. While some of these issues have been navigated before in isolation, when combined, they become an unchartered territory, in particularly for patients and regulators. A comprehensive and interdisciplinary approach is required to guide hybrid beta-cell replacement therapy in the clinic in an acceptable and ethically sound manner. Researchers should collaborate across disciplinary fields and engage in dialogue, involving not only scientists but also patients, clinicians, citizens and policymakers. Patient engagement is particularly essential in this clinical translation process to ensure the acceptance, uptake and adoption of such treatments in routine clinical practice.

AUTHOR CONTRIBUTIONS

DdJ, RT, AC, EB, and EM contributed to conception and design of the manuscript. DdJ and RT wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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